Molecular studies of New Zealand Fucales: Phylogeography, phylogeny and taxonomy in *Carpophyllum* and *Cystophora* (Phaeophyceae)

by

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A thesis submitted to the Victoria University of Wellington in fulfilment of the requirements for the degree of Doctor of Philosophy in Marine Biology

School of Biological Sciences
Victoria University of Wellington
Te Whare Wānanga o te Ūpoko o te Ika a Māui

2011

Previous page: Main axis and laterals of *Cystophora scalaris* J. Agardh.

On the rocky shore of the island in the offing, the lovely seaweed – I will miss it when it goes under the flowing tide.

– Yamabe no Akahito
composed in the fifth of the tenth month, 724.

Abstract

Genetic variation in *Carpophyllum* Greville and *Cystophora* J. Agardh (Fucales, Phaeophyceae) was investigated at a variety of scales. An extensive survey of mitochondrial spacer variation in *Carpophyllum maschalocarpum* from 32 populations around New Zealand shows strong population differentiation at relatively small scales (50–100 kilometres), but also pathways of long distance dispersal that connect populations over much greater distances. In addition, historical climate change appears to have restricted *C. maschalocarpum* to the northern North Island during the last glacial maximum, with subsequent southward range expansion revealed by low genetic diversity in southern populations. These results are consistent with limited dispersal at the gamete and zygote stage, expected in fucalean algae, but with occasional long distance dispersal by detached floating thalli. The genetic signature suggests these two modes of dispersal are decoupled.

Internal Transcribed Spacers sequences show little differentiation between *C. maschalocarpum* and *C. angustifolium*, and hybridisation was found in several populations where these species are broadly sympatric. In the Bay of Plenty the two species had different ITS ribotypes, but most *C. angustifolium* specimens had a mitochondrial spacer haplotype that clustered with *C. maschalocarpum* haplotypes. This indicates mitochondrial introgression from *C. maschalocarpum* into *C. angustifolium*. In Northland species were difficult to separate by morphology or molecular markers, and some populations appear to be comprised entirely of hybrids.

Genetic distances between different species of *Cystophora* are very variable, and in some cases intra-species distances are similar to inter-species distances. This is problematic for DNA barcoding methods that rely on thresholds between inter-species and intra-species genetic distances. In some (but not all) cases, the absence of molecular differentiation can be attributed to oversplitting of *Cystophora* species by

morphological methods, and I synonymise *C. congesta* with *C. retroflexa*, and *C. distenta* with *C. scalaris*. These studies exemplify the difficulties of delimiting species in brown algae: Morphology is often misleading or uninformative; genetic differentiation of species is very variable and often low; and species' histories show complex patterns of isolation and secondary contact. I argue for an explicitly historical concept of species, with species' history included in species descriptions.

Acknowledgements

Many people contributed to the completion of this thesis in a variety of ways. My supervisor, Joe Zuccarello, provided tireless support, advice and enthusiasm, both for the preparation of the thesis and in all matters phycological. I could not have found a better person to oversee this project. Pete Ritchie undertook the role of secondary supervisor and was always available when advice was needed.

Maria McMillan provided tireless support for me, my work and my family during this time. Abbie and Lily provided welcome distraction.

Wendy Nelson, Tracy Farr, Kate Neill, Peter Martin, Erasmo Macaya, Fiona Hodge, Kyatt Dixon and Maria helped with collecting specimens. Chris Hepburn, Rochelle Dewdney, Francisco “Pancho” Neira and an anonymous Australian surfer were helpful in directing me to some excellent collecting sites.

Transport and accommodation during fieldwork were provided by Claire Haglund, Simon Hart and Andrea McMillan, Woodford Rouse and family, Maraehako Backpackers, Stuart and Nancy McMillan, Marty Smith and Phil (Napier) and Department of Conservation Chatham Islands staff.

Martin Thiel provided advice on Chapter 2. Alan Millar from the Sydney Royal Botanic Gardens Herbarium and Jenn Dalen from Te Papa Tongarewa (WELT) assisted with access to herbaria. Members of the Wellington phycological group and the VUW Plant Club provided feedback and advice at different stages of the project.

Financial assistance was provided by the Victoria University of Wellington Vice-Chancellor’s Strategic Research Scholarship and by the Education New Zealand Post Graduate Study Abroad Scheme. The Department of Conservation assisted with fieldwork costs.

Many Victoria University staff assisted in many different ways, in particular I would like to thank Cam, Jo, Chris, Mary and Patricia for logistical support.

Essential to completion of this project was the assistance with childcare. In particular, I would like to thank the entire VUW crèche staff, Jane Buchanan, Lenka Rocheford and Pip, and Pip Adam.

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Chapter 1
Introduction and background

1.1 Introduction

THE BROWN ALGAE, or Phaeophyceae, are a monophyletic class within the Stramenopiles (Baldauf 2008). They are distantly related to the other eukaryote lineages that have evolved complex multicellular thalli (Metazoans, Fungi, Archaeplastida) and have unique characteristics (Charrier *et al*. 2007). In this project I investigate evolutionary processes in two genera of brown algae, *Carpophyllum* Greville and *Cystophora* J. Agardh. By comparing evolutionary processes in different lineages of life we can progress towards a general understanding of the variation and commonalities in evolutionary processes.

In a review of the state of knowledge of New Zealand marine macroalgae, Nelson (1994) wrote: "Speciation and endemism, and our lack of understanding about rates and processes involved, remain as outstanding issues." Fucoid algae are ideal organisms to investigate evolutionary processes as: (1) They are diverse, Australasia is the probable origin and centre of diversity of the Fucales (Clayton 1984); (2) New Zealand has endemic species and genera and others that are shared with Australia (Parsons 1985); and (3) Fucalean algae have a relatively simple life history, with a single life stage (Chapman 1995). This last character simplifies interpretation of genetic variation, as ecological and evolutionary processes are not acting differently on the dominant macrothallus phase and on the cryptic and phenotypically distinct microthallus phase.

Carpophyllum is endemic to New Zealand, *Cystophora* is found in New Zealand and southern Australia. Species of both genera are among the most common shallow marine macroalgae of New Zealand (Shears & Babcock 2007). Stands of *Carpophyllum* dominate the shallow sub-tidal around a large part of rocky New Zealand coastline. *Cystophora* species are common in the shallow subtidal, rock pools and lower intertidal. Species of both genera provide structure and shelter for other marine organisms and are a significant contributor to primary productivity (Schiel 1990).

Some difficulties arise in species identifications in both genera (Adams 1994). Some forms appear morphologically intermediate between species (Lindauer *et al*. 1961) and ecological studies often identify specimens to generic

level only. This lack of taxonomic information can lead to problems in identifying introduced species, recording distributional changes in species resulting from climate change and in investigating ecological and phylogenetic relationships of these algae.

This project aims to use molecular techniques to investigate genetic variation in these algae at different scales, to provide insights into processes effecting speciation. Specifically, I investigate phylogeny, taxonomy, dispersal and hybridisation. Here I review the taxonomic background and ecology of these genera and outline some questions that will be addressed.

1.2 *Carpophyllum* Greville 1830

Carpophyllum has four species[1], all endemic to New Zealand (Adams 1994). A fifth species of *Carpophyllum* is under consideration based on two specimens collected in Antarctica (M. Clayton & W. Nelson pers. comm.). *Carpophyllum flexuosum* (Esper) Greville is the most widespread species, extending throughout the North and South Islands, Stewart Island, and the Chatham Islands. *Carpophyllum maschalocarpum* Turner (Greville) is the most abundant species (Shears & Babcock 2007) and is found around the North Island, Chatham Islands and South Island as far south as Fiordland and Banks Peninsula. *Carpophyllum plumosum* (A. Richard) J. Agardh and *C. angustifolium* J. Agardh have more restricted distributions. *Carpophyllum plumosum* is found only on the eastern coasts of the North Island, from Northland to the Wairarapa Coast, and on the Chatham Islands. *Carpophyllum angustifolium* is found only on the east coast of the North Island, from Northland to just north of East Cape. Adams & Nelson (1985) also recorded *C. angustifolium* and *C. plumosum* from the Three Kings Islands,[2] off the northern tip of New Zealand's North Island. *Carpophyllum* is generally absent from the sub-Antarctic islands, although *C. flexuosum* has been recorded from the Auckland Islands (Papenfuss 1964), but Adams (1994) suggests

[1] Algaebase (Guiry & Guiry 2011) includes *Carpophyllum macrophyllum* as a current species. This was created by Montagne (1845) from material from the Auckland Islands. According to Lindauer *et al*. (1961) *C. macrophyllum* is a synonym for *C. flexuosum*.

[2] I examined specimens of *C. angustifolium* collected from the Three Kings Islands held in WELT and consider them to be *C. angustifolium* × *C. maschalocarpum* hybrids.

reports from the sub-Antarctic islands are from drift material. Reports of *Carpophyllum* species from the Kermadec Islands appear to be based only on drift material (Adams & Nelson 1984).

Carpophyllum is placed in the Sargassaceae and is related to some species of *Sargassum* (Draisma *et al.* 2010). The phylogenetic relationships within *Sargassum* have yet to be completely elucidated (Mattio *et al.* 2008, Draisma *et al.* 2010), and *Carpophyllum*'s relationship to the various sub-groupings of *Sargassum* is not entirely clear. BLAST searches and preliminary trees using my sequence data suggest *Carpophyllum* is monophyletic and most closely related to some species of the *Sargassum* subg. *Phyllotricha*, with *S. verruculosum* the closest relative found (albeit with limited species sampling). Draisma *et al.* (2010), in the only published phylogeny that included *Carpophyllum*, placed *Carpophyllum maschalocarpum* as sister species to *S. decurrens* (in the clade *Sargassum*-2 in their system) in a phylogeny inferred from 23S rDNA and *psa*A chloroplast DNA (they did not include *S. verruculosum* in their dataset). My ITS data suggest a close relationship between *S. verruculosum* and *S. decurrens.* Draisma *et al.* (2010) also show a close relationship between *Carpophyllum* and *Nizamuddinia zanardinii* from Oman (suggested by Nelson 1994).

Carpophyllum was established by Greville (1830, xxxii), with two species, *C. maschalocarpum* and *C. flexuosum*. His description is brief: "Frons plana vel compresso-plana. Folia ramiformia, distichia. Receptacula minuta, tuberculata, cylindracea, in racimes marginalibus." The description emphasises the flattened thallus (found in all species except *C. angustifolium*) and distichous laterals. Marginal receptacles are only found in *C. flexuosum,* in other species receptacles are axillary. Lindauer *et al.* (1961, p. 306) listed six characters that, in combination, separate *Carpophyllum* from *Cystophora* and *Sargassum*: "1. the flattened stem; 2. elongate flattened holdfast with short haptera at intervals on edges; 3. rounded or elongate vesicles; 4. leafy appendages; 5. reproductive organs on special branchlets; 6. absence of special basal leaves (these are present in *C. flexuosum*)." I consider that only the flattened holdfast reliably separates *Carpophyllum* from *Sargassum*. *Carpophyllum* appears to have affinities with the distichous members of *Sargassum* subg. *Phyllotricha* (based on unpublished

molecular data[3]). The holdfast of all *Carpophyllum* species is flattened, creeping over the substrate, with haptera on the edges. In all species except *C. angustifolium,* the holdfast gives rise to a flattened main axis, with no intermediate terete stipe. In *C. angustifolium* the holdfast is flattened and gives rise to a terete stipe. Holdfasts in *Sargassum* subg. *Phyllotricha* species are conical or discoid and give rise to an initially terete stipe. In some species this stipe then becomes flattened and distichous (Womersley 1954, Rainbo Dixon, personal communication, January 16, 2011).

Carpophyllum species were collected by early visitors to New Zealand (Laing 1926) and returned to Europe for description. *Carpophyllum flexuosum* was described by Esper (1802) as *Fucus flexuosus*. *Carpophyllum maschalocarpum* was described by Turner (1811) as *Fucus maschalocarpus*. *Carpophyllum plumosum* was described by A. Richard (1832) as *Sargassum plumosum,* from material collected on Dumont D'Urville's second voyage to New Zealand. *Carpophyllum angustifolium* was described by J. Agardh (1877), from material supplied by Sven Berggren. Synonyms and other taxonomic details are recorded in Lindauer *et al.* (1961).

Some descriptive work on *Carpophyllum*, mainly related to the reproductive anatomy was undertaken by Delf (1939), Dawson (1940), and Naylor (1954). I can find no systematic treatment of *Carpophyllum* since the compilation of early work in Lindauer *et al*. (1961), although Dromgoole's (1973) largely ecophysiological study included some taxonomic notes. Other studies have investigated the chemistry of *Carpophyllum maschalocarpum* (Glombitza & Li 1991a, 1991b, Li & Glombitza 1991) and *C. angustifolium* (Glombitza & Schmidt 1999).

A major survey of macroalgal abundance (Shears & Babcock 2007) found *Carpophyllum maschalocarpum* was the second most abundant macroalgal species around New Zealand (after *Ecklonia radiata*). Other *Carpophyllum* species are locally abundant. Ecological studies have investigated relationships between *Carpophyllum* species and invertebrates, especially effects of urchin grazing, in northern New Zealand (Choat & Schiel 1982, Andrew 1988), the

[3] These and other data suggests *Phyllotricha* should be elevated to a distinct genus (Rainbo Dixon, Pers. Comm. 3 May 2011). A paper addressing this is in preparation.

Chatham Islands (Schiel *et al*. 1995) and Fiordland (Villouta *et al.* 2001). Taylor (1998a and b) investigated epifauna on *Carpophyllum*. A study by Cole *et al.* (2001) investigated recruitment of *C. flexuosum* around Northland. Various studies have recorded *Carpophyllum* distribution and abundances in local areas (refs. in Hurd *et al*. 2004).

Carpohyllum species are all dioecious, Fertile thalli have axillary clusters of receptacles. *Carpophyllum flexuosum* also has marginal receptacles on some lateral lamina (Lindauer *et al.* 1961).

1.3 *Cystophora* J. Agardh 1848

Womersley (1964) monographed *Cystophora*, describing 23 species, separated mainly by characters of the vegetative morphology and the arrangement of conceptacles on the receptacle. Adams (1994) lists six species of *Cystophora* from New Zealand: *Cystophora torulosa* (R. Brown) J. Agardh and *C. retroflexa* (Labillardière) J. Agardh are widespread. *Cystophora distenta* J. Agardh, *C. platylobium* (Mertens) J. Agardh, *C. scalaris* J. Agardh, and *C. congesta* Womersley & Nizamuddin ex Womersley are found from Cook Strait southwards. Two species (*C. scalaris* and *C. distenta*) are reported from the Auckland Islands (Adams 1994). Other species extend south as far as Stewart Island and east to the Chatham Islands (Nelson 1994).

Lindauer *et al.* (1961) also list *C. dumosa* (Greville) J. Agardh, a species that Womersley (1964) rejected as a *nomen confusum* under the then current ICBN rules. Womersley (1964) found most of the material used to establish *Cystophora dumosa* consisted of fragments of *C. monilifera*. The earliest valid description of *C. dumosa* is by Sonder (1846). According to Womersley, Sonder's material is a mix of fragments of *C. monilifera* and probably *C. brownii*. Doubtful reports of Australian species from New Zealand include *Cystophora* (= *Caulocystis*) *cephalornithos, C. monilifera, C. paniculata* and *C. retorta.* These are either misidentifications or Australian specimens with localities mislabelled (Lindauer *et al*. 1961, Womersley 1964, 1987).

Womersley (1964) created a new species, *Cystophora congesta* Womersley & Nizamuddin that includes specimens recorded as *C. dumosa*,

including those from New Zealand. *Cystophora congesta* is reported from the New Zealand mainland from Wellington southwards and from Stewart Island and the Chatham Islands. According to Womersley (1964) it is closely related to *C. retroflexa* and "occasional intergrades occur." Hybridisation has been reported in other species of *Cystophora.* Lindauer *et al.* (1961) states: "Within the New Zealand species [of *Cystophora*] a degree of hybridization occurs in certain areas where some of the species overlap. *Cystophora platylobium* seems quite distinct, but *C. torulosa, C. scalaris, C. retroflexa* and *C. dumosa* [=*distenta*] seem to intergrade. A full analysis of the degree of this variation needs to be made."

Several studies have recorded ecological interactions involving *Cystophora. Cystophora* species are settled preferentially by some reef fish (McDermott & Shima 2005) and shelter higher numbers of invertebrates than other, less ramified, large brown algae (Taylor & Cole 1994). Other workers have investigated the chemistry of *Cystophora* (Glombitza *et al*. 1997). Amico (1995) included several *Cystophora* species in a chemotaxonomic study of the Cystoseiraceae.

Several phylogenetic studies have included specimens of *Cystophora.* Rousseau & De Reviers' (1999) phylogeny of the order Fucales, based on rDNA LSU and SSU, included three species of *Cystophora* and other Australian fucalean species. Cho *et al.* (2006) included a single (unidentified) specimen of *Cystophora* from Brighton Beach, New Zealand, in a phylogeny of the Fucales inferred from the *psa*A gene. A multi-locus phylogeny of the "crown" group of brown algae (Silberfield *et al.* 2010) included the Australian species *Cystophora retorta* and *C. grevillei*. Draisma *et al.* (2010) included two species of *Cystophora* in a multi-gene phylogeny. These studies have not elucidated relationships within *Cystophora*, but have identified *Landsburgia* as the sister genus to *Cystophora*, and placed *Cystophora* among other Cystoseiraceae, as well as showing the Cystoseiraceae are polyphyletic.

Most *Cystophora* species have bisexual conceptacles, although sometimes individual conceptacles have mainly oogonia or antheridia. Womersley (1964) notes that *C. monilifera* and *C. racemosa* can have unisexual conceptacles, but either single receptacles bear both types of conceptacle, or adjoining receptacles

bear different types. Only *C. siliquosa* is fully dioecious, with separate male and female plants.

1.4 Research questions

1.4.1 Hybridisation

Hybrids have been reported in *Carpophyllum* (Hodge *et al.* 2010) and *Cystophora* (Womersley 1964). Hybridisation has been extensively investigated in the northern hemisphere fucalean genus *Fucus.* Morphological variation in *Fucus* has been ascribed to both environmental (Chapman 1995) and genetic factors (Anderson & Scott 1998), and natural and artificial hybrids have been studied extensively (McLachlan *et al.* 1971, Coyer *et al*. 2002, Coyer *et al*. 2010).

Two questions immediately arise: (1) Are intermediate forms hybrids?; and (2) are hybrid zones widespread or geographically limited? A number of additional questions are likely to arise: If hybrids are widespread how are the species maintained? If they are limited do they represent zones of secondary contact following speciation due to climate change, geological processes or long distance dispersal (e.g., from Australia)? Further, if hybrids have persisted, is there evidence of hybridisation leading to speciation (reticulate evolution)?

1.4.2 Genetic variation and population structure

Large, negatively buoyant and non-motile eggs are assumed to limit dispersal ability at the gamete and zygote stage in fucalean algae, such as *Carpophyllum* and *Cystophora*, but drifting detached thalli might facilitate longer dispersal distances (Coleman & Brawley 2005, Coleman & Kelaher 2009, Fraser *et al.* 2009). Dispersal might also be extended by the retention of sporelings on the receptacles followed by release and rapid attachment (Schiel & Foster 2006).

Dispersal ability should be closely related to genetic variation within and between populations. Broad-scale studies of population genetic structure in macroalgae often find high population structure, and this is related to (1) limited dispersal ability (Dayton 1973, Coleman & Brawley 2005); and (2) historical

processes, especially historical climate change (Hoarau *et al*. 2007, Maggs *et al*. 2008, Coyer *et al*. 2010). These processes have mostly been studied in the northern hemisphere, especially Europe, and there is little understanding of the effect of these processes in New Zealand, although some phylogeographic work has been done on fish (Hickey *et al*. 2009) and invertebrates (Goldstien *et al*. 2006, 2009).

All *Carpophyllum* species are endemic to New Zealand, which simplifies sampling across the range of the species, and allows population level studies to capture the history of the species. I will use mitochondrial markers to attempt to discern historical patterns of range expansion and contemporary patterns of gene flow by dispersal. Trans-Tasman distributions of some species of *Cystophora* are presumed to arise from long-distance dispersal (Parsons 1985), but the degree of isolation of New Zealand and Australian populations has not been studied. This raises the question of whether there have been multiple dispersal events between New Zealand and Australia or whether dispersal events are rare and ancient, with subsequent genetic differentiation.

1.4.3 Phylogeny

The relationships between species of *Cystophora* and *Carpophyllum* are not clear. In *Carpophyllum* characters such as the terete main axis of *C. angustifolium* or the large basal lamina of *C. flexuosum* might link *Carpophyllum* to a shared common ancestor with *Sargassum*. Alternatively these might be derived characters relating to the sheltered habitat of *C. flexuosum* and the exposed habitat of *C. angustifolium.*

Womersley (1964) produced a tentative phylogeny of *Cystophora* based on the arrangement of branching of the main axis and laterals, and Amico (1995) used a chemotaxonomical approach to group *C. torulosa, C. congesta, C. scalaris* and *C. monilifera* together, separate from a second group containing *C. expansa* and *C. platylobium* together, with *C. monilifera* (which Amico (1995) considered "more developed") distinct from both of these. It is not clear whether characters such as branching from the edge (rather than the face) of main axes are shared or

derived characters. In both genera a molecular phylogeny might shed light on the development of these characters.

1.4.4 Taxonomic questions

Characters used to separate New Zealand species of *Cystophora* vary between authors. Lindauer *et al*. (1961) recognise *C. dumosa* as a species with characters intermediate between *C. scalaris*, *C. retroflexa* and *C. torulosa.* Womersley (1964) created a new species, *C. congesta,* which included some specimens assigned to *C. dumosa*, but Womersley's characters are hardly intermediate between the above three species. Adams (1994) also recognised *C. congesta,* but her illustration (Adams 1994, p. 141) does not agree with Womersley's description, and her choice of characters provided in a key for New Zealand species appears closer to Lindauer *et al.*'s (1961) than Womersley's (1964). I have found this key difficult to use, with ambiguous outcomes for several specimens. Molecular markers might be useful for testing species limits in *Cystophora* and assessing the value of different morphological characters.

1.5 Structure of this thesis

In Chapter 2 I investigate the phylogeography of *Carpophyllum maschalocarpum* using extensive sampling of a mitochondrial spacer marker from 32 populations. In Chapter 3 I look at patterns of hybridisation between *Carpophyllum maschalocarpum* and *C. angustifolium* from several populations from the north-eastern North Island. In Chapter 4 I infer a phylogeny for *Cystophora* and compare this to species delimited using a DNA barcoding methodology. Chapter 5 examines the taxonomy of New Zealand species of *Cystophora* in some detail, resulting in the synonymisation of two pairs of species. Finally in Chapter 6 I present a summary of my findings, together with some additional data, in the context of approaches taken by other workers to species delimitation in brown algae.

Because this thesis was written as a series of independent papers, there is some unavoidable repetition of introductory material and general information in each chapter.

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Chapter 2

Decoupling of short and long distance dispersal pathways in the endemic New Zealand seaweed *Carpophyllum maschalocarpum* (Phaeophyceae, Fucales).

2.1 Abstract

The processes that produce and maintain genetic structure in organisms operate at different time scales and on different life history stages. In marine macroalgae, gene flow occurs through gamete/zygote dispersal and rafting by adult thalli. Population genetic patterns arise from this contemporary gene flow interacting with historical processes. Spatial patterns of mitochondrial DNA variation were analysed to investigate contemporary and historical dispersal patterns in the New Zealand endemic fucalean brown alga *Carpophyllum maschalocarpum* (Turner) Greville. Populations bounded by habitat discontinuities were often strongly differentiated from adjoining populations over scales of tens of kilometres and intra-population diversity was generally low, except for one region of north-east New Zealand (the Bay of Plenty). There was evidence of strong connectivity between the northern and eastern regions of New Zealand's North Island and between the North and South Islands of New Zealand and the Chatham Islands (separated by 650 km of open ocean). Moderate haplotypic diversity was found in Chatham Islands populations while other southern populations showed low diversity consistent with Last Glacial Maximum retreat and subsequent recolonisation. I suggest that ocean current patterns and prevailing westerly winds facilitate long distance dispersal by floating adult thalli, decoupling genetic differentiation of Chatham Island populations from dispersal potential at the gamete/zygote stage. This study highlights the importance of encompassing the entire range of a species when inferring dispersal patterns from genetic differentiation, as realised dispersal distances can be contingent on local or regional oceanographic and historical processes.

Abbreviations:
AMOVA, Analysis of Molecular Variation; LGM, Last Glacial Maximum; CTAB, Cetyltrimethylammonium Bromide; SSCP, Single Stranded Conformational

Polymorphism; SAMOVA, Spatial Analysis of Molecular Variance; SST, Sea Surface Temperature; TBE, Tris-Borate-EDTA.

2.2 Introduction

MULTIPLE dispersal mechanisms are found in many organisms. In many plant species most seed dispersal is limited to local populations, but alternative dispersal mechanisms connect distant populations (Higgins *et al.* 2003). Short dispersal distances are also found in many sessile or demersal marine species, but occasional long distance dispersal events connect populations across wide habitat disjunctions (Pakker *et al.* 1996, Reed *et al.* 1988, Fraser *et al.* 2009). Dispersal distance in marine organisms is often correlated with the duration of a pelagic larval stage (Bohonak 1999), but many marine organisms have the potential to disperse as adults, either because of inherent buoyancy or by rafting on floating substrata (e.g., drift wood, algae or pumice) (Thiel & Gutow 2005a).

Long distance dispersal mechanisms differ from short distance mechanisms in two ways: Firstly, long distance dispersal mechanisms are often mediated by large scale processes (climate patterns, ocean currents, seasonal migrations of animal vectors), rather than local conditions (turbulence, wave regimes, inshore currents). Secondly, long distance dispersal events are rare, stochastic and will seldom be observed directly, but will become evident in a species' range or population genetic structure over long time scales. Consequently, factors that mediate long distance dispersal are decoupled from factors that mediate most observed dispersal (Kinlan *et al.* 2005, Thiel & Haye 2006).

Dispersal by floating thalli might be an important dispersal mechanism for macroalgae (Deysher & Norton 1982, van den Hoek 1987, Thiel & Gutow 2005b, Fraser *et al.* 2009). Floating macroalgal thalli are abundant and can release viable propagules for a considerable time (Kingsford 1992, Smith 2002, Macaya *et al.* 2005, Hernandez-Carmona *et al.* 2006, McKenzie & Bellgrove 2008), allowing dispersal between populations separated by habitat disjunctions such as soft sediment coasts or large stretches of open water (Dethier *et al.* 2003). Genetic evidence for long distance dispersal in macroalgae across open ocean is accumulating but still rare (Tatarenkov *et al.* 2007, Fraser *et al.* 2009).

Fucalean algae (Phaeophyceae) have a monophasic life history with dispersal from maternal thalli by non-motile, negatively buoyant eggs or zygotes (Kendrick & Walker 1991, 1995, Norton 1992, Reed *et al.* 1992, Chapman 1995). Populations are predicted to show strong genetic differentiation because of limited dispersal potential at this stage. Population genetic studies of northern hemisphere Fucaceae have shown population differentiation (Coyer *et al.* 2003, Coleman & Brawley 2005a, Hoarau *et al.* 2007, Tatarenkov *et al.* 2007), but often at larger scales than predicted from direct measurements of propagule dispersal. Long distance dispersal by detached, floating thalli is often invoked to explain population connectivity at large scales (van den Hoek 1987, Coleman & Brawley 2005b, Thiel & Gutow 2005b), especially where local population differentiation is high (Muhlin *et al.* 2008, Coleman & Kelaher 2009),

More generally, the use of molecular markers has uncovered higher than expected genetic population structure in marine organisms. Currents, upwelling, habitat disjunctions, and larval behaviour limit dispersal even for organisms with long pelagic duration (Sotka *et al.* 2004). Historical events are also evident in genetic structure. Marko (2004), for example, found different biogeographical histories in two species of the intertidal gastropod *Nucella* in the northeastern Pacific, despite similar dispersal modes. Retreat from high latitudes following glacial cooling, followed by recolonisation from either northern or southern refugia is a common pattern (Hoarau *et al.* 2007, Maggs *et al.* 2008) although marine species appear to show more variable responses to global cooling than terrestrial species (Wares & Cunningham 2001, Marko *et al.* 2010).

Carpophyllum maschalocarpum (Turner) Greville is a common fucalean alga that is endemic to New Zealand. All *Carpophyllum* species are dioecious. Female thalli produce large (c. 200 μm diameter) non-motile eggs that are extruded to the surface of receptacles but retained by a mucilaginous stalk during fertilisation (Naylor 1954, Clayton 1992). Following fertilisation, zygotes remain attached to the maternal thallus for up to 28 days before release (Delf 1939, Dromgoole 1973). As eggs and zygotes are negatively buoyant, the species is expected to have low dispersal ability at these life stages, but long distance dispersal is possible as adult thalli usually possess pneumatocysts and are

frequently found floating on the sea surface (Kingsford 1992). Floating female thalli bearing zygotes or germlings could establish new populations in the absence of fertile males.

Carpophyllum maschalocarpum forms extensive stands in the shallow subtidal of New Zealand's rocky coasts, where it is the second most abundant algal species by biomass and an important structuring organism (Shears & Babcock 2007). The species is present around both main islands of New Zealand and the Chatham Islands, but is absent from the south-east of the South Island and the sub-Antarctic islands (Morton & Miller 1968, Shears & Babcock 2007, Nelson *et al.* 1991, Schiel *et al.* 1995).

Carpophyllum maschalocarpum's extensive range, potential multiple dispersal modes, ecological importance and ubiquity in rocky habitats make it an ideal organism for investigating phylogeographic processes. The species' range is interrupted by significant habitat breaks (Fig. 2.1), including extensive soft sediment coast around both main islands of New Zealand and stretches of deep water between the main islands (Cook Strait), and between the mainland and the Chatham Islands, 650 km east of the mainland (Morton & Miller 1968, Nelson 1994, Shears *et al.* 2008). The factors mediating the two mechanisms of dispersal should be a major determinant of genetic structure in *Carpophyllum maschalocarpum*. If dispersal by floating thalli is rare, high population differentiation is expected, especially between populations separated by discontinuities in habitat. If floating dispersal is common, connectivity is expected to be mediated by current and wind patterns.

The aim of this study is to determine if the population genetic variation of *C. maschalocarpum* is congruent with dispersal modes. Specifically I compare connectivity between adjacent (10s of kilometres) and distant (100s of kilometres) populations, test for congruence between phylogeographic structure and biogeographic provinces proposed by Shears *et al.* (2008) (Fig. 2.1), and test for genetic isolation by distance and population expansion.

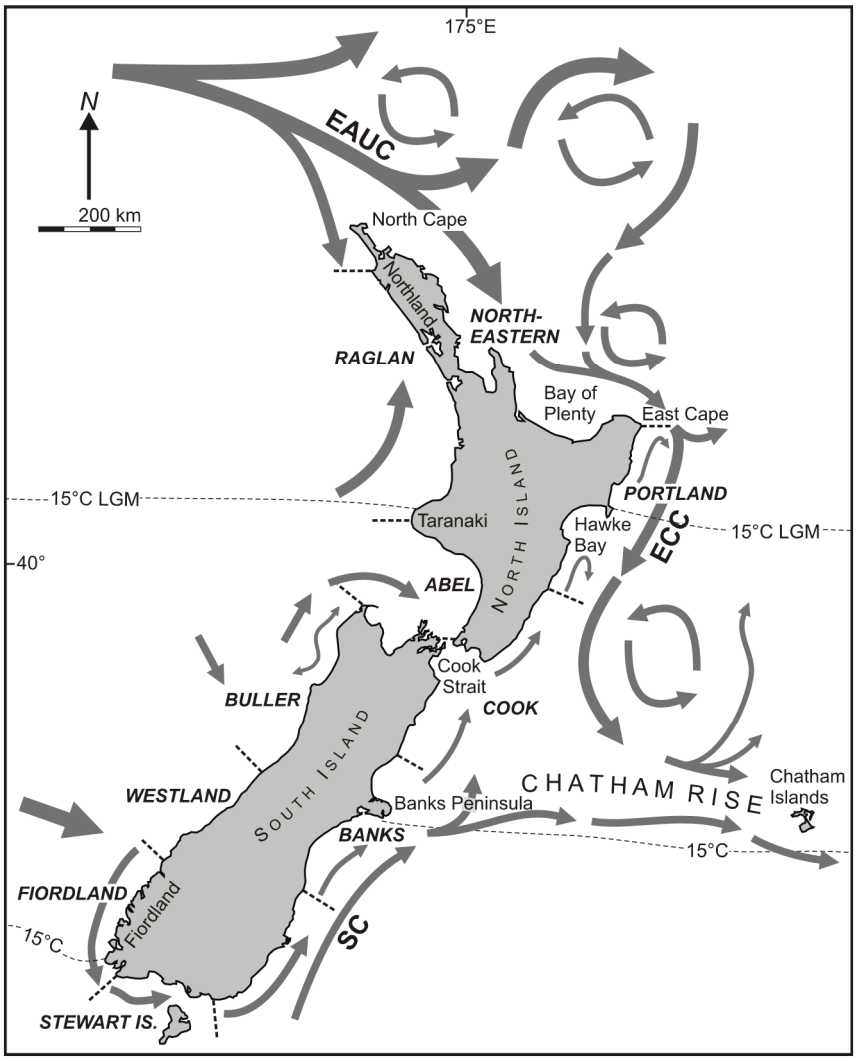

Fig. 2.1 New Zealand map showing major currents (Heath 1985) and locations referred to in the text. Boundaries of bioregions proposed by Shears *et al.* 2008 are indicated by dashed lines and named in italics. EAUC: East Auckland Current; ECC: East Cape Current; SC: Southland Current. Dashed lines show present day 15ºC SST isotherm and estimated 15ºC isotherm for the Last Glacial Maximum (Barrows & Juggins 2005).

2.3 Materials and Methods

Sampling and DNA extraction

Specimens identified in the field as *Carpophyllum maschalocarpum* were collected from 32 sites around New Zealand (Table 1.1), representing the entire range of *C. maschalocarpum* with the exception of Fiordland, where the species is relatively rare (Shears & Babcock 2007). Thalli were collected haphazardly, at least 1 m apart. Tips of thalli for DNA extraction were rapidly dried and stored in silica gel. In addition, one or more typical thalli from each species/site were prepared as voucher specimens. Five *Carpophyllum angustifolium* J. Agardh specimens and a number of putative *C. angustifolium* × *C. maschalocarpum* hybrid thalli were also included as outgroup specimens and to assist in genetic identification of hybrid specimens. DNA was extracted from approximately 2–5 mg of dry tissue using a modified CTAB buffer procedure (Zuccarello & Lokhorst 2005), with the addition of 1% polyvinylpolypyrrolidone to the extraction buffer.

PCR conditions and primers

The 23S-tRNA Lys mitochondrial spacer (225 bp) was amplified from a total of 651 samples, using primers (forward: fuc2625F 5′-GCTGTGAGGTTTTTAGCTGACC-3′ and reverse: fuc3141R 5′-TCCACCACTCTAACCAACTGAG-3′) designed from alignments of the *Fucus*, *Laminaria* and *Desmarestia* mitochondrial genomes (Oudot-Le Seq *et al.* 2002, 2006).

PCR amplifications used a touchdown routine with an initial denaturation step of 94ºC for 4 min, followed by 5 cycles of 1 min at 94ºC, 1 min at 55ºC (−1ºC/cycle) and 1 min at 72ºC, followed by 30 cycles of 1 min at 94ºC, 1 min at 50ºC and 1 min at 72ºC, with a final extension of 72ºC for 10 min. The PCR mix contained 1 µL genomic DNA, 0.5 U Taq DNA polymerase (New England Biolabs, MA. USA) 1X ThermoPol reaction buffer (NEB), 7.5 pmoles of each primer, 200 nmoles dNTP, 5% DMSO and 0.01% BSA. Amplified products were checked for length and yield on 1% agarose gels stained with ethidium bromide.

Populations were screened for variable haplotypes by SSCP (Zuccarello *et al.* 1999, Sunnucks *et al.* 2000). 3–4 µL PCR product was mixed with 9 µL 98% formamide, 10 mM NaOH, 0.025% bromophenol blue, 0.025% xylene cyanol. Samples were denatured at 95–100^{o}C for 5 min, then snap cooled on ice before loading. Gels (225 mm long and 0.75 mm thick, D-Code System, BioRad, Hercules, CA. USA) contained 9% 37.5:1 acrylamide/bis-acrylamide (Sigma Aldrich, St Louis, MO. USA) in 0.5X TBE buffer with the addition of 10% glycerol. Electrophoresis was carried out for 18–20 hrs at 7W in 0.5X TBE buffer at 4ºC. After electrophoresis, gels were silver stained following protocols in Bassam *et al*. (1991) and banding patterns scored by eye. One or more of each haplotype indicated by SSCP was sequenced from each population, as well as any samples giving ambiguous SSCP profiles. Amplification for sequencing used the same PCR conditions and reverse primer as above, but used a novel forward primer (fuc2512F: 5′-CCGGGTAGCTACATCGAGAA-3′) that anneals approximately 100 bp further toward the 5′ end of the fragment, to ensure accurate reads of the 5′ end of the spacer.

Generally SSCP banding and sequencing results were concordant. In very few cases haplotypes were not easily distinguished by SSCP and all samples were sequenced. PCR products were cleaned with ExoSAP-IT (USB, Cleveland, OH, USA) enzymes and sequenced commercially (Macrogen Inc., Seoul, South Korea). Sequences from 210 samples were included in the alignment.

Data analysis

Sequences were aligned using the ClustalW algorithm in MEGA 3.1 (Kumar *et al.* 2004) and checked by eye. Alignment was straightforward, requiring insertion of a single gap to accommodate outgroups. Haplotype networks were generated in TCS 1.21 (Clement *et al.* 2000). Arlequin 3.11 (Excoffier *et al.* 2005) was used to generate summary population genetic statistics and perform neutrality tests (Tajima's D, Fu & Li's F* and D*, and Fu's F_s). Neutrality indices were tested against 2000 coalescence simulations assuming demographic stability and neutrality.

Pairwise Φ_{st} values were calculated in Arlequin 3.11 for Bay of Plenty populations (in which shared haplotypes were reasonably frequent) and tested for

significance by 1023 permutations. Mantel tests, also implemented in Arlequin 3.11, were used to determine isolation by distance, first for all populations then for mainland populations (excluding Chatham Island populations). A matrix of shortest over-water geographic distances between populations was calculated and tested for correlation with pairwise Φ_{st}, with significance tested against 2000 permutations. A second test was made using only the north-south component of these distances.

AMOVAs, implemented in Arlequin 3.11, were used to compare population partitions found in *C. maschalocarpum* with proposed marine biogeographic regions (Shears *et al.* 2008). These were compared to genetic structure indicated by SAMOVA (Spatial Analysis of Molecular Variance, Dupanloup *et al.* 2002) implemented in SAMOVA 1.0. This uses a simulated annealing process to group adjoining populations into K-groups so as to maximise genetic distance between groups (maximum F_{ct}), while minimising distance within groups (minimum F_{sc}). K was iterated in successive runs until a maximum F_{ct} was calculated, and significance of fixation indexes tested by 1000 permutations.

Mismatch distribution analysis (Rogers & Harpending 1992), implemented in Arlequin 3.11, was used to test for signatures of spatial expansion in frequency distributions of pairwise sequence differences. Analyses were applied to the total data and separately to populations partitioned into two groups as indicated by SAMOVA and tested against 5000 coalescent simulations assuming spatial expansion. Results were interpreted in the context of simulations using stepping stone and infinite island models. Here unimodal frequency distributions of pairwise mutational distances are regarded as evidence for past population expansions, but this signature is weakened or removed where populations are subdivided with low migration rates (Ray *et al.* 2003, Excoffier 2004).

Table 2.1: Geographic locations, numbers of specimens sampled, mtDNA spacer haplotype designations and population diversity indices for *Carpophyllum maschalocarpum.* Hyphens in gene diversity and nucleotide diversity columns indicate populations where substantial introgression by *C. angustifolium* was detected and values were not calculated.

Map ID (Fig 2.2)	**Site**	**N**	**Latitude**	**Longitude**	**Herbarium number of exemplars**	**Haplotypes present (n)**	**Gene diversity *h* ±** S.D.	**Nucleotide diversity *π* ± S.D.**
1	Hooper Point, Northland	47	34° 24′ 56″ S	172° 51′ 13″ E	-	21 (10), 34 (34), 65 (1), 66 (1), 67 (1)	-	-
2	North Cape, Northland	10	34° 24′ 57″ S	173° 03′ 09″ E	WELT A031664	30 (8), 31 (1), 32 (1)	-	-
3	Matai Bay, Northland	18	34° 49′ 37″ S	173° 24′ 54″ E	-	68 (18)	0	0
4	Ahipara, Northland	25	35° 10′ 10″ S	173° 06′ 30″ E	-	40 (1), 52 (24)	0.0800 ± 0.0722	0.0004 ± 0.0008
5	Wekarua, Northland	19	34° 56′ 42″ S	173° 39′ 12″ E	WELT A031665	30 (7), 33 (1), 47 (7), 50 (4)	-	
6	Cape Wiwiki, Northland	21	35° 09′ 22″ S	174° 07′ 20″ E	-	21 (21)	0	0
7	Bland Bay, Bay of Islands	20	35° 20′ 31″ S	174° 21′ 58″ E	WELT ASG290	13 (20)	0	0
8	Poor Knights Islands	10	35° 27′ 40″ S	174° 44′ 20″ E	WELT A031311, A031317, A031319	21 (10)	0	0
9	Waterfall Reef, Leigh, Northland	11	36° 16′ 08″ S	174° 47′ 53″ E	WELT A031318, A031320	01 (2), 13 (8), 36 (1)	0.4727 ± 0.1617	0.0067 ± 0.0049
10	Matheson Bay, Northland	34	36 18′ 05″ S	174° 47′ 58″ E	-	01 (2), 13 (27), 55 (1), 56 (1), 57 (2), 66 (1)	0.3708 ± 0.1042	0.0063 ± 0.0044
11	Sailors Grave, Bay of Plenty	20	36° 57′ 37″ S	175° 50′ 43″ E	WELT A031312–A031316, A031331	20 (1), 21 (1), 25 (2), 27 (1), 28 (13), 29 (1), 70 (1)	0.5842 ± 0.1270	0.0084 ± 0.0056

Table 2.1 (Continued)

Map ID (Fig 2.2)	**Site**	**N**	**Latitude**	**Longitude**	**Herbarium number of exemplars**	**Haplotypes present (n)**	**Gene diversity *h* ±** S.D.	**Nucleotide diversity *π* ± S.D.**
12	Otanga, Bay of Plenty	25	37° 32′ 59″ S	178° 09′ 41″ E	-	14 (3), 16 (3), 21 (2), 22 (1), 26 (3), 28 (9), 39 (3), 43 (1)	0.8367 ± 0.0539	0.0152 ± 0.0090
13	Maraehako Bay, Bay of Plenty	25	37° 40′ 20″ S	177° 47′ 48″ E	-	14 (15), 15 (3), 16 (2), 17 (1), 18 (1), 19 (2), 23 (2), 38 (2), 71 (1), 72 (1)	0.7425 ± 0.0813	0.0065 ± 0.0045
14	Maketu, Bay of Plenty	24	37° 44′ 41″ S	176° 28′ 13″ E	WELT A031333	58 (21), 59 (1), 60 (2)	0.2355 ± 0.1093	0.0017 ± 0.0019
15	Opape, Bay of Plenty	21	37° 58′ 20″ S	177° 25′ 15″ E	-	01 (1), 14 (19), 69 (1),	0.1857 ± 0.1102	0.0021 ± 0.0021
16	Horoera, East Cape	24	37° 38′ 17″ S	178° 28′ 29″ E	WELT A031310	21 (19), 52 (1), 61 (1), 62 (1), 64 (2)	0.3768 ± 0.1224	0.0050 ± 0.0038
17	Whale Bay, Waikato	20	37° 49′ 22″ S	174° 48′ 02″ E	-	01 (17), 06 (1), 35 (1), 37 (1)	0.2842 ± 0.1284	0.0013 ± 0.0016
18	Kiritehere, Waikato	20	38° 19′ 31″ S	174° 42′ 07″ E	WELT A024176	01 (17), 02 (1), 04 (1), 05 (1)	0.2842 ± 0.1284	0.0022 ± 0.0022
19	Kaiti Beach, Gisborne	23	38° 40′ 56″ S	178° 01′ 46″ E	WELT A031332	21 (21), 63 (1), 67 (1)	0.1700 ± 0.1025	0.0054 ± 0.0040
20	Waipatiki, Hawke Bay	23	39° 18′ 19″ S	176° 58′ 29″ E	WELT A031324, A031325	08 (21), 11 (2)	0.1660 ± 0.0976	0.0015 ± 0.0017
21	Ahuriri St, Port Napier	15	39° 28′ 42″ S	176° 54′ 13″ E	WELT A031323	08 (15)	0	0
22	Pihama, South Taranaki	21	39° 31′ 17″ S	173° 54′ 55″ E	WELT A031321, A031322, A031330	01 (18), 07 (3)	0.2571 ± 0.1104	0.0011 ± 0.0015
23	Pukerua Bay, Wellington	20	41° 01′ 29″ S	174° 54′ 06″ E	WELT A031309	01 (20)	0	0

Table 2.1 (Continued)

Map ID (Fig 2.2)	**Site**	**N**	**Latitude**	**Longitude**	**Herbarium number of exemplars**	**Haplotypes present (n)**	**Gene diversity *h* ±** S.D.	**Nucleotide diversity *π* ±** S.D**.**
24	Riversdale, Wairarapa	15	41° 06′ 25″ S	176° 04′ 11″ E	WELT A031308	01 (12), 12 (3)	0.3429 ± 0.1278	0.0046 ± 0.0036
25	Wharanui, Kaikoura	23	41° 55′ 20″ S	174° 06′ 00″ E	*	01 (23)	0	0
26	Waipapa Point, Kaikoura	17	42° 12′ 41″ S	173° 52′ 29″ E	*	01 (17)	0	0
27	Ripapa Island, Banks Peninsula	24	43° 37′ 07″ S	172° 45′ 13″ E	*	01 (24)	0	0
28	Cape Three Points, Banks Peninsula	16	43° 49′ 50″ S	172° 54′ 30″ E	*	01 (16)	0	0
29	Wharekauri, Chatham Islands	10	43° 42′ 18″ S	176° 34′ 55″ W	WELT ASG257	24 (10)	0	0
30	Point Dorset, Chatham Islands	24	43° 49′ 34″ S	176° 42′ 21″ W	WELT A031328	01 (16), 03 (6), 24 (2)	0.5072 ± 0.0929	0.0060 ± 0.0043
31	Waitangi, Chatham Islands	22	43° 56′ 42″ S	176° 33′ 42″ W	WELT A031329	01 (6), 08 (1), 09 (3), 10 (5), 24 (4), 39 (3)	0.8398 ± 0.0368	0.0206 ± 0.0117
32	Manukau Reef, Chatham Islands	5	37° 44′ 41″ S	176° 28′ 13 ″ W	WELT A024213	08 (5)	0	0

* Vouchers for these sites were lost as a result of a chemical spillage accident.

2.4 Results

Haplotypic diversity and population structure

SSCP and subsequent sequencing identified 67 haplotypes (Genbank numbers HM070070 to HM070166; Appendix 1). While SSCP might underestimate haplotypic diversity, sequencing of a large proportion of samples should reduce the likelihood of significant underestimation of diversity. A statistical parsimony network (Fig. 2.2b) grouped haplotypes into three clusters, each separated by five or more steps. The largest cluster (labelled (1) in Fig. 2.2b) joined 52 haplotypes. These are considered to arise from the *C. maschalocarpum* mitochondrial lineage. A second cluster (labelled (2) in Fig. 2.2b) joined four closely related haplotypes from North Cape (pop. 2 in Fig. 2.2a) and Wekarua (pop. 5). This cluster was five steps removed from the nearest *C. maschalocarpum* haplotype and six steps removed from the nearest haplotype of the sister species *C. angustifolium*. It is unclear whether these haplotypes arise from the *C. angustifolium* or *C. maschalocarpum* maternal lineage. A third cluster (labelled (3) in Fig. 2.2b), joined eleven haplotypes from *C. angustifolium* and putative hybrid specimens, and was six steps removed from the nearest *C. maschalocarpum* haplotype. Haplotypes from *Carpophyllum angustifolium* and any specimens considered to have hybrid origin (including all of pops. 1, 2 and 5), are not included in population genetic analyses. Three haplotypes (15, 23 and 36) were found only in specimens identified morphologically as either *C. angustifolium* or hybrids, but which differed from haplotypes found in specimens morphologically identified as *C. maschalocarpum* by only one mutational step. These are considered to have arisen in the *C. maschalocarpum* mitochondrial lineage and are included in subsequent analyses.

Geographically restricted haplotypes were common (Fig. 2.2a). Forty-one *C. maschalocarpum* haplotypes were private (sampled from a single population only). At two sites, Matai Bay (pop. 3 in Fig 2.2a) and Maketu (pop. 14) all haplotypes sampled were private. At Ahipara (pop. 4) both haplotypes sampled were private aside from a single specimen of haplotype 52 also sampled at Horoera (pop. 16). Haplotypes from Bay of Plenty and Hawke Bay populations were not shared with neighbouring regions, apart from one Hawke Bay haplotype that was also sampled in the Chatham Islands. Four haplotypes were endemic to the Chatham Islands. Population differentiation was moderate to strong between populations with shared haplotypes (Table 2.2).

A small number of haplotypes were widely distributed (Fig. 2.2a). Haplotype 01 was sampled in Northland (pops. 9 and 10), Bay of Plenty (pop. 15), Riversdale (pop. 24), the west coast of the North Island (all populations except 4), the Chatham Islands (pops. 31 and 30) and in all South Island populations. Haplotype 01 was the only haplotype sampled from the South Island. Three haplotypes were shared between Northland and East Coast populations. Haplotype 21 was sampled frequently in both regions. Also shared were haplotype 52 (pop. 4 and a single sample from pop. 16) and haplotype 67 (pops. 1 and 19). Haplotype 67 appears to be derived from the *C. angustifolium* lineage and is 14 mutational steps removed from the closest *C. maschalocarpum* haplotype. Three Chatham Island haplotypes were shared with mainland New Zealand: haplotype 39 was sampled in Otanga (pop. 12) and Waitangi (pop. 31) only; haplotype 08 was shared with two Hawke Bay sites (pops. 20 and 21); and the common southern haplotype 01 was sampled at two Chatham Islands sites (pops. 30 and 31).

Haplotype and nucleotide diversity (Table 2.1) was highest in three Bay of Plenty populations: Sailors Grave, Otanga and Maraehako Bay (pops. 11, 12 and 13 respectively), and in two Chatham Island populations: Waitangi and Point Dorset (pops. 31 and 30). Diversity was lowest in the South Island (a single haplotype sampled in four populations).

Biogeographic regions

SAMOVA analyses reached maximum F_{ct} at K = 14 (F_{ct} = 0. 811425). At K = 14 populations were partitioned into five groups and nine singletons (Fig. 2.3). The largest group of ten populations, dominated by the common haplotype 01, included all South Island sites, Riversdale (pop. 24), Point Dorset (pop. 30) and all west coast North Island

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Fig. 2.2. (opposite) (a) Distribution of mitochondrial haplotypes of *Carpophyllum maschalocarpum*. Area of pie charts is proportional to sample size. Locations are described in Table 2.1. (b) Statistical parsimony network inferred by TCS software (95% confidence limits). Area of circles is proportional to number of haplotypes sampled. Small black circles represent extinct or unsampled haplotypes. Dashed line indicates an alternative but unlikely connection.

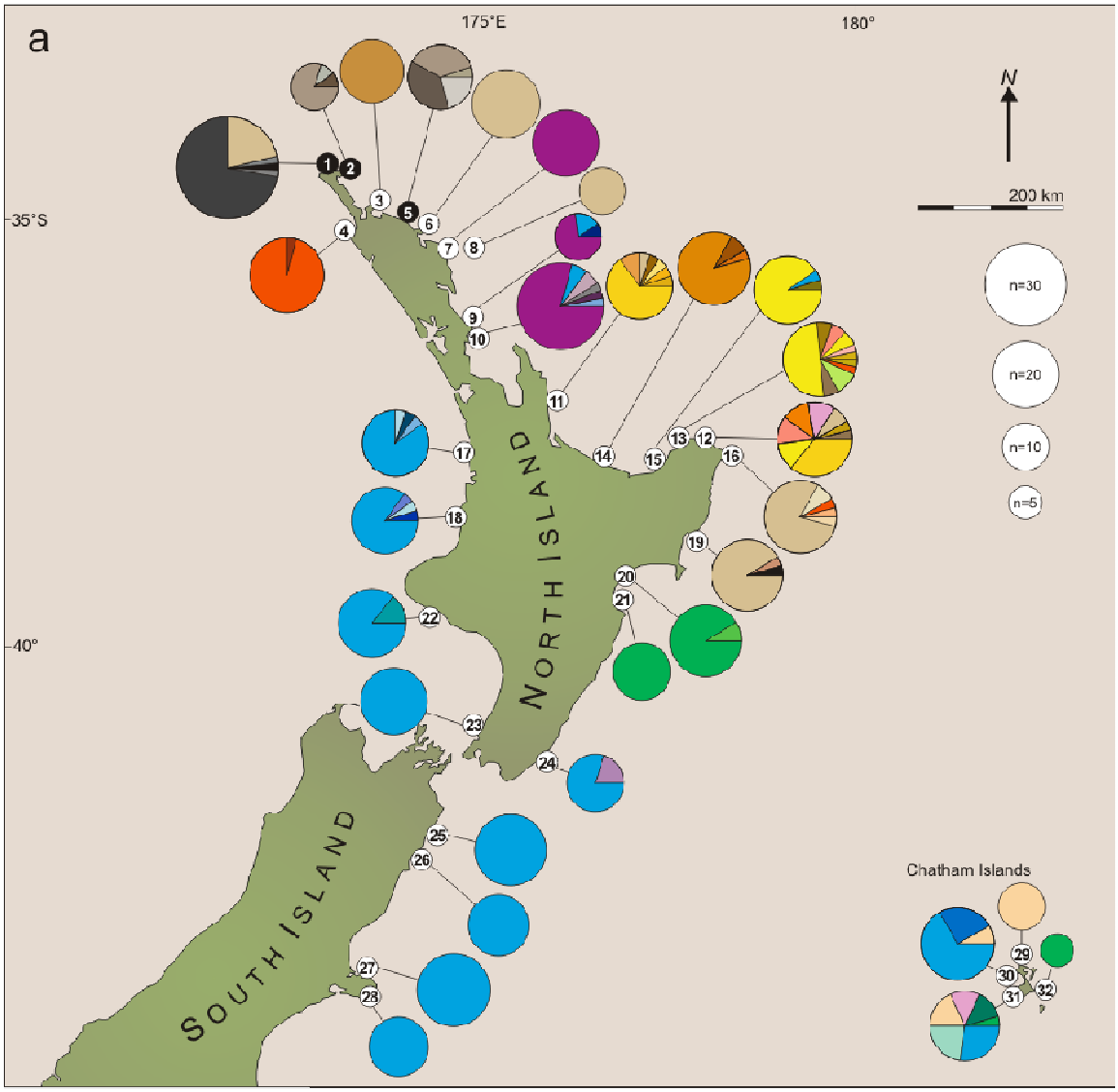
a
175°E
180°
35°S
40°
N
200 km
n=30
n=20
n=10
n=5
NORTH ISLAND
SOUTH ISLAND
Chatham Islands
b
n=2
n=3
n=5
n=15
(1) C. maschalocarpum
(2)
(3) C. angustifolium

Table 2.2. Pairwise Φ_{st} estimates from mtDNA spacer data (below diagonal) and minimum round coast distances (km, above diagonal) for Bay of Plenty populations of *Carpophyllum maschalocarpum*. Significance shown as: ** = $p \leq 0.01$ and * = $p \leq 0.05$ after sequential Bonferroni correction for multiple tests. N. S. = No significant difference. Population numbers refer to Table 2.1/Fig. 2.2.

	Sailors Grave	Maketu	Opape	Maraehako Bay	Otanga
Sailors Grave (pop. 11)	-	103	179	190	215
Maketu (pop. 14)	0.54931**	-	87	117	151
Opape (pop. 15)	0.46280**	0.84906**	-	48	86
Maraehako Bay (pop. 13)	0.42232**	0.68024**	N. S.	-	38
Otanga (pop. 12)	0.12573*	0.26357**	0.11334*	0.12548**	-

sites except Ahipara (pop. 4). This group was unchanged for all iterations of K > 2. Chatham Island populations did not group together despite their proximity. Only Waitangi and Manukau Reef (pops. 31 and 32) were retained in the same group above K=3 and these two populations separated into singletons at K = 13 and above. Wharekauri (pop. 29) grouped with Northland populations and two populations south of East Cape, then formed a singleton at K = 8. Point Dorset (pop. 30) remained grouped with South Island and southern North Island populations.

AMOVA based on biogeographic regions and provinces proposed by Shears *et al.* (2008) explained considerably less between-group variance than the maximum obtained by SAMOVA (Table 2.3). The low haplotype diversity in South Island and southern North Island samples precluded showing the North/South disjunction found in other studies. A biogeographic break at East Cape was not shown in *C. maschalocarpum*, rather the data showed connectivity between two populations south of East Cape (pops. 16 and 19) and Northland.

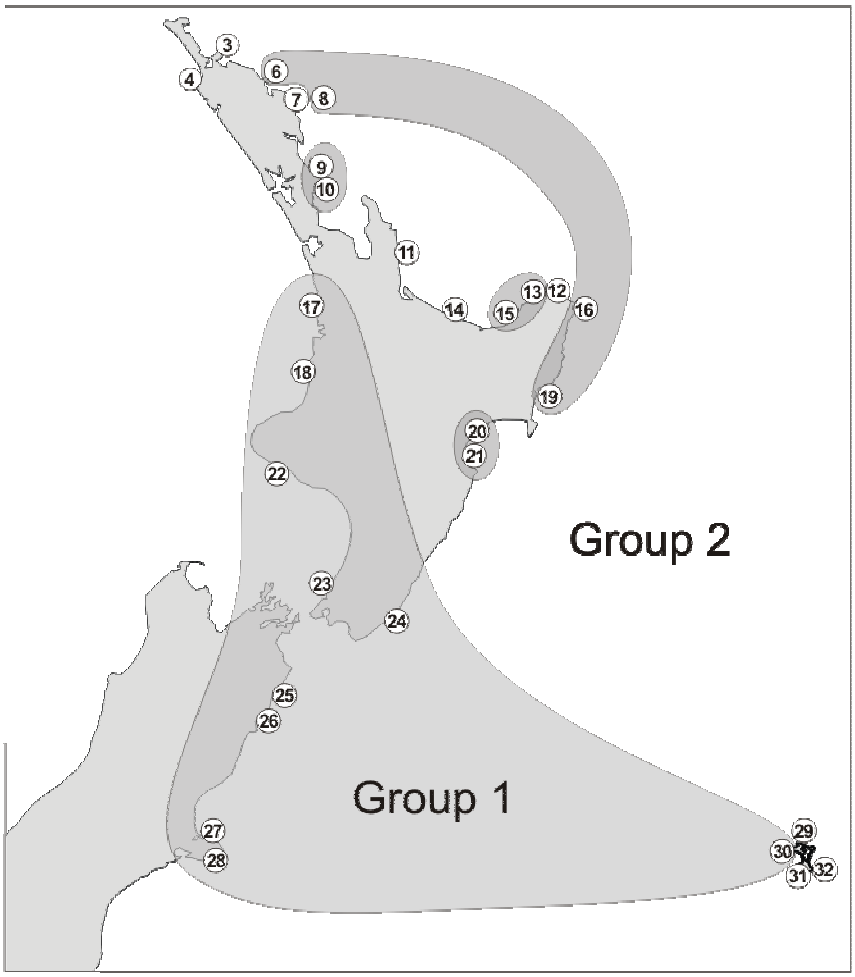

Fig. 2.3. Grouping of populations of *Carpophyllum maschalocarpum* inferred by SAMOVA to maximise F_{ct}. Shaded areas show groups inferred with K = 14. Division into two groups shown was used for mismatch distribution analyses (Fig. 2.4).

Departures from neutrality

Neutrality tests compare estimates of θ using different parameters. Data from demographically stable populations using neutral markers should estimate similar values of θ. Discrepancies in estimates of θ indicate departure from neutrality, with different tests sensitive to different underlying factors (Fu 1997).

Tajima's D value (Tajima 1989) was negative (D = −0.7109), which suggests population expansion, but not significant (P = 0.268). Fu and Li's D* test (Fu & Li 1993) was significant (D* = −2.3973, P = 0.016). Fu and Li's F* test (Fu & Li 1993) was also significant (F* = −2.1275, P = 0.024). Fu's F_s value, which is sensitive to population

Table 2.3: Hierarchical partitioning of molecular variance estimated by AMOVA from *Carpophyllum maschalocarpum* mtDNA spacer data. (a) Variation explained by grouping in biogeographic regions proposed by Shears *et al.* (2008); and (b) groupings at maximum F_{ct} inferred by SAMOVA.

a. Groupings by Biogeographic Regions

Source of variation	*d.f.*	*Sum of squares*	*Variance*	*Percentage of variation*
Among groups	6	603.55	V_a= 1.026	33.05
Among populations	22	626.90	V_b= 1.402	45.14
Within populations	552	373.84	V_c= 0.677	21.81
Fixation indices: F_{sc}= 0.674, F_{st}=0.782, F_{ct}=0.331				

b. Groupings by SAMOVA (Fig. 2.3)

Source of variation	*d.f.*	*Sum of squares*	*Variance*	*Percentage of variation*
Among groups	13	804.64	V_a= 1.652	81.14
Among populations	15	6.94	V_b= 0.004	0.21
Within populations	550	208.93	V_c= 0.380	18.65
Fixation indices: F_{sc}= 0.011, F_{st}=0.814, F_{ct}=0.811				

expansion (Fu 1997), was strongly negative, and highly significant (F_s = −25.2996, P = 0.0002). F_s tests were also carried out on data split into two groups as indicated by SAMOVA. Group 1 (North Island west coast/South Island/Riversdale/Port Dorset) and Group 2 (all other samples) both gave negative and significant F_s values (F_s = −11.7910, P < 0.0001 and F_s = −23.1464, P = 0.0002 respectively).

Isolation by distance

Correlation between geographic distance and genetic distance was low but significant (r^2 = 0.10388, P < 0.0001). Recalculating distances to exclude east-west distances gave

similar results ($r^2 = 0.10820$, $P < 0.0001$). Stronger correlation ($r^2 = 0.20768$, $P < 0.0001$) was obtained by excluding Chatham Islands samples from the analysis.

Mismatch analysis

Mismatch analyses showed an approximation to the expected distribution under a model of sudden expansion (Fig. 2.4). Separation of data into two groups as indicated by SAMOVA resulted in two distinct distributions: an L-shaped distribution in Group 1 (North Island west coast/South Island/Riversdale/Port Dorset) and a unimodal distribution in Group 2 (all other populations). No group showed significant departure from the expected distribution under a model of sudden expansion (Group 1: $P = 0.355$; Group 2: $P = 0.881$; combined populations: $P = 0.624$). M (the parameter for gene flow where $M = 2Nm$ and N is the deme size and m is the migration rate) is low in Group 1 ($M = 0.035$) and high in Group 2 ($M = 12.156$).

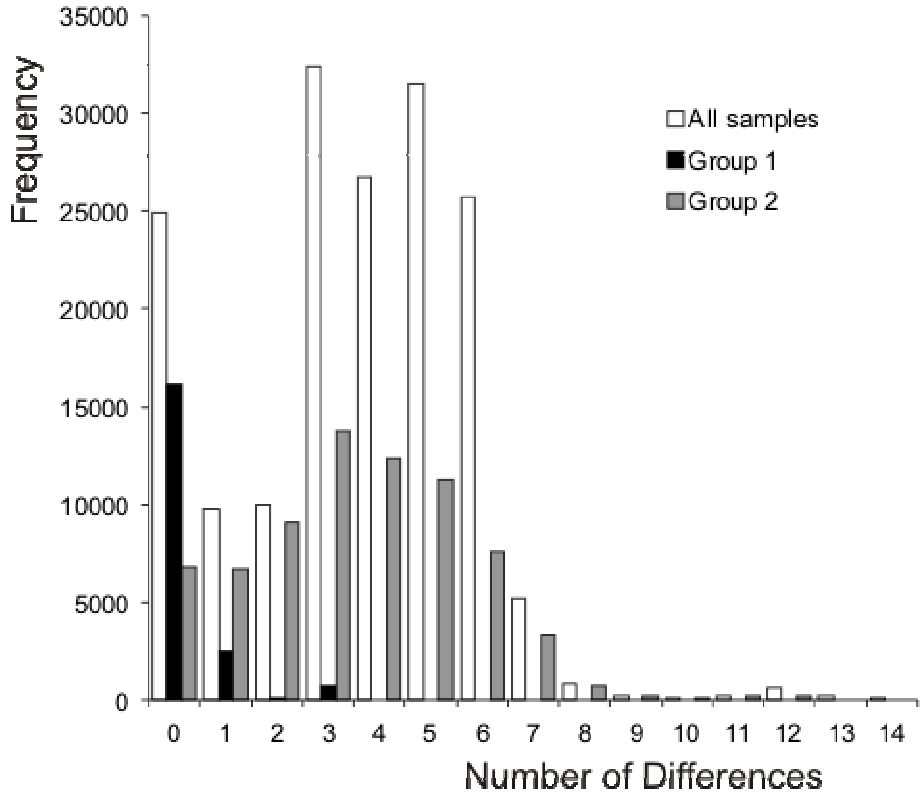

Fig. 2.4 Mismatch distributions: Group 1 (South Island/North Island West Coast, Riversdale and Point Dorset populations): black bars; Group 2 (all other populations): Grey bars; Combined populations: open bars.

2.5 Discussion

Patterns of gene flow that arise from multiple dispersal modes are complex as dispersal patterns might overlap, especially when each mode is facilitated by different physical processes and influenced by separate historical events. In these data three main patterns are evident: (1) Often high genetic differentiation between populations, including adjacent populations; (2) Connectivity between some distant populations, with only slight overall isolation by distance; and (3) Generally low southern diversity, with the notable exception of the Chatham Islands. These patterns are consistent with fucalean morphological and life history characteristics that limit dispersal at the gamete/zygote stage, but facilitate occasional long distance dispersal (Thiel & Haye 2006). Historical climatic changes also appear to have influenced the distribution of *C. maschalocarpum*.

Genetic differentiation

Genetic differentiation between many populations of *Carpophyllum maschalocarpum* was in the order of that expected for fucalean algae (Coleman & Brawley 2005b, Hoarau *et al.* 2007, Tatarenkov *et al.* 2007, Coleman & Kelaher 2009). Strong genetic differentiation was found between some adjacent populations that were separated by habitat discontinuities, even where distances were relatively short (for example, Cape Wiwiki (pop. 6) and Bland Bay (pop. 7), 39 kilometres apart but separated by the estuarine Bay of Islands). These discontinuities would be expected to provide a barrier to gametic or zygotic dispersal, but not to dispersal of floating thalli. Despite the abundance of floating thalli (Kingsford 1992), it appears that this mode of dispersal seldom contributes to gene flow.

Explanations for limited gene flow by floating thalli include dioecy, small target areas for immigrant floating algae (e.g., Maketu a short, 1 km, rocky peninsula flanked by long stretches of soft sediment coast) and density blocking (Deysher & Norton 1982, Hewitt 1996, Austerlitz *et al.* 2000), where relatively rare immigrants seldom contribute alleles to established stands of algae. High regional haplotype endemism might reflect isolation of water masses, for example limited current transport into embayments such as Hawke Bay and Bay of Plenty (Ridgway 1960, 1962, Stanton *et al.* 1997). Direct studies of transport of floating algae have not been undertaken in New Zealand. A high prevalence of westerly winds might mean surface floating species are usually exported and lost from nearshore habitat.

Long distance dispersal

Shared haplotypes in Northland and North Island east coast populations, and North Island and Chatham Island populations, show some haplotypes have dispersed over long distances. Haplotype 21 is common in Northland and East Coast populations, and it is improbable that this haplotype arose independently in two areas. Further, haplotype 67, which clusters with *C. angustifolium*, was only sampled at Hooper Point (pop. 1) and Kaiti Beach (pop. 19). Kaiti is well outside of the range of *C. angustifolium*, which does not extend south of East Cape (Morton & Miller 1968 and my observations). It appears this haplotype entered the *C. maschalocarpum* gene pool by hybridisation and dispersed to southern populations (Hodge *et al.* 2010). This connection, and the connection between the mainland and the Chatham Islands, is congruent with contemporary currents and surface winds. Northland and the East Coast are connected by the East Auckland/East Cape Current. This current does not extend to the inner area of the Bay of Plenty (Heath 1985, Stanton *et al*. 1997) and my data suggest Bay of Plenty populations occupy an isolated eddy. Despite their spatial separation, the outer populations (Sailors Grave-pop. 11 and Otanga-pop. 12) are more connected to each other than to inner populations. Haplotype 28 occurs in high frequencies in these populations, but was absent from the other Bay of Plenty populations.

Four lineages are present in Chatham Island populations, with haplotypes in each lineage the same or closely related to haplotypes found in North and South Island populations, suggesting at least four dispersal events between mainland New Zealand and the Chatham Islands. High connectivity between the mainland and the Chatham Islands is surprising as many Chatham Island marine algae are endemic (Nelson *et al.* 1991, Schiel *et al.* 1995), several common mainland species are absent (e.g., the non-buoyant laminarian species *Ecklonia radiata* and *Lessonia variegata*) (Nelson *et al.* 1991, Nelson 1994), and studies in other organisms have shown genetic isolation in Chatham Island populations (Goldstien *et al.* 2009). My data suggest a very different pattern for algae capable of dispersal by floating, with prevailing westerly winds and currents facilitating eastward dispersal from the mainland to the Chatham Islands (Chiswell & Booth 2008). Removing the East-West component of geographic distance between populations did not affect correlation with genetic connectivity. These data

suggest wind and currents facilitate long distance transport to the Chatham Islands, while North to South movement proceeds mostly by stepping stone dispersal.

The North/South disjunction found in other New Zealand studies (Apte & Gardner 2002, Sponer & Roy 2002, Waters & Roy 2004, Ayres & Waters 2005, Goldstien *et al.* 2006) was evident in the sudden reduction in haplotype diversity in southern populations, but this occurs further north than the break found in previous studies. A barrier to dispersal by upwelling or current transport of propagules offshore around the north of the South Island has been suggested, but evidence for this is equivocal (Ross *et al.* 2009). I suggest historical climate change explains the pattern in these data better than a dispersal barrier and discuss this below. Bioregions proposed by Shears *et al.* (2008) are partly reflected in the distribution of *C. maschalocarpum* haplotypes (Table 2.3). In particular, disjunctions just south of Ahipara and south of Hawke Bay (pops. 4 and 20/21, Fig. 2.3) in these data are congruent with boundaries of bioregions in Shears *et al.* (2008). Conversely, the connection between Northland and the East coast, and the relative isolation of the Bay of Plenty and Hawke Bay populations of *C. maschalocarpum* is not evident in data obtained by Shears *et al.* (2008). It is not clear whether this arises from sampling differences, the stochastic nature of floating dispersal or factors unique to *C. maschalocarpum*.

This pattern of population differentiation and connectivity is consistent with a species that is generally restricted in dispersal, but with intermittent long distance dispersal by floating (Thiel & Haye 2006). Leapfrog dispersal, where more distant populations show greater connectivity than adjacent populations, is a predicted outcome of intermittent floating dispersal (Thiel & Haye 2006) and has been found in a variety of organisms (Snyder & Gooch 1973, Bockelmann *et al.* 2003, Colson & Hughes 2004). In *C. maschalocarpum*, leapfrog dispersal appears to be overlaid on patterns of low dispersal or stepping stone dispersal.

Low southern diversity and post-LGM recolonisation

The abundant centre model (Eckert *et al.* 2008) predicts greatest genetic diversity in the centre of a species' range, with a decline toward peripheral populations. My data are broadly consistent with this model. Diversity is highest in the Bay of Plenty in North-East New Zealand and generally declines with distance from this region. But there are two departures from this pattern: (1) Haplotypically diverse populations in the far north

(pops. 1, 2 & 5); and (2) high haplotypic diversity in the Chatham Islands. I invoke both contemporary factors and historical factors to explain these departures.

Far north diversity appears to have hybrid origin, with gene flow between *C. angustifolium* and *C. maschalocarpum*. Far north populations have been identified as either species by different workers (e.g., *C. maschalocarpum* by Shears & Babcock 2007, *C. angustifolium* by Hay & Grant 2003). Hybrids have been reported between *Carpophyllum* species based on intermediate morphology (Lindauer *et al.* 1961, Dromgoole 1973, Shears & Babcock 2007) and molecular data (Hodge *et al.* 2010), and hybridisation in fucalean species has been widely reported (Scott & Hardy 1994, Kim *et al.* 1997, Coyer *et al.* 2002, Engel *et al.* 2005).

The pattern of high northern diversity and low southern diversity suggests relatively recent southward expansion with warming of the ocean waters following the Last Glacial Maximum (LGM). Water temperature is a major determinant of species range in many macroalgae (Lüning 1990, Adey & Steneck 2001). Low diversity at high latitudes is often reported in species that have recolonised high latitudes during post-glacial warming (Coyer *et al.* 2003, Marko 2004, Hoarau *et al.* 2007, Maggs *et al.* 2008, Fraser *et al.* 2009). The present day distribution of *Carpophyllum maschalocarpum* extends south to Fiordland (46^{o} S) on the South Island's west coast (Nelson *et al.* 2002), where warm currents push in from the Tasman Sea, but only as far south as Banks Peninsula (43^{o} 45′ S) on the east coast, where cold currents push up from the south (Adams 1994). This southern limit is correlated with the 15°C Sea Surface Temperature (SST) isobar for the warmest month of the year (Fig. 2.1). While there are few direct data on thermal tolerances in *C. maschalocarpum*, the correlation between southern limit and SST on both coasts suggests this is a low temperature limit. Records of specimens of *C. maschalocarpum* from driftlines on Stewart Island and Sub-Antarctic Islands (Dromgoole 1973) show thalli are transported further south, but populations have not established.

Climate reconstructions of the LGM (Barrows & Juggins 2005) place the 15°C warmest month SST isobar north of Taranaki on the New Zealand's west coast and north of Hawke Bay on the east coast (Fig. 2.1). These temperatures would have restricted *C. maschalocarpum* to the northern half of the North Island during the LGM. Recolonisation southwards down the North Island west coast, through the reopened

Cook Strait (Lewis *et al.* 1994) and down the east coast of the South Island would explain the low diversity and the different patterns of population expansion indicated by mismatch distributions.

Genetic diversity in northern hemisphere algae has been shown to retain the signature of past climate events – in particular the last glaciation, which removed algae from higher latitudes (reviewed by Maggs *et al.* 2008). Similar patterns have been shown for marine species in the southern hemisphere (Hickey *et al.* 2009) including algae (Fraser *et al.* 2009). The distribution of haplotype diversity in my data is strikingly similar to patterns of the same marker in *Fucus serratus* from Europe (Hoarau *et al.* 2007), and is similar to distributions interpreted as showing post-glacial range expansion for a number of species (Hewitt 1999, Provan *et al.* 2005, Maggs *et al.* 2008).

I offer two explanations for the presence of four endemic haplotypes in the Chatham Islands. Either this is further evidence for the ease of dispersal to the Chatham Islands, with these haplotypes either extinct or not sampled on the main islands of New Zealand, and with dispersal pathways facilitating co-dispersal of closely related haplotypes (e.g., haplotypes 08, 09 and 10); or Chatham Island populations of *C. maschalocarpum* have persisted for a considerable time, allowing the accumulation of unique mutations. At present summer water temperatures around the Chatham Islands are elevated as seasonal north/south movement of the sub-tropical convergence is constrained by an area of shallow bathymetry, the Chatham Rise (Stanton 1997). Paleoclimate studies indicate warm summer water temperatures around the Chatham Islands might have persisted during the LGM (Nelson *et al.* 2000, Fenner *et al.* 1992). Hoarau *et al.* (2007) estimated a mutation rate of 2% to 3.4%/Myr for this mitochondrial spacer in *Fucus*. While I would be cautious in applying this rate directly to *C. maschalocarpum*, a much faster mutation rate would be required if four endemic haplotypes arose following post-LGM recolonisation.

The pattern of local population differentiation, but with specific pathways for gene flow between distant populations, overlaid on a historical pattern of retreat and recolonisation is consistent with the intermittent and stochastic dynamics of long distance dispersal by floating (Thiel & Haye 2006), and might be a general pattern for buoyant algae (Fraser *et al.* 2009) and organisms rafting on these algae. An important finding is that connectivity between distant populations can be high, even where local

connectivity is often low. This highlights the importance of sampling across species' ranges when investigating dispersal, as large scale processes driving long distance dispersal might not be apparent in studies at local scales.

2.6 Acknowledgements

I thank Wendy Nelson, Kate Neill, Tracy Farr, Nick Shears, Peter Martin, Erasmo Macaya, Fiona Hodge and Chatham Islands Department of Conservation staff for help with collections, and Peter Ritchie for help with early drafts of the manuscript. Dr Martin Thiel provided many useful comments. I also thank the New Zealand Department of Conservation for funding assistance.

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Chapter 3
Patterns of hybridisation in *Carpophyllum* (Phaeophyceae, Fucales)

3. 1 Abstract

Hybrid zones can vary in time and space, even where the same species are involved. *Carpophyllum maschalocarpum* (Phaeophyceae, Fucales) and *C. angustifolium* hybridise in sites in north-eastern New Zealand where the two species are broadly sympatric, with *C. angustifolium* limited to more wave-exposed habitats. Patterns of hybridisation were investigated by sampling across this zone of sympatry, from the northern tip of New Zealand to near the south-eastern limit of *C. angustifolium*. Specimens were assigned to species using morphological criteria. Hybrids have an intermediate morphology, specifically, a stipe width intermediate between the two parental species. Ribotypes and haplotypes were determined for ITS2 and a mtDNA spacer. In the southern range of *C. angustifolium,* most hybrids have heterozygous ITS2 and ribotypes associated with both parent species, and are interpreted as F1 hybrids, with a low frequency of backcrossing. Mitochondrial haplotypes were species specific in southern populations, but one haplotype found only in *C. angustifolium* and hybrids clustered with haplotypes found only in *C. maschalocarpum,* suggesting previous mitochondrial introgression in *C. angustifolium*. In the northern range of *C. angustifolium,* shared ITS ribotypes suggest backcrosses are more common, and all specimens in some populations resemble hybrids with parent plants apparently absent. Unlike *Fucus,* where asymmetrical hybridisation results from different mating systems between sister species, *Carpophyllum* is dioecious and either species can act as the maternal parent in the production of hybrids. High frequencies of *C. angustifolium*-associated mtDNA haplotypes in hybrids probably arise from imbalances in population sizes of parent species.

Abbreviations:
CTAB, Cetyltrimethylammonium Bromide; ITS, Internal Transcribed Spacer; SSCP, Single Stranded Conformational Polymorphism; TBE, Tris-Borate-EDTA.

3.2 Introduction

Natural interspecific hybridisation events are natural experiments that can provide insight into evolutionary processes that lead to divergence and speciation (Hewitt 1988). Hybridisation can enable gene flow between lineages (Arnold 2006, Mallet 2005), reversing species divergence, and can produce novel phenotypes and initiate new species (Riesberg 1997, 2006). Gametic contact between potentially hybridising species is usually restricted in space creating hybrid zones, where genetically distinct populations overlap (Barton & Hewitt 1985, 1989, Arnold 1993, Gardner 1997). Hybrid zones can arise from secondary contact following natural range expansion (Hewitt 2001, Neiva *et al.* 2010) or anthropogenic introductions (Coyer *et al.* 2002a, 2006b, Rhymer & Simberloff 1996). Early studies assumed hybrid zones were an ephemeral stage in species divergence, occurring when development of reproductive barriers was incomplete (Howard *et al.* 2003). Subsequent theoretical work suggested hybrid zones might be stable, either maintained by a balance of selection against hybrids and dispersal into the zone (Bigelow 1965, Barton & Hewitt 1985), or by greater fitness of hybrids in some habitats (Moore 1977). Recently, a more complex picture has emerged, with variable hybrid genotypes (Arnold & Hodges 1995) interacting with patchy and dynamic environmental conditions to create a mosaic hybrid zone, with multiple potential evolutionary outcomes (Butlin *et al.* 2008, Jiggins & Mallet 2000, Schwenk *et al.* 2008). Consequently, all hybrid zones are not all alike, even where the same species are involved (Riginos & Cunningham 2005). In the marine environment, hybridisation is probably common (Gardner 1997) but has been extensively studied in only a few systems (Rawson & Hilbish 1998, Rawson *et al.*1999, Coyer *et al*. 2004, Lewis 1996, Coyer *et al*. 2007). Fucalean brown algae are a useful system for studying hybridisation as they have a simple life history, with no alternation of generations and predominantly sexual reproduction (Chapman 1995). Various scenarios lead to hybridisation in *Fucus*: anthropogenic secondary contact (Coyer *et al.* 2002a, 2006b, 2007); marginal habitat (Wallace *et al.* 2004, Coyer *et al.* 2006a); and natural sympatry (Billard *et al.* 2005, Engel *et al.* 2005, Neiva *et al.* 2010). Species integrity in *Fucus* appears to be maintained by selection against hybrids (Coyer *et al.* 2007) and contrasting mating systems (Billard *et al.* 2005, Engel *et al.* 2005), but some populations show extensive

introgression (Neiva *et al*. 2010), and possible incipient hybrid speciation (Coyer *et al.* 2010 in press).

In New Zealand, species of the endemic genus *Carpophyllum* Greville typically dominate the shallow rocky sub-tidal (Adams 1994, Shears & Babcock 2007). Hybridisation, inferred from observations of morphologically intermediate specimens where two species are in contact, has been reported between *Carpophyllum maschalocarpum* Turner (Greville) and the three other species in the genus (Lindauer *et al.* 1961, Dromgoole 1973). *Carpophyllum maschalocarpum* is a common species, growing in dense stands around the Chatham Islands and most of mainland New Zealand (Adams 1994). A sister species, *Carpophyllum angustifolium* J. Agardh, has a more restricted range, extending from the northern tip of New Zealand's North Island to just short of East Cape (Adams 1994). These species are broadly sympatric, but *C. angustifolium* is absent from sheltered areas and replaces *C. maschalocarpum* in sites exposed to strong wave action (Shears & Babcock 2007). Morphologically, they can be distinguished by their main axes, broad and flattened in *C. maschalocarpum* and narrow and terete in *C. angustifolium* (Adams 1994).

Hodge *et al.* (2010, Appendix 8) combined ribosomal DNA and morphological data to show that hybridisation occurred between *Carpophyllum maschalocarpum* and *C. angustifolium* J. Agardh from two populations in the eastern Bay of Plenty, New Zealand. Specimens were morphologically assigned to species, primarily by stipe width (stipe < 2mm = *C. angustifolium*, stipe 2–5 mm = hybrid, stipe >5mm = *C. maschalocarpum*). Parent species had homozygous ITS2, with one of two species-specific ribotypes. In contrast, ITS2 electropherograms from morphologically intermediate specimens showed a pattern of double peaks consistent with the presence of both ribotypes in each individual. A small number of individuals had morphological characters consistent with *C. angustifolium*, but had hybrid (heterozygous) ITS2 sequences. Hodge *et al.* (2010) suggested these were backcrosses, possibly indicating asymmetrical introgression.

Hybrids have also been reported from Auckland localities (Dromgoole 1973) and Shears & Babcock (2007) reported what they considered to be a slender form of *C. maschalocarpum* from sites in the far north of New Zealand. These specimens somewhat resembled *C. angustifolium* and were found in wave

exposed locations, but where the authors considered *C. angustifolium* was absent. As hybrids collected from the Bay of Plenty (Hodge *et al.* 2010) could be described as resembling "slender" *C. maschalocarpum*, I consider it likely that specimens observed by Shears & Babcock (2007) are also hybrids. Hay & Grant (2003) reported only *C. angustifolium* from the northern tip of the North Island, whereas Shears & Babcock (2004) reported only *C. maschalocarpum*. This situation is unsatisfactory and the taxonomic status of these populations needs to be clarified.

Here I investigate evidence for hybridisation across the range of *C. angustifolium*, by comparing populations from Northland and the Bay of Plenty, New Zealand. In addition to ribotyping by ITS2, a mitochondrial intergenic spacer (Hoarau *et al.* 2007) was used to determine the maternal species of putative hybrid specimens. The utility of this spacer was shown in an extensive survey of *C. maschalocarpum* and *C. angustifolium* (Chapter 2) where haplotypes formed three clusters, two associated with *C. angustifolium* and one associated with *C. maschalocarpum*. The aim of this study is to determine if other hybridising populations of *Carpophyllum* follow the same patterns as those investigated by Hodge *et al.* (2010), and how species boundaries are maintained in *Carpophyllum*.

3.3 Materials and Methods

Sampling

Carpophyllum maschalocarpum specimens were collected from around New Zealand, and *C. angustifolium* and putative *C. maschalocarpum* × *C. angustifolium* hybrids from northern New Zealand (Table 3.1 and Fig. 3.2). More intensive sampling of parent species and putative hybrids, identified by morphological characters, was done at four sites: the Poor Knights Islands (N=36) and Leigh (N=29) in Northland, and Maraehako Bay (N=75) and Otanga (N=66) in the eastern Bay of Plenty. In addition, morphologically intermediate thalli were collected at Wekarua (N=7) and Hooper Point (N=14), both Northland sites where parental forms were not found.

Thalli were collected haphazardly, at least 1 metre apart. Tips of thalli for DNA extraction were dried with silica gel. In addition, one or more typical thalli from each species/site were prepared as voucher specimens. *A priori* species assignments were made using morphological characters developed by Hodge *et al.*

(2010), primarily a wide (>5mm) flattened primary axis in *C. maschalocarpum* and a narrow terete axis in *C. angustifolium*. Putative hybrids had a flattened primary axis intermediate in width (2–5 mm).

Molecular methods

DNA was extracted from approximately 2–5 mg of dry tissue in a modified CTAB buffer following procedures described in Zuccarello & Lokhorst (2005), with the addition of 1% polyvinylpolypyrrolidone to the extraction buffer. The ITS2 rDNA region and a variable mitochondrial spacer region were amplified and screened for variation by SSCP and sequencing. Amplification and sequencing of ITS2 (448–450 bp) used KP5 (forward) and KG4 (reverse) primers (Lane *et al*. 2006). Two further primers, ITS86F (5′–ACAGCTTCGGGTTCGATCT–3′) and ITS424R (5′–ACCGGTCTCTCTCCGGTATT–3′), were developed from preliminary *Carpophyllum* ITS2 sequences to amplify a 337–339 bp fragment for SSCP analyses. This shorter fragment produced clearer SSCP profiles while retaining all known variable positions.

A 222 bp mitochondrial DNA fragment, containing 78 bp of the 3′ end of the 23S rRNA gene and 144 bp of spacer was amplified from Frasers Landing (Poor Knights Islands) and Maraehako Bay specimens with primers from Chapter 2. In addition, mtDNA sequences from Hooper Point and Wekarua specimens (Chapter 2) are included in the dataset.

Table 3.1. Exemplars of specimens sampled with ITS2 ribotypes and locality information.

ITS2 ribotype	**Specimen field number**	**Herbarium number[5]**	**Species**	**Site**	**Latitude/ Longitude**	**GenBank Accession number**
B	A220	WELT ASG290#1	*Carpophyllum maschalocarpum*	Bland Bay, Bay of Islands, Northland	35.342°S 174.366°E	JF313148
G×N	A397	WELT A031674a	*Carpophyllum angustifolium*	Cavalli Passage, Northland	35.051°S 173.943°E	JF313160
G	A398	WELT A031674b	*Carpophyllum angustifolium*	Cavalli Passage, Northland	35.051°S 173.943°E	JF313161
I	A404	WELT A031673	*Carpophyllum maschalocarpum* × *C. angustifolium* hybrid	Cavalli Passage, Northland	35.051°S 173.943°E	JF313162
B	A561	PKI008	*Carpophyllum maschalocarpum*	Frasers Landing, Poor Knights Islands	35.483°S 174.745°E	JF313165
G	A563	WELT A031326	*Carpophyllum angustifolium*	Frasers Landing, Poor Knights Islands	35.483°S 174.745°E	JF313166

[5] Specimens are awaiting accession at WELT. Herbaria number will be inserted when the accession process is complete.

Table 3.1 (Continued)

ITS2 ribotype	Specimen field number	Herbarium number	Species	Site	Latitude/ Longitude	GenBank Accession number
D	A885	-	*Carpophyllum maschalocarpum* × *C. angustifolium* hybrid	Hooper Point, Northland	34.416°S 172.854°E	JF313175
B	A905	-	*Carpophyllum maschalocarpum* × *C. angustifolium* hybrid	Hooper Point, Northland	34.416°S 172.854°E	JF313176
B	A725	-	*Carpophyllum maschalocarpum*	Otanga, Bay of Plenty	37.550°S 178.161°E	JF313168
A	A1000	-	*Carpophyllum maschalocarpum*	Otanga, Bay of Plenty	37.550°S 178.161°E	JF313178
B	A758	BOP339	*Carpophyllum maschalocarpum* × *C. angustifolium* hybrid	Maraehako Bay, Bay of Plenty	37.672°S 177.797°E	JF313169
B	A766	-	*Carpophyllum maschalocarpum*	Maraehako Bay, Bay of Plenty	37.672°S 177.797°E	JF313170
B	A808	BOP350	*Carpophyllum maschalocarpum* × *C. angustifolium* hybrid	Maraehako Bay, Bay of Plenty	37.672°S 177.797°E	JF313171

Table 3.1 (Continued)

ITS2 ribotype	Specimen field number	Herbarium number	Species	Site	Latitude/ Longitude	GenBank Accession number
B	A809	BOP351	*Carpophyllum maschalocarpum* × *C. angustifolium* hybrid	Maraehako Bay, Bay of Plenty	37.672°S 177.797°E	JF313172
B	A639	-	*Carpophyllum maschalocarpum*	Matts Crack, Poor Knights Islands	35.476°S 174.741°E	JF313167
G	A378	WELT A031669	*Carpophyllum angustifolium*	Flat Island, Northland	34.984°S 173.865°E	JF313155
G	A379	WELT A031669	*Carpophyllum angustifolium*	Flat Island, Northland	34.984°S 173.865°E	JF313156
D	A423	WELT A031670	*Carpophyllum maschalocarpum* × *C. angustifolium* hybrid	North Cape, Northland	34.416°S 173.053°E	JF313164
C	A935	WELT A031313	*Carpophyllum angustifolium*	Sailor's Grave, Coromandel	36.960°S 175.845°E	JF313177
I	A387	WELT A031671	*Carpophyllum maschalocarpum* × *C. angustifolium* hybrid	Stephenson Island, Northland	34.965°S 173.777°E	JF313157

Table 3.1 (Continued)

ITS2 ribotype	Specimen field number	Herbarium number	Species	Site	Latitude/ Longitude	GenBank Accession number
D	A388	WELT A031672	*Carpophyllum maschalocarpum* × *C. angustifolium* hybrid	Stephenson Island, Northland	34.965°S 173.777°E	JF313158
G	A390	WELT A031672	*Carpophyllum maschalocarpum* × *C. angustifolium* hybrid	Stephenson Island, Northland	34.965°S 173.777°E	JF313159
G	A408	WELT A031666	*Carpophyllum angustifolium*	Stephenson Island, Northland	34.965°S 173.777°E	JF313163
B	A341	-	*Carpophyllum maschalocarpum*	Waterfall Reef, Leigh, Northland	36.269°S 174.798°E	JF313149
B	A342	-	*Carpophyllum maschalocarpum*	Waterfall Reef, Leigh, Northland	36.269°S 174.798°E	JF313150
F	A345	-	*Carpophyllum angustifolium*	Waterfall Reef, Leigh, Northland	36.269°S 174.798°E	JF313151
F	A869	WELT A031320	*Carpophyllum maschalocarpum*	Waterfall Reef, Leigh, Northland	36.269°S 174.798°E	JF313173

Table 3.1 (Continued)

ITS2 ribotype	Specimen field number	Herbarium number	Species	Site	Latitude/ Longitude	GenBank Accession number
B	A870	-	*Carpophyllum angustifolium*	Waterfall Reef, Leigh, Northland	36.269°S 174.798°E	JF313174
H	A350	WELT A031668	*Carpophyllum maschalocarpum* × *C. angustifolium* hybrid	Wekarua, Northland	34.945°S 173.653°E	JF313152
D	A353	WELT A031667	*Carpophyllum maschalocarpum* × *C. angustifolium* hybrid	Wekarua, Northland	34.945°S 173.653°E	JF313153
E	A358	WELT A031667	*Carpophyllum maschalocarpum* × *C. angustifolium* hybrid	Wekarua, Northland	34.945°S 173.653°E	JF313154
B×G	A562	WELT A031326	*Carpophyllum angustifolium*	Frasers Landing, Poor Knights Islands	35.483°S 174.745°E	-
H×L	A349	WELT A031320	*Carpophyllum maschalocarpum* × *C. angustifolium* hybrid	Waterfall Reef, Leigh, Northland	36.269°S 174.798°E	-
B×G	A810	BOP352	*Carpophyllum maschalocarpum* × *C. angustifolium* hybrid	Maraehako Bay, Bay of Plenty	37.672°S 177.797°E	-

Table 3.1 (Continued)

ITS2 ribotype	**Specimen field number**	**Herbarium number**	**Species**	**Site**	**Latitude/ Longitude**	**GenBank Accession number**
B×G	A814	BOP356	*Carpophyllum maschalocarpum* × *C. angustifolium* hybrid	Maraehako Bay, Bay of Plenty	37.672°S 177.797°E	-

The PCR mix contained 1µl genomic DNA, 0.5 U Taq DNA polymerase (New England Biolabs), 1X Thermopol reaction buffer (NEB), 7.5 pmoles each primer, 200 nM dNTP, 5% DMSO and 0.01% BSA. All PCR amplifications used a touchdown protocol with an initial denaturation step of 94ºC for 4 min, followed by 5 cycles of 1 min at 94ºC, 1 min at 55ºC (−1ºC/cycle) and 1 min at 72ºC, followed by 30 cycles of 1 min at 94ºC, 1 min at 50ºC and 1 min at 72ºC, with a final extension of 72ºC for 10 min. Amplified products were checked for length and yield on 1% agarose gels stained with ethidium bromide.

SSCP screening

3µl PCR product was mixed with 9 µL 98% formamide, 10 mM NaOH, 0.025% bromophenol blue, 0.025% xylene cyanol, denatured at 100^{o}C for 5 min, then snap cooled on ice before loading. Gels were 225 mm long and 0.75 mm thick (BioRad D-Code System) and contained 10% Mutation Detection Acrylamide (Sigma A5934, Sigma Aldrich, St Louis, USA) for ITS2 or 9% 37.5:1 acrylamide/bis-acrylamide (Sigma Aldrich) for the mitochondrial spacer, in 0.5X TBE buffer with the addition of 10% glycerol. Electrophoresis was carried out for 16–18 hrs at 8W in 0.5X TBE buffer at 4^{o}C. After electrophoresis, gels were silver stained following protocols in Bassam *et al*. (1991) and banding patterns scored by eye.

Sequences were obtained from random samples of each ITS2 ribotype or mitochondrial haplotype indicated by SSCP analyses, as well as any samples that showed ambiguous SSCP patterns. In addition, sequences were obtained from other populations where low sampling did not warrant use of SSCP (Table 3.1). PCR products were cleaned with ExoSAP-IT (USB, Cleveland, Ohio) enzymes and sequenced commercially (Macrogen Inc., Korea).

ITS2 Cloning

Four hybrid specimens were selected for cloning, two from Leigh and one each from Maraehako Bay and Otanga, to determine if both parental ribotypes could be obtained. The complete ITS1–5.8S–ITS2 cistron was amplified using KP1 and KG4 primers. Purified PCR product was cloned using the pGEM-T Easy vector kit (Promega Corporation, Madison, Wisconsin USA) following the manufacturer’s protocols.

Transformed colonies were sub-cultured and plasmid preparations carried out by alkali lysis (Sambrook *et al*. 1989). Inserts were sequenced commercially using the pUC M13 forward and reverse primers.

Data analysis

Homozygous sequences were aligned using ClustalW in MEGA 3.1 (Kumar *et al.* 2004) and checked by eye (Appendix 2). A statistical parsimony map was inferred from ITS2 data using TCS 1.21 (Clement *et al.* 2000) from 83 sequences from parental specimens, and six cloned sequences from hybrid specimens.

Heterozygous ITS2 sequences were assumed to result from inheritance of only two ribotypes. Electropherograms were examined and assigned the most likely parental ribotypes required to produce the observed heterozygosity (Table 3.1, Fig. 3.2). Three heterozygous sequences could not be explained as progeny of sampled homozygous ribotypes. Parental ribotypes of these could be inferred from heterozygous sequences as these all differed from frequently sampled ribotypes by a single heterozygous position. These inferred parental ribotypes were included in the statistical parsimony analysis. Mitochondrial spacer haplotypes were all identical to haplotypes obtained in a previous study (Chapter 2). Haplotypes were assigned to species according to clusters inferred by statistical parsimony in that study.

3.4 Results

Morphology

Specimens from Bay of Plenty sites (Maraehako Bay and Otanga), Leigh and the two Poor Knights Islands sites were able to be assigned to three morphological classes (*C. maschalocarpum*, *C. angustifolium* or putative hybrids) based on stipe width (Hodge *et al.* 2010). All three classes were sampled at these sites All specimens sampled fromWekarua and Hooper Point showed intermediate (2–5 mm) stipe width and no specimens from these sites were assigned to parental species (Table 3.1, Fig. 3.1)..

SSCP data

SSCP successfully distinguished mitochondrial spacer haplotypes in all populations. Variation in the longer ITS2 fragment was less well resolved by SSCP, but ribotypes

could be distinguished among populations of low ribotype diversity (Bay of Plenty, Poor Knights Islands). Assigning ribotypes from SSCP profiles was difficult in Northland populations where ribotype diversity was higher and differences were often single base changes only. I therefore rely on sequence data for most Northland populations.

ITS2

Nine ITS2 ribotypes were sampled by direct sequencing from homozygous specimens, three of these were common (Fig. 3.1). Ribotype G was associated with *C. angustifolium*, and was widespread, from Otanga to Stephenson Island. Ribotypes D and B were widespread and mainly associated with *C. maschalocarpum*. Ribotype D is predominantly northern, from Leigh to Hooper Point; ribotype B was found throughout New Zealand. Two ribotypes, A and E, were found in single specimens only. Other uncommon ribotypes appeared to be fairly local: Ribotype C was found only in *C. angustifolium* from Sailor's Grave; ribotype I from parental species and hybrids from North Cape to Cavalli Passage in Northland; ribotype H from *C. angustifolium* from Wekarua and one hybrid from the Poor Knights Islands; and ribotype F from *C. angustifolium* from Leigh and the Alderman Islands.

Four ribotypes (L, M, N and O, enclosed by dashed lines in Fig. 3.1) were not sampled in homozygous specimens but were obtained by inferring parental ribotypes from heterozygous specimens. Ribotypes M, N and O were each inferred from single specimens that differed from common ribotypes by one heterozygous position. Ribotype L was inferred from sequences from six heterozygous specimens from Leigh (Fig. 3.2) and two heterozygous specimens sequenced from North Cape (Table 3.1). Ribotype L was also found in cloned sequences from a hybrid specimen from Leigh (Table 3.2).

Statistical parsimony analysis did not cluster ribotypes with morphological species assignments (Fig. 3.1). Ribotypes G and H were predominantly found in *C. angustifolium* or hybrids and were separated by two substitutions, but ribotypes F, I and

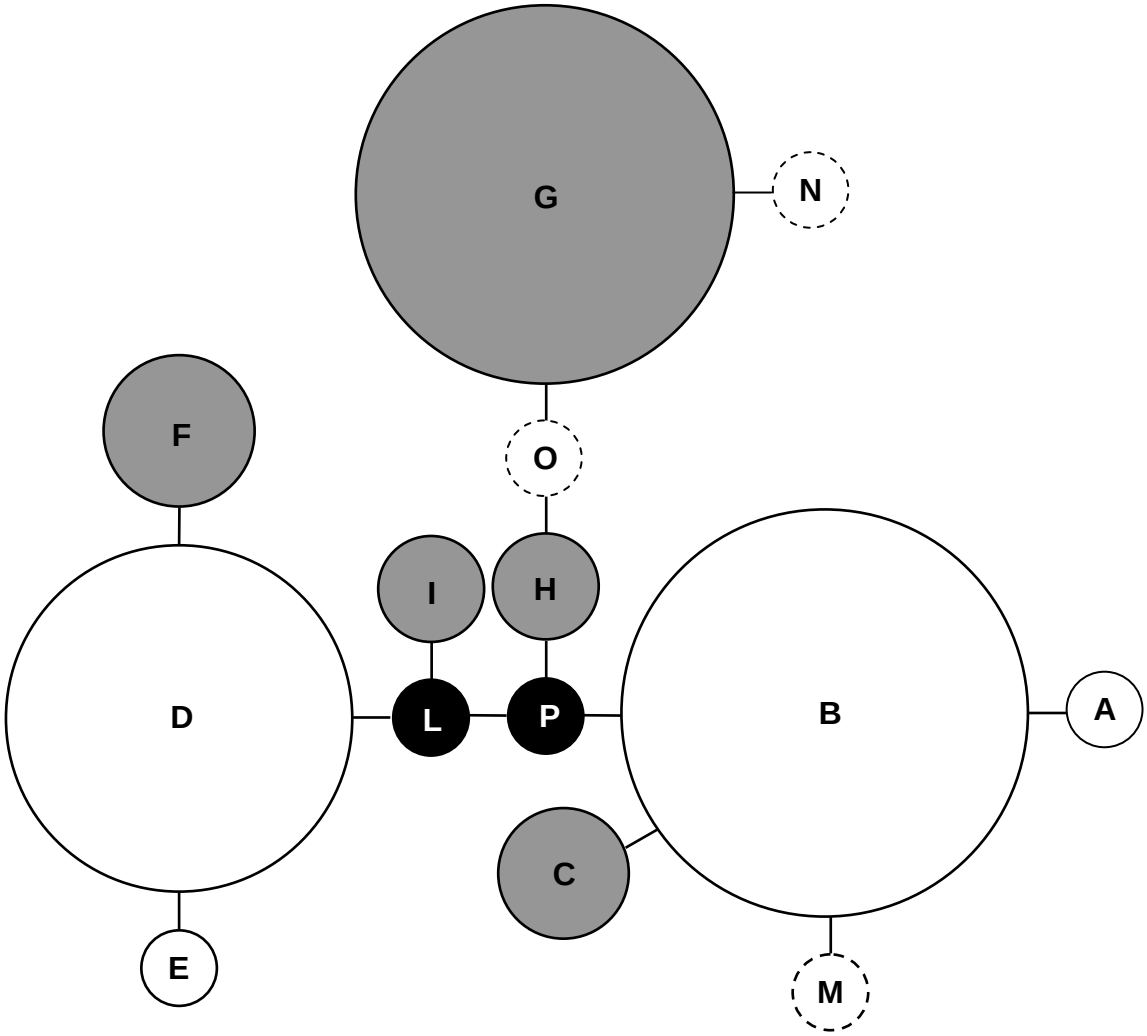

Fig. 3.1. Statistical parsimony map inferred from 83 homozygous ITS2 sequences and six cloned sequences from *Carpophyllum maschalocarpum*, *C. angustifolium* and putative hybrids. Shaded circles are predominantly *C. angustifolium*; clear circles are predominantly *C. maschalocarpum*. Black circles represent ribotypes sampled only by cloning. Dashed circles indicate ribotypes not found in homozygotes but are parental ribotypes required to produce heterozygotic sequences sampled in some specimens. Area of circles is proportional to number of samples with that haplotype. Connections are made at 95% probability.

C were also predominantly found in *C. angustifolium* but were connected by single changes to ribotypes sampled extensively in *C. maschalocarpum*. The most common *C. maschalocarpum* ribotypes (B and D) were separated by three changes, and by ribotypes only sampled from cloned specimens.

Hybrids

With three exceptions, all specimens from Bay of Plenty and Poor Knights Islands sites that had intermediate morphologies also had heterozygous ITS2 (Fig. 3.2). In heterozygous specimens, double peaks were visible at particular sites in electropherograms. Both sequences could be read from these electropherograms. One ribotype was identical to ribotypes from *C. maschalocarpum* specimens from the same population, and the other ribotype was identical to ribotypes from *C. angustifolium* specimens from the same population. At Leigh, six hybrid specimens were heterozygous but one ribotype (ribotype L in Fig. 3.1) was not sampled in any non-hybrid specimens. Ribotype L differed from a common Northland *C. maschalocarpum*-associated ribotype by one substitution (Fig. 3.1). Three specimens with hybrid morphology from Maraehako Bay were homozygous for ribotype B, the ribotype found in all *C. maschalocarpum* specimens from this site. Specimens from Wekarua and Hooper Point were all assigned to hybrids based on morphology but nearly all specimens were homozygous for ITS2, except for two heterozygous specimens from Wekarua. All specimens sampled at these two sites had ribotypes associated with *C. maschalocarpum*, apart from two Wekarua specimens that had the *C. angustifolium*-associated Ribotype H (Fig. 3.1).

At Frasers Landing (Poor Knights Islands) and Leigh, two specimens morphologically assigned to *C. maschalocarpum* and seven specimens assigned to *C. angustifolium* also had heterozygous ITS2. These specimens all possessed the common *C. maschalocarpum*-associated ribotype B (Fig. 3.1) and *C. angustifolium*-associated ribotypes F or G.

ITS cloning

Eight to eleven clones containing PCR products were sequenced for each specimen selected for cloning (Table 3.2; Appendix 3). Single base changes that appear in one cloned sequence only, and were not detected in any directly sequenced sample, are

regarded as copy errors from PCR, and are excluded from further analysis. Multiple copies of expected parental ribotypes were obtained from all cloned specimens. In addition, two ribotypes (L and P, shown as black circles in Fig. 3.1) obtained from one specimen from Leigh (A347 in Table 3.2) were not sampled by direct sequencing of homozygous specimens. Both these ribotypes were predicted as unsampled ribotypes by statistical parsimony analysis using homozygous sequences, and ribotype L was inferred as one ITS2 variant in heterozygous specimens from Leigh and North Cape.

Table 3.2: Frequency of ribotypes from cloned ITS2 sequences from heterozygous specimens of *Carpophyllum*. Ribotype designation is shown in Fig.3.1.

	Ribotype						
Specimen/site	B	D	F	G	H	L	P
A980 Otanga	2	-	-	9	-	-	-
A810 Maraehako Bay	3	-	-	5	-	-	-
A868 Leigh	4	1	3	-	-	-	-
A347 Leigh	-	-	-	-	5	3	1

Table 3.3. Mitochondrial haplotype frequency from *Carpophyllum maschalocarpum, C. angustifolium* and hybrids from northern New Zealand. Haplotypes associated with *C. angustifolium* are shaded. Other haplotypes were associated with *C. maschalocarpum*. Haplotype numbers are from Chapter 2, Fig. 2.2.

Site	MorphoSpecies	Haplotype	N
Maraehako Bay	*C. angustifolium*	48	3
		23	17
	C. maschalocarpum	14	15
		16	2
		17	1
		18	1
		19	2
		38	2
		71	1
		72	1
	Hybrids	14	3
		15	3
		16	3
		23	14
		48	3
Frasers Landing	*C. angustifolium*	45	7
	C. maschalocarpum	21	6
	Hybrids	21	6
Hooper Point	Hybrids	21	10
		34	34
		65	1
		66	1
		67	1
Wekarua	Hybrids	30	7
		33	1
		47	7
		50	4
		67	1

Mitochondrial DNA

Mitochondrial DNA haplotypes associated with both *C. angustifolium* and *C. maschalocarpum* (Chapter 2) were found in morphologically intermediate specimens from the Bay of Plenty and Hooper Point (Table 3.3). At Frasers Landing (Poor Knights Islands) all hybrids had *C. maschalocarpum*-associated mtDNA haplotypes. At all other sites the ratios of *C. angustifolium*-associated haplotypes:*C. maschalocarpum*-associated haplotypes were > 0.5. Departure from a 0.5 ratio was significant at Hooper Point (Binomial Test, $P = 0.01$) and Wekarua ($P << 0.01$).

Morphologically intermediate specimens from the most northern sites (Wekarua and Hooper Point) had predominantly *C. maschalocarpum*-associated ITS2 ribotypes but *C. angustifolium*-associated mtDNA haplotypes. At Wekarua, five out of seven specimens sequenced had *C. maschalocarpum*-associated ITS2 ribotypes, but all specimens had *C. angustifolium*-associated mtDNA haplotypes. At Hooper Point, all 14 specimens sequenced showed *C. maschalocarpum*-associated ITS2 ribotypes, but ten of these had *C. angustifolium*-associated mtDNA haplotypes.

3.5 Discussion

These data suggest hybridisation with *C. maschalocarpum* is occurring across the range of *C. angustifolium*, but patterns of hybridisation differ between localities. Three patterns are evident: (1) *Carpophyllum* populations from the Bay of Plenty and the Poor Knights Islands show strong congruence between intermediate morphology and ITS heterozygosity. I interpret this as interspecies crossing resulting in morphologically distinct hybrids with few backcrosses. (2) At Leigh, specimens with intermediate morphology and heterozygous ITS2 were also present, but four (out of ten) specimens assigned morphologically to *C. angustifolium* had heterozygous ITS2, with one ribotype inferred from electropherograms associated with *C. maschalocarpum*. These data suggest backcrosses between hybrids and *C. angustifolium*, resulting in *C. maschalocarpum*-associated ITS2 ribotypes being found in specimens resembling *C. angustifolium*. (3) At Hooper Point and Wekarua, all specimens were morphologically similar to hybrids found in other populations, and parental species were not seen, and are assumed to be rare or absent. Specimens were predominantly homozygous at ITS2, with *C. maschalocarpum*-associated ribotypes, but the majority of samples had *C.*

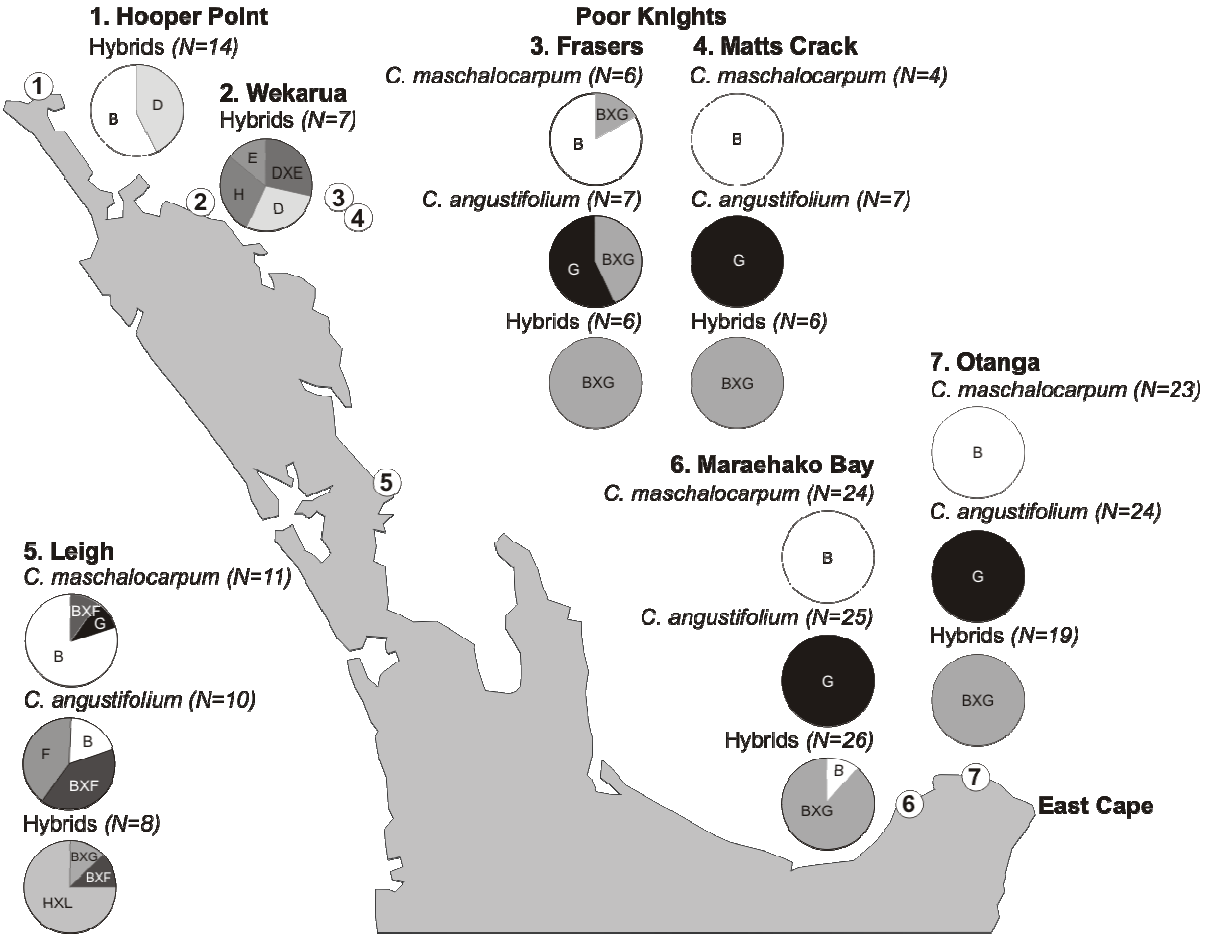

Fig. 3.2. Geographical patterns of ITS2 ribotypes in *Carpophyllum maschalocarpum*, *C. angustifolium* and hybrids from northern New Zealand populations. Pie charts show proportion of ribotypes sampled at each site.

angustifolium mtDNA haplotypes. These data suggest a population with hybrid origins, but where ITS homogenisation has taken place.

In the Bay of Plenty, hybrids are found predominantly in areas of moderate wave exposure, where *C. maschalocarpum* begins to be replaced by the more wave exposure-tolerant *C. angustifolium* (Hodge 2009). These hybrid zones are consistent with Barton & Hewitt's (1985) tension model, where hybrids are selected against relative to parents, but hybrid zones are maintained by migration into the zone. Hybrid populations at Hooper Point and Wekarua appear to persist without obvious sources of immigrants, but also without competition from parent populations. Either local environmental conditions favour hybrid morphologies (Moore 1977) or hybrid populations have established through founder effects (Neiva *et al.* 2010). Further research is needed to clarify this.

These ITS2 data from the Bay of Plenty are broadly in agreement with Hodge *et al.* (2010) who sampled *Carpophyllum* from Maraehako Bay and the adjoining Uncles Bay. I extended ITS2 data by including a third, more distant site (Otanga, near Lottin Point). Two ITS2 ribotypes dominated Bay of Plenty sites, although an additional ITS2 ribotype (A) was sampled in one Otanga specimen. With the exception of three specimens from Maraehako Bay, morphology is a reliable predictor of ITS2 ribotype in these populations: in homozygous specimens one ribotype was associated with *C. angustifolium*, the other with *C. maschalocarpum*. Specimens with intermediate morphology from these sites were heterozygous, with both parental ribotypes, and could be interpreted as F1 hybrids.

Hodge *et al.* (2010) found six specimens that were assigned to *C. angustifolium* on the basis of morphology, but had heterozygous ITS2, and suggested these were backcrosses between F1 hybrids and *C. angustifolium*. No specimens with *C. maschalocarpum* morphology were found with *C. angustifolium*-associated ribotypes, suggesting backcrossing might be asymmetrical. No thalli with heterozygous ribotypes and parental morphology were found in the Bay of Plenty in this study. Instead, three specimens had both an intermediate hybrid morphology and homozygous ITS2, with the *C. maschalocarpum*-associated ribotype B. I interpret these as F2 or later progeny of hybrid parents, where ITS2 DNA has homogenised to a single ribotype. ITS homogenisation, where a single, randomly selected ITS variant is fixed within the

individual (Elder & Turner 1995), is expected to remove the signature of hybridisation. However, the rate of homogenisation in brown algae is not known. Curiously, these three specimens had a unique mitochondrial spacer haplotype (haplotype 15 in Chapter 2), which was not sampled in any other population in this study (or in Chapter 2). Possible explanations are that this is a quirk of stochastic sampling and this haplotype is more widespread, or these might represent an incipient hybrid lineage, such as is found in the low-shore variety of *Fucus spiralis* (Coyer *et al*. 2010).

Biparental inheritance of ribosomal DNA provides a useful tool for the detection of hybridisation (Coleman 2002, Carine *et al.* 2007), but its value is limited (Álvarez & Wendel 2003) because of homogenisation. ITS heterozygosity is expected in F1 hybrids, but the signature of backcrosses will disappear if F1 hybrids homogenise to the same ribotype as the parental species involved in the backcross. Hence backcrosses will be underestimated by this method, especially where ribotype diversity is low, such as the Bay of Plenty. In addition, direct sequencing might not reveal the intra-individual diversity of ITS where homogenisation is incomplete and some ribotypes are retained in low copy number. One solution is to use cloning to estimate intra-individual ITS diversity. In my data, Ribotype L was predicted by statistical parsimony analysis as an intermediate haplotype, and inferred from heterozygous specimens, but was only confirmed in one cloned specimen, from Leigh. This specimen also provided a single cloned sequence of ribotype P, also predicted by statistical parsimony analysis. PCR artifacts can be a problem during cloning, but it is unlikely that copy errors would produce five copies of predicted ribotypes. This suggests backcrossing might be more frequent in Bay of Plenty populations, but other markers, such as microsatellites need to be developed to accurately estimate rates.

Patterns of hybridisation in Bay of Plenty populations are relatively straightforward. A more complicated pattern was found in Northland sites. At Frasers Landing and Leigh, three and four specimens (out of seven and ten samples, respectively) specimens had *C. angustifolium* morphology but heterozygous ITS2, with one ribotype that is usually associated with *C. maschalocarpum.* Two specimens from Leigh had *C. angustifolium* morphology and were homozygous, but had *C. maschalocarpum*-associated ribotypes (Fig. 3.1). Conversely, only a single specimen from each site had *Carpophyllum maschalocarpum* morphology and heterozygous ITS2,

and only one *C. maschalocarpum* specimen (from Leigh) was homozygous with a *C. angustifolium*-associated ribotype. I interpret this mis-match between morphology and ITS sequence(s) as backcrosses. Backcrosses mostly had *C. angustifolium* morphology, a result also found by Hodge *et al*. (2010), suggesting hybrids are more likely to backcross with *C. angustifolium*.

Maternal mitochondrial inheritance seems to be ubiquitous in oogamous algae, such as the Fucales, although rare heteroplasmy does occur (Coyer *et al.* 2002b, Coyer *et al.* 2004, Hoarau *et al*. 2009). There was no evidence of heteroplasmy in my samples, and I consider mitochondrial data to reflect maternal lineages. My mitochondrial data show that either *Carpophyllum* species can act as the maternal parent in hybridisation events, but in all populations except the Poor Knights Islands, hybrids had a higher frequency of *C. angustifolium* mtDNA (Table 3.3). The simplest explanation for this is a greater frequency of motile *C. maschalocarpum* male gametes (sperm) available for contact with immotile *C. angustifolium* female gametes (eggs). *Carpophyllum angustifolium* is usually restricted to the most wave exposed areas, often on the exposed parts of headlands, whereas *C. maschalocarpum* occupies a wide zone, from moderately exposed to moderately sheltered areas and is more abundant at most sites (Shears & Babcock 2007). This should result in a greater likelihood of *C. maschalocarpum* sperm being carried into stands of *C. angustifolium* than *vice versa*, and a greater potential for hybrids with *C. angustifolium* mtDNA. In contrast, hybrids from Frasers Landing had exclusively *C. maschalocarpum* mtDNA. This pattern might arise stochastically from low sampling at this site, but in an extensive survey of macroalgal abundance in New Zealand, the Poor Knights Islands were the only locality where *C. angustifolium* occurred more frequently than *C. maschalocarpum* (Shears & Babcock 2007). This might reverse the usual imbalance in gamete availability.

Haplotype 23 was common in *C. angustifolium* and hybrids from Maraehako Bay (Table 3.3). This haplotype was not sampled in *C. maschalocarpum* in this study or a previous study (Chapter 2), so I consider it indicative of a *C. angustifolium* maternal parent. However, statistical parsimony analysis in Chapter 2 places haplotype 23 in a cluster with *C. maschalocarpum* haplotypes, and just one change from a common haplotype found in *C. maschlocarpum* from Northland and the east coast of New Zealand's North Island. This suggests this haplotype or its ancestor has introgressed into

C. angustifolium populations in the past, and is now either extinct or rare in *C. maschalocarpum*. Founder effects might account for its high frequency at Maraehako Bay. A similar pattern is found in Europe, where a mitochondrial haplotype associated with *F. vesiculosus* is found in *F. ceranoides* throughout the latter species' northern range. This extensive introgression is believed to result from repeated founder effects (Neiva *et al.* 2010)

The high frequency of *C. angustifolium*-associated mtDNA haplotypes but mostly *C. maschalocarpum*-type ITS ribotypes in morphologically intermediate specimens from the most northern populations of Wekarua and Hooper Point suggest that these populations have hybrid origin, rather than being a wave-exposed ecotype of *C. maschalocarpum* as suggested by Shears & Babcock (2007). As no parent specimens were found at these sites, they appear to be self-sustaining populations of hybrid origin that have undergone ITS homogenisation, but retain hybrid morphology.

Mitochondrial spacer haplotypes are usually diagnostic for *Carpophyllum* species (Chapter 2). In contrast, only some ITS2 ribotypes had strong species associations, and most had exceptions (Table 3.1, Fig. 3.1). Most mitochondrial haplotypes also formed species-specific clusters (Chapter 2), whereas ITS2 ribotypes did not. It is not clear from these data whether ITS paraphyly is a consequence of gene transfer by hybridisation, or if this results from incomplete lineage sorting, with ancestral polymorphisms retained in both species. These results agree with the prediction that lineage sorting will proceed at a faster rate in mitochondrial DNA compared to nuclear DNA, due to a smaller effective population size in the former (Avise 2000). However, this prediction is probabilistic and cannot be applied to individual lineages with certainty (Hudson & Turelli 2003).

Carpophyllum populations in the Bay of Plenty are strongly segregated by habitat (Hodge 2009), with *C. maschalocarpum* occupying relatively sheltered embayments, but being replaced by *C. angustifolium* on the exposed headlands. Hybrids are predominantly found in a zone between the most exposed and sheltered areas. Species integrity in the Bay of Plenty might be maintained by segregation by habitat and limited gamete dispersal, with episodic selection against hybrids, possibly by physical removal of hybrids during storm events and overgrowth by *C. maschalocarpum* during calm periods. Habitat segregation is a factor in maintaining

species integrity in hybridising *Fucus* (Coyer *et al.* 2007, 2010). Northland sites appeared to have less sheltered habitat than Bay of Plenty sites. This might limit habitat segregation and increase the frequency of hybridisation events and backcrosses. Unlike *Fucus*, all *Carpophyllum* species are dioecious, which is expected to increase the frequency of hybridisation.

Three main findings arise from my data: (1) Hybridisation between *C. angustifolium* and *C. maschalocarpum* is widespread; (2) Patterns of hybridisation differ across the range of *C. angustifolium*; and (3) Initial hybridisation events appear symmetrical, but limited data suggests backcrosses are more likely between hybrids and *C. angustifolium*. Further ecological research and additional genetic markers are needed to determine if hybrids are under selection and if this varies between sites. While mitochondrial introgression from *C. angustifolium* into *C. maschalocarpum* appears rare (Chapter 2), the high frequency of haplotype 23 in *C. angustifolium* from Maraehako Bay suggests introgression in *C. angustifolium* might be more common. Further mitochondrial DNA data for *C. angustifolium* populations is needed to elucidate this pattern.

3.6 Acknowledgements

I thank Nick Shears, Wendy Nelson, Franz Smith, Kate Neill, Tracy Farr, Fiona Hodge and Peter Martin for assistance with collections.

3.7 References

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Chapter 4

Barcoding brown algae: Does *cox*1 identify species of *Cystophora* (Fucales, Phaeophyceae)?

4.1 Abstract

Many brown macroalgal species can be confused because of limited morphological differentiation and plasticity. DNA barcoding has been proposed as an alternative to morphological species identification. In barcoding, short (c. 600 bp) mitochondrial DNA sequences are analysed by distance methods and discontinuities between intraspecific and interspecific mtDNA variation are used as indicators of species boundaries. The utility of *cox*1 DNA barcoding was evaluated by comparing 108 sequences from 13 species of *Cystophora*, an Australasian genus of brown algae. A 615 base pair fragment of the *cox*1 gene was adequate to assign most specimens to species and resolved some morphologically difficult specimens, but molecular distance between some species was low – often only slightly greater than intraspecific distances. Morphologically ambiguous specimens of *C. subfarcinata*, *C. retorta* and *C. siliquosa* could be identified by *cox*1 sequences. Neither *cox*1 nor ITS data supported the maintenance of *C. congesta* or *C. distenta* as independent species. I suggest *cox*1 alone might be too conserved for reliable barcoding in the brown algae.

4.2. Introduction

DNA BARCODING uses phenetic differences between short mtDNA sequences from a single region for species identification (Hebert *et al.* 2003, 2004; Ratnasingham & Hebert 2007). Proponents of DNA barcoding have suggested that the technique can fill gaps in taxonomic knowledge, document biodiversity loss, facilitate ecological studies and allow objective delimitation of species (Hebert *et al.* 2003). The approach attracted controversy (Will & Rubinoff 2004; Rubinoff *et al.* 2006), in part because both proponents and detractors have failed to distinguish barcoding from DNA taxonomy, species discovery, and molecular phylogenetics. In practice, establishing databases of species tagged sequences has required use of additional markers and morphological and ecological data to assign species names to specimens, effectively engaging in integrative taxonomy (Dayrat 2005), and early suggestions that the approach might supplant traditional taxonomy have abated (Schindel & Miller 2005; Rubinoff 2006). Large

DNA barcoding programmes have been established, particularly in animal groups (Frézal & Leblois 2008; Janzen *et al.* 2009; Golding *et al.* 2009; Ward *et al.* 2009).

Most barcoding studies have delimited species using a discontinuity between intraspecific and interspecific genetic distances (either uncorrected p-distance or Kimura 2-parameter). Hebert *et al.* (2004) proposed a species level threshold of 10-times mean intraspecific variation. Using mean distances inflates discontinuities and the smallest distance should be used (Meier *et al*. 2008). Ten-times thresholds cannot be rigidly applied, as genetic distance between congeners is variable, depending on the time since speciation, generation time, the strength of different speciation processes, population size and connectivity, and mutation and repair rate variation between lineages (Ferguson 2002; Will & Rubinoff 2004; Kumar 2005; Hickerson *et al.* 2006). One solution is to test species divergence using coalescence models (Papadopoulou *et al.* 2008; Baker *et al.* 2009). Character-based species delimitation has been proposed as an alternative to distance methods (DeSalle *et al*. 2005), but its application, so far, is limited (Rach *et al.* 2008).

Spatial heterogeneity of intraspecific variation (Avise 2000; Hoarau *et al.* 2007; Maggs *et al.* 2008) is problematic for barcoding as even geographically widespread sampling might not encompass the most variable populations (Zuccarello *et al.* 2006), and the limited sampling of conspecifics in many barcoding studies might seriously underestimate intraspecific variation and overestimate the number of fixed differences between species. This will produce misleading estimates of genetic distance between species. Alternatively, where genetic structure is high, extensive sampling might result in demes being identified as species (Trewick 2008).

As a technical method of assigning specimens to species, either character-based or distance-based barcoding procedures might assist species identification if a preliminary study shows sufficient interspecific distance or fixed characters to separate closely related (i.e. sister) species, based on adequate sampling over the geographical range of the species; and if interspecific distances or fixed characters are corroborated in a preliminary study with morphological or other characters (i.e. integrated taxonomy, Dayrat 2005).

Most barcoding studies aim to evaluate the efficacy of a marker to delineate species within a group, and to establish a reference database of species tagged

sequences to identify samples from subsequent studies. Specimens are assigned to species according to existing taxonomic criteria; the congruence of disjunctions in genetic variation with *a priori* species assignments is assessed; and, if necessary, taxonomic revisions are made on the basis of corroborated information. I suggest four criteria for a conservative evaluation of a locus for species identification: (1) does the marker resolve discrete clusters within a genus? (2) Are these clusters concordant with species boundaries indicated by other taxonomic information (e.g., morphology, anatomy, ecology, chemistry, physiology, mating studies)? (3) Is sampling sufficient to represent intraspecific variation across the range of each species? (4) Does sampling include closely related species?

The 5′ end of the cytochrome oxidase 1 mtDNA gene (*cox*1) is the preferred locus for barcoding in vertebrates and arthropods (Hebert *et al.* 2004; Linares *et al*. 2008; Ward *et al*. 2008; Golding *et al.* 2009). This region is too conserved at the species level in plants and green algae, and various combinations of regions have been proposed as potential barcodes for terrestrial plants (Chase *et al.* 2007; Kress & Erickson 2007; Lahaye *et al.* 2008; Ford *et al.* 2009; Seberg & Petersen 2009) or green algae (Presting 2006; Engelmann *et al.* 2009). Barcoding has been attempted in red algae (Saunders 2005, 2008, 2009; Robba *et al.* 2006; Yang *et al.* 2008), dinoflagellates (Litaker *et al.* 2007), diatoms (Evans *et al.* 2007; Moniz & Kaczmarska 2009, 2010), and brown algae (Lane *et al.* 2007; Kucera & Saunders 2008; McDevit & Saunders 2009). The *cox*1 region has been proposed as a suitable locus for barcoding red and brown algae (Saunders 2005; Robba *et al.* 2006).

To evaluate *cox*1 as a locus for species identification in brown algae, I compare a barcoding approach to phylogenetic and morphological species delimitation in the genus *Cystophora* J. Agardh (Sargassaceae, Fucales), with an emphasis on New Zealand representatives. *Cystophora* is an ideal genus for testing the barcoding approach as a thorough morphological taxonomy is available (Womersley 1964, 1987), the genus is relatively speciose (23 species listed in Womersley 1964), and includes species with highly disjunct morphologies and others that are morphologically similar.

Barcoding of brown algae by *cox*1 has had limited success. Lane *et al.* (2007) attempted a *cox*1 barcoding study of *Alaria* species from the north-east Pacific. Intergeneric variation was relatively low and there was no clear threshold between inter-

clade and intra-clade variation. In addition, clusters of *cox*1 mitotypes were not congruent with *a priori* morphological species assignments, nor with clades inferred from nrDNA internal transcribed spacer (ITS) sequences. ITS variation was relatively high, but did not resolve species level groups. Lane *et al.* (2007) suggest that widespread introgressive hybridisation precludes molecular delimitation of *Alaria* species in the study region. Kucera & Saunders (2008) used *cox*1 sequences to delineate samples of *Fucus* into three clusters, but it is not clear whether these results support wider use of *cox*1 in brown algae, as sampling did not extend across the species' ranges, some morphological assignments were not congruent with sequence data and one cluster included two species. McDevit & Saunders (2009) reported *cox*1 barcodes for 29 species of brown algae, but did not assess intraspecific or intrageneric diversity. Most genera were represented by a single species and sampling of congeners or conspecifics was geographically restricted. This study shows adequate *cox*1 variation to separate genera, but does not address the separation of sister species.

These studies leave the efficacy of *cox*1 barcoding of brown algae uncertain. This study aims to further evaluate *cox*1 species identification in brown algae by comparing *cox*1 species delimitation of *Cystophora* species against morphological criteria and combined *cox*1 and ITS phylogenetic analysis.

4.3 Materials and Methods

Cystophora species and close relatives were collected from 51 sites in New Zealand and southern Australia (Table 4.1, Fig. 4.1). Thalli were pressed as voucher specimens. Clean apices were detached and rapidly dried and stored in silica gel for subsequent DNA extraction. Morphological identifications used characters specified by Womersley (1964, 1987). Key diagnostic characters are listed in Table 4.2.

DNA was extracted from approximately 2–5 mg of dry tissue using a modified CTAB buffer procedure (Zuccarello & Lokhorst 2005), with the addition of 1% polyvinylpolypyrrolidone to the extraction buffer.

PCR and primers

A 615 bp fragment from the 5′ end of the *cox*1 mitochondrial gene was amplified using primer pairs GAZF2 and GAZR2 (Lane *et al.* 2007) or GAZF2 and a new primer,

fcox808R (5′-TAAACTTCGGGGTGTCCAAA-3′). The latter primer was designed from alignments of the *Fucus*, *Laminaria* and *Desmarestia* mitochondrial genomes (Oudot-Le Seq *et al.* 2002, 2006). Amplification of the ITS1-5.8S-ITS2 cistron used either AFP2(F) (Peters & Burkhardt 1998) or P1 (Tai *et al.* 2001) forward primers and KG4 reverse primer (Lane *et al*. 2006).

*Cox*1 amplification used a PCR routine with an initial denaturation step of 94ºC for 4 min, then 35 cycles of 1 min at 94ºC, 30 seconds at 50ºC and 1 min at 72ºC, with a final extension of 72ºC for 7 min. ITS amplifications used a touchdown PCR routine with an initial denaturation step of 94ºC for 4 min, then 5 cycles of 1 min at 94ºC, seconds at 55ºC (−1ºC/cycle) and 1 min at 72ºC, followed by 30 cycles of 1 min at 94ºC,

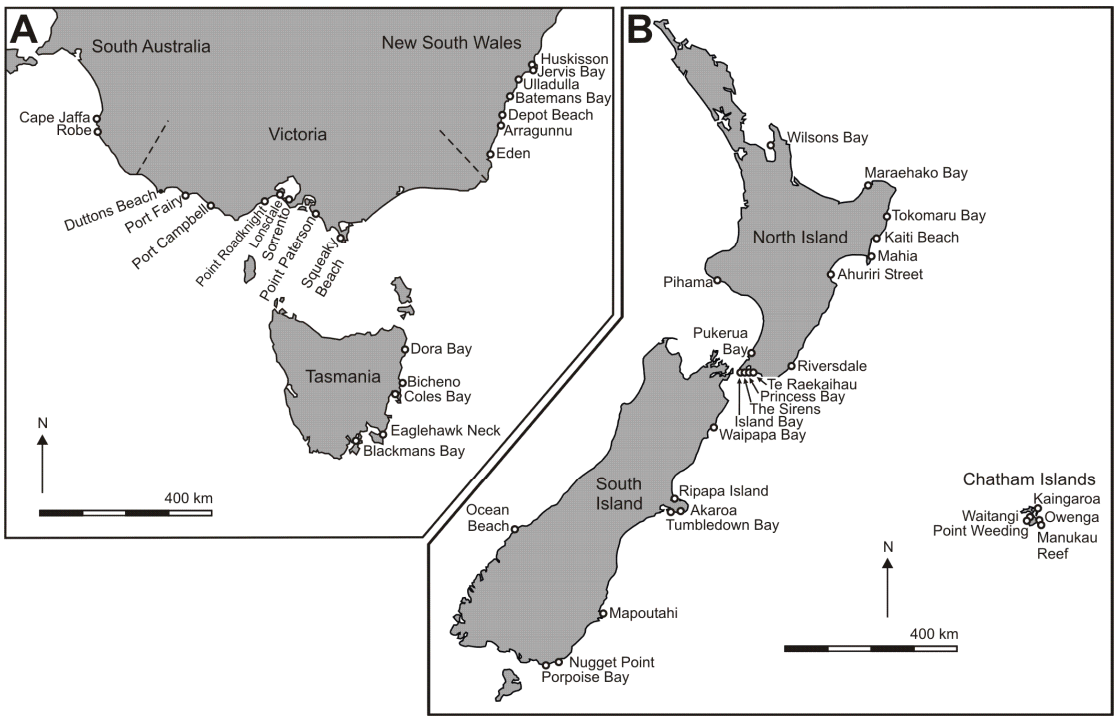

Fig. 4.1. Map showing sampling locations (for details see Table 4.1). **A.** Southern Australia. **B.** New Zealand.

45 seconds at 50ºC and 1 min at 72ºC, with a final extension of 72ºC for 10 min. The PCR mix for both regions contained 1µl genomic DNA, 0.5 U Taq DNA polymerase (New England Biolabs) 1× ThermoPol reaction buffer (NEB), 7.5 pmoles each primer,

200 nM dNTP and 0.01% BSA. Amplified products were checked for length and yield on 1% agarose gels stained with ethidium bromide.

PCR products were cleaned with ExoSAP-IT (USB, Cleveland, Ohio) enzymes and sequenced commercially (Macrogen Inc., Seoul, Korea). Sequencing *cox*1 fragments used the same primers as amplification. ITS sequencing used the additional internal primers KP5 and KIR1 (Lane *et al.* 2006).

Data analysis

*Cox*1 fragments were aligned using the ClustalW algorithm in MEGA3 (Tamura *et al*. 2007). Alignment was straightforward with no gaps (Appendix 4). A neighbour-joining tree was inferred in PAUP*4.10 (Swofford 2002), with the parameters estimated from MODELTEST 3.7 (Posada & Crandall 1998) (HKY85 + Γ +I, transition/transversion ratio = 3.6467, kappa = 7.489067, nucleotide frequencies: A = 0.1948, C = 0.1909, G = 0.2191, T= 0.3952, invariable sites = 0.6392, Γ = 0.9596). Confidence limits of branches were tested with 1000 bootstrap replications.

ITS sequences were aligned using the ClustalW algorithm in MEGA3, and the alignment edited manually (Appendix 5). A partition homogeneity test for incongruity between *cox*1 and ITS datasets was executed in PAUP* (1000 replicates). Maximum-parsimony trees were inferred from the combined ITS and *cox*1 sequences (2074 bp) in PAUP* with the parameters estimated from MODELTEST (HKY+I+Γ, transition/transversion ratio = 2.5289, nucleotide frequencies: A = 0.1801, C = 0.2663, G = 0.3050, T= 0.2486, invariable sites = 0.5809, Γ = 0.7626). 1000 maximum likelihood trees were inferred using GARLI 1.0 (Zwickl 2006) using the same parameters, and a consensus tree was calculated in PAUP*. A Bayesian tree for the combined data was inferred using MrBayes 3.1.2 (Huelsenbeck & Ronquist 2001) with ITS and *cox*1 regions partitioned, and each *cox*1 codon position partitioned.

Table 4.1. Voucher numbers of specimens used in analyses, collection locations, latitude and longitude and GenBank accession numbers of sequences.

Herbarium voucher number	Species	Location	Latitude, Longitude	GenBank Accession numbers	
				*cox*1	ITS
WELT A031507	*C. congesta*	Lonsdale VIC	38°17′5.30″S, 144°36′54.74″E	GU289263	GU289363
WELT A031510	*C. congesta*	Lonsdale VIC	38°17′5.30″S, 144°36′54.74″E	GU289264	GU289364
WELT A031509	*C. congesta*	Port Campbell VIC	38°37′14.84″S, 142°59′32.69″E	GU289281	-
WELT A031512	*C. congesta*	Robe SA	37° 9′23.33″S, 139°44′56.32″E	GU289330	-
WELT A031511	*C. congesta*	Robe SA	37° 9′23.33″S, 139°44′56.32″E	GU289285	GU289374
WELT A031508	*C. congesta*	Robe SA	37° 9′23.33″S, 139°44′56.32″E	GU289286	GU289375
WELT A030927	*C. congesta*	The Sirens, Wellington NZ	41°20′56.91″S, 174°45′51.05″E	GU289316	GU289388
WELT A031340	*C. distenta*	Manukau Reef, Chatham Islands	44° 2′4.90″S, 176°19′44.31″W	GU289317	GU289389
WELT A030933	*C. distenta*	Mapoutahi, Otago Peninsula NZ	45°44′5.57″S, 170°37′4.53″E	GU289338	-
WELT A031343	*C. distenta*	Owenga, Chatham Islands	44° 2′0.30″S, 176°19′58.80″W	GU289318	GU289390
WELT A031497	*C. monilifera*	Eden NSW	37° 4′16.80″S, 149°54′21.85″E	GU289255	GU289356
WELT A031500	*C. monilifera*	Huskisson, NSW	35° 2′34.31″S, 150°40′38.77″E	GU289248	GU289352
WELT A031501	*C. monilifera*	Lonsdale VIC	38°17′5.30″S, 144°36′54.74″E	GU289266	-
WELT A031498	*C. monilifera*	Ulladulla, NSW	35°21′37.65″S, 150°29′5.55″E	GU289251	-

Table 4.1. (continued)

Herbarium voucher number	Species	Location	Latitude, Longitude	GenBank Accession numbers	
				*cox*1	ITS
WELT A031499	*C. monilifera*	Ulladulla, NSW	35°21′37.65″S 150°29′5.55″E,	GU289306	GU289387
WELT A031496	*C. moniliformis*	Blackmans Beach, Tasmania	43° 0′27.26″S, 147°19′44.06″E	GU289291	GU289379
WELT A031495	*C. moniliformis*	Jervis Bay, NSW	35° 8′11.52″S 150°44′28.69″E	GU289249	GU289353
WELT A031493	*C. platylobium*	Cape Paterson VIC	38°40′29.26″S, 145°37′2.65″E	GU289257	GU289358
WELT A031494	*C. platylobium*	Duttons Beach, Portland VIC	38°19′42.74″S, 141°36′22.27″E	GU289279	-
WELT A030929	*C. platylobium*	Porpoise Bay, Otago NZ	46°39′39.37″S, 169° 6′29.32″E	GU289237	GU289348
WELT A031492	*C. polycystidea*	Cape Paterson VIC	38°40′29.26″S, 145°37′2.65″E	GU289261	GU289361
WELT A031489	*C. polycystidea*	Cape Paterson VIC	38°40′29.26″S, 145°37′2.65″E	GU289262	GU289362
WELT A031491	*C. polycystidea*	Coles Bay, Tasmania	42° 7′35.52″S, 148°17′32.09″E	GU289298	GU289383
WELT A031490	*C. polycystidea*	Port Fairy VIC	38°23′42.65″S, 142°14′35.99″E	GU289284	GU289373
WELT ASZ243A	*C. retorta*	Cape Jaffa SA	36°56′30.94″S, 139°40′30.93″E	GU289309	-
WELT ASZ243B	*C. retorta*	Cape Jaffa SA	36°56′30.94″S, 139°40′30.93″E	GU289310	-
WELT A031546	*C. retorta*	Cape Paterson VIC	38°40′29.26″S, 145°37′2.65″E	GU289258	-
WELT A031545	*C. retorta*	Cape Paterson VIC	38°40′29.26″S, 145°37′2.65″E	GU289259	GU289359

Table 4.1. (continued)

Herbarium voucher number	Species	Location	Latitude, Longitude	GenBank Accession numbers	
WELT A031547	*C. retorta*	Coles Bay, Tasmania	42° 7′35.52″S, 148°17′32.09″E	GU289297	-
WELT A031550	*C. retorta*	Point Roadknight VIC	38°25′40.72″S, 144°11′7.78″E	GU289273	-
WELT A031548	*C. retorta*	Point Roadknight VIC	38°25′40.72″S, 144°11′7.78″E	GU289275	GU289369
WELT A031549	*C. retorta*	Squeaky Beach, Wilsons Promontory VIC	39° 1′45.89″S, 146°18′24.24″E	GU289278	-
WELT A030919	*C. retroflexa*	Akaroa, Banks Peninsula, NZ	43°49′49.58″S, 172°54′30.03″E	GU289246	-
WELT A031488	*C. retroflexa*	Blackmans Beach, Tasmania	43° 0′27.26″S, 147°19′44.06″E	GU289288	GU289377
WELT A031534	*C. retroflexa*	Coles Bay, Tasmania	42° 7′35.52″S, 148°17′32.09″E	GU289296	-
WELT A031537	*C. retroflexa*	Coles Bay, Tasmania	42° 7′35.52″S, 148°17′32.09″E	GU289324	-
WELT A031531	*C. retroflexa*	Depot Beach NSW	35°37′37.91″S, 150°19′37.27″E	GU289252	-
WELT A030917	*C. retroflexa*	Island Bay, Wellington, NZ	41°20′51.22″S, 174°46′9.77″E	GU289329	-
WELT A030934	*C. retroflexa*	Kaiti Beach, Gisborne NZ	38°40′55.97″S, 178° 1′45.79″E	GU289333	-
WELT A030918	*C. retroflexa*	Mahia, East Coast Road, Eastland NZ	39° 5′14.33″S, 177°55′46.40″E	GU289331	-
WELT A031533	*C. retroflexa*	Point Lonsdale VIC	38°17′20.52″S, 144°36′4.42″E	GU289269	-

Table 4.1. (continued)

Herbarium voucher number	Species	Location	Latitude, Longitude	GenBank Accession numbers	
WELT A030928	*C. retroflexa*	Porpoise Bay, Otago NZ	46°39′39.37″S, 169° 6′29.32″E	GU289340	-
WELT A031536	*C. retroflexa*	Port Fairy VIC	38°23′42.65″S, 142°14′35.99″E	GU289342	-
WELT A030914	*C. retroflexa*	Princess Bay, Wellington NZ	41°20′43.05″S, 174°47′14.08″E	GU289325	-
WELT A030913	*C. retroflexa*	Princess Bay, Wellington NZ	41°20′43.05″S, 174°47′14.08″E	GU289326	-
WELT A030926	*C. retroflexa*	Princess Bay, Wellington NZ	41°20′43.05″S, 174°47′14.08″E	GU289232	GU289345
A085	*C. retroflexa*	Princess Bay, Wellington NZ	41°20′43.05″S, 174°47′14.08″E	GU289233	GU289346
WELT A031535	*C. retroflexa*	Sorrento, VIC	38°20′5.35″S, 144°44′33.96″E	GU289268	-
WELT A031530	*C. retroflexa*	Sorrento, VIC	38°20′5.35″S, 144°44′33.96″E	GU289267	GU289366
WELT A030900	*C. retroflexa*	Te Raekaihau, Wellington NZ	41°20′50.92″S, 174°47′29.01″E	GU289238	GU289349
WELT A030932	*C. retroflexa*	Tokomaru Bay, Eastland, NZ	38° 6′35.83″S, 178°21′18.27″E	GU289328	-
WELT A030930	*C. sp.* cf. *scalaris*	Ahuriri Street, Napier NZ	39°28′42.28″S, 176°54′12.89″E	GU289312	-
WELT A030919	*C. scalaris*	Akaroa, Banks Peninsula NZ	43°49′49.58″S, 172°54′30.03″E	GU289245	-
WELT A031346	*C. scalaris*	Kaingaroa, Chatham Islands	43°43′51.20″S, 176°16′2.26″W	GU289303	GU289385
WELT ASG207	*C. scalaris*	Manukau Reef, Chatham Islands	44° 2′4.90″S, 176°19′44.31″W	GU289241	GU289350

Table 4.1. (continued)

Herbarium voucher number	Species	Location	Latitude, Longitude	GenBank Accession numbers	
WELT A030922	*C. scalaris*	Ocean Beach, Jackson Bay NZ	43°57′54.44″S, 168°36′21.70″E	GU289243	GU289351
WELT A031345	*C. scalaris*	Point Weeding, Chatham Islands	43°56′32.08″S, 176°34′14.74″W	GU289302	-
WELT A030909	*C. scalaris*	Point Weeding, Chatham Islands	43°56′32.08″S, 176°34′14.74″W	GU289335	-
WELT A030908	*C. scalaris*	Point Weeding, Chatham Islands	43°56′32.08″S, 176°34′14.74″W	GU289336	-
WELT A030910	*C. scalaris*	Point Weeding, Chatham Islands	43°56′32.08″S, 176°34′14.74″W	GU289337	-
WELT A031345	*C. scalaris*	Point Weeding, Chatham Islands	43°56′32.08″S, 176°34′14.74″W	GU289319	GU289391
WELT A030925	*C. scalaris*	Pukerua Bay NZ	41° 1′28.36″S, 174°54′5.54″E	GU289235	-
A596*	*C. scalaris*	Ripapa Island, Banks Peninsula NZ	43°37′7.12″S, 172°45′13.27″E	GU289244	-
WELT A030921	*C. scalaris*	Riversdale, Wairarapa NZ	41° 6′24.49″S, 176° 4′10.73″E	GU289339	-
B176*	*C. scalaris*	Auckland Islands, NZ	50°41′5.44″S, 166°17′55.93″E	GU289301	-
WELT A031342	*C. scalaris*	Te Raekaihau, Wellington NZ	41°20′50.92″S, 174°47′29.01″E	GU289313	-
WELT A031341	*C. scalaris*	Te Raekaihau, Wellington NZ	41°20′50.92″S, 174°47′29.01″E	GU289314	-
WELT A031344	*C. scalaris*	Te Raekaihau, Wellington NZ	41°20′50.92″S, 174°47′29.01″E	GU289315	-

* Vouchers for these sites were lost as a result of a chemical spillage accident.

Table 4.1. (continued)

Herbarium voucher number	Species	Location	Latitude, Longitude	GenBank Accession numbers	
A318*	*C. scalaris*	Tumbledown Bay, Banks Peninsula NZ	43°51′27.57″S, 172°46′13.01″E	GU289242	-
A154*	*C. scalaris*	Waipapa Point, Kaikoura NZ	42°12′40.90″S, 173°52′28.64″E	GU289236	GU289347
WELT A030911	*C. scalaris*	Waitangi, Chatham Islands	43°56′41.58″S, 176°33′41.78″W	GU289334	-
WELT A031502	*C. siliquosa*	Duttons Beach, Portland VIC	38°19′42.74″S, 141°36′22.27″E	GU289307	-
WELT A031506	*C. siliquosa*	Lonsdale VIC	38°17′5.30″S, 144°36′54.74″E	GU289265	GU289365
WELT A031505	*C. siliquosa*	Point Roadknight VIC	38°25′40.72″S, 144°11′7.78″E	GU289274	-
WELT A031503	*C. siliquosa*	Port Campbell VIC	38°37′14.84″S, 142°59′32.69″E	GU289280	GU289371
WELT A031504	*C. siliquosa*	Robe SA	37° 9′23.33″S, 139°44′56.32″E	GU289322	GU289392
WELT A028492	*C. sp. cf. C. scalaris*	Wilson Bay, Coromandel NZ	36°53′22.06″S, 175°25′32.12″E	GU289320	-
WELT A031526	*C. subfarcinata*	Bicheno, Tasmania	41°52′29.89″S, 148°18′40.76″E	GU289293	GU289381
WELT A031518	*C. subfarcinata*	Cape Paterson VIC	38°40′29.26″S, 145°37′2.65″E	GU289260	GU289360
WELT A031521	*C. subfarcinata*	Dora Bay, Tasmania	41°15′16.35″S, 148°19′4.67″E	GU289295	GU289382

Table 4.1. (continued)

Herbarium voucher	Species	Location	Latitude, Longitude	GenBank Accession numbers	

number					
WELT A031522	*C. subfarcinata*	Duttons Beach, Portland VIC	38°19′42.74″S, 141°36′22.27″E	GU289276	-
WELT A031520	*C. subfarcinata*	Duttons Beach, Portland VIC	38°19′42.74″S, 141°36′22.27″E	GU289341	-
WELT A031528	*C. subfarcinata*	Lonsdale VIC	38°17′5.30″S, 144°36′54.74″E	GU289323	-
WELT A031532	*C. retroflexa*	Mimosa Rocks, Arragunnu NSW	36°35′2.07″S, 150° 3′15.18″E	GU289254	GU289355
WELT A031523	*C. subfarcinata*	Observation Head, Batemans Bay NSW	35°43′45.50″S, 150°12′30.26″E	GU289253	GU289354
WELT A031519	*C. subfarcinata*	Point Roadknight VIC	38°25′40.72″S, 144°11′7.78″E	GU289272	-
WELT A031523	*C. subfarcinata*	Point Roadknight VIC	38°25′40.72″S, 144°11′7.78″E	GU289271	GU289368
WELT A031525	*C. subfarcinata*	Port Campbell VIC	38°37′14.84″S, 142°59′32.69″E	GU289343	-
WELT A031514	*C. subfarcinata*	Port Campbell VIC	38°37′14.84″S, 142°59′32.69″E	GU289287	GU289376
WELT A031517	*C. subfarcinata*	Port Fairy VIC	38°23′42.65″S, 142°14′35.99″E	GU289344	-
WELT A031516	*C. subfarcinata*	Port Fairy VIC	38°23′42.65″S, 142°14′35.99″E	GU289282	GU289372
WELT A031524	*C. subfarcinata*	Port Fairy VIC	38°23′42.65″S, 142°14′35.99″E	GU289289	GU289378
WELT A031527	*C. subfarcinata*	Squeaky Beach, Wilsons Promontory VIC	39° 1′45.89″S, 146°18′24.24″E	GU289321	-
WELT A031515	*C. subfarcinata*	Squeaky Beach, Wilsons Promontory VIC	39° 1′45.89″S, 146°18′24.24″E	GU289277	GU289370

Table 4.1. (continued)

Herbarium voucher number	Species	Location	Latitude, Longitude	GenBank Accession numbers	
WELT A031529	*C. subfarcinata*	Ulladulla, NSW	35°21′37.65″S, 150°29′5.55″E	GU289250	-
WELT A031544	*C. torulosa*	Cape Paterson VIC	38°40′29.26″S, 145°37′2.65″E	GU289308	-
WELT A031543	*C. torulosa*	Coles Bay, Tasmania	42° 7′35.52″S, 148°17′32.09″E	GU289294	-
WELT A031540	*C. torulosa*	Dora Bay, Tasmania	41°15′16.35″S, 148°19′4.67″E	GU289305	-
WELT A031538	*C. torulosa*	Eaglehawk Neck, Tasmania	43° 0′32.91″S, 147°55′59.87″E	GU289292	GU289380
WELT A030912	*C. torulosa*	Maraehako Bay, Bay of Plenty NZ	37°40′19.74″S, 177°47′47.80″E	GU289300	-
WELT A030915	*C. torulosa*	Nugget Point, Otago NZ	46°26′26.91″S, 169°48′1.78″E	GU289240	-
WELT A030920	*C. torulosa*	Pihama, Taranaki, NZ	39°31′16.48″S, 173°54′54.98″E	GU289304	GU289386
WELT A031539	*C. torulosa*	Point Roadknight VIC	38°25′40.72″S, 144°11′7.78″E	GU289270	GU289367
WELT A031541	*C. torulosa*	Port Fairy VIC	38°23′42.65″S, 142°14′35.99″E	GU289283	-
WELT A030916	*C. torulosa*	Princess Bay, Wellington NZ	41°20′43.05″S, 174°47′14.08″E	GU289327	-
WELT A030924	*C. torulosa*	Pukerua Bay NZ	41° 1′28.36″S, 174°54′5.54″E	GU289234	-
WELT A031542	*C. torulosa*	Squeaky Beach, Wilsons Promontory VIC	39° 1′45.89″S, 146°18′24.24″E	GU289256	GU289357

Table 4.1. (continued)

Herbarium voucher number	Species	Location	Latitude, Longitude	GenBank Accession numbers	
WELT A031347	*C. torulosa*	Te Raekaihau, Wellington NZ	41°20′50.92″S, 174°47′29.01″E	GU289239	-
WELT A030931	*Landsburgia quercifolium*	Pihama, Taranaki, NZ	39°31′16.48″S, 173°54′54.98″E	GU289299	GU289384

Table 4.2. Key morphological characters used in species assignments.

Species	Primary axis	Primary branching	Branching of laterals	Receptacles	Vesicles
C. congesta	Fairly straight, compressed, 4–6(–10) mm broad × 2–4 mm thick	Alternately distichously branched from face of the main axes, Secondary axes usually 20–50 mm long forming crowded tufts	Irregularly branched on all sides	Slightly compressed, 10–30 mm long × 1–1.5 (–2) mm wide, usually with sterile apical awn	Subspherical, 3–7(–10) mm diameter, occasionally absent
C. distenta	Strongly zig-zag, flattened, 2–5 mm broad × 1.5–2 mm thick	Alternately distichously branched from face of the main axes	Alternately distichously branched with rounded axils	Compressed, torulose with rows of prominent conceptacles, 10–40(–50) mm long × 1.5–3 mm broad	Spherical to subspherical, 4–10 mm diameter
C. expansa	Fairly straight, flattened, 2–5(–10) mm broad × 1–2(–4) mm thick	Alternately distichously branched from the face of the main axes	Tristichously branched from a three-sided rhachis	Terete to slightly compressed, slender, 0.5–1(–1.5) mm long × 0.5–1(–1.5) mm diameter	Small, elongate-ellipsoid, 3–5(–7) long × 1–2 mm broad
C. monilifera	Flattened, 3–8(–15) mm broad × 1–2 mm thick, with narrow edges	Alternately distichously branched from the face of the main axes	Tristichously branched from a subterete rhachis	Terete, slender, (5–)10–20(–30) long × 0.5–1 mm diameter, constricted between conceptacles	Subspherical to ovoid, 3–6(–8) mm diameter
C. moniliformis	Straight, strongly compressed, 5–20 mm broad × 2–3(–7) mm thick	Alternately distichously branched from the edge of the main axes	Alternately distichously branched, fasciculate	Moniliform to torulose, 5–15(–40) long × 0.5–1 mm diameter	Absent

Table 4.2 (Continued)

Species	Primary axis	Primary branching	Branching of laterals	Receptacles	Vesicles
C. platylobium	Strongly compressed, flexuous, (3–)5–10(–15) mm broad × 2–4(–5) mm thick	Alternately distichously branched from the edge of the main axes	Alternately distichously branched, complanate, once pinnate	Strongly flattened, lanceolate to elliptic-lanceolate, basally stalked, 10–30 mm long × 3–8 (–10) mm broad	Spherical to ovoid, 5–15 mm diameter
C. polycystidea	Compressed, quadrangular, 2–7 mm broad × 2–5 mm thick	Alternately distichously branched from the face of the main axes	Radially and spirally branched on all sides	Distantly moniliform, 5–20 mm long and 0.4–0.7(– 1) mm diameter at conceptacles	Numerous, small, elongate, tapering at both ends, in clusters, (2–)3–5(–7) mm long × (1–)2–3(–4) mm diameter
C. retorta	Relatively straight, strongly compressed, 2–5(–7) mm broad × 1–2 mm thick	Alternately distichously branched from the face of the main axes	Alternately distichously branched, becoming subdichotomous with broadly rounded axils	Terete to slightly compressed, (10–)20–50(–80) long × 1–2 mm diameter, conceptacles bisexual, often separated by considerable sterile tissue	Usually absent
C. retroflexa	Straight to zig-zag, compressed, 3 – 10 mm broad ×2 – 5 mm thick, narrow edged	Alternately distichously branched from the face of the main axes	Terete, irregularly branched on all sides	Compressed, smooth to slightly torulose, 20 – 50(– 60) mm long × 1 – 2(– 2.5) mm broad, apex attenuate with sterile awn, ostiole in axils of conceptacle swelling.	Ovoid to subspherical, 4–10 mm long ×3–6 mm broad
C. scalaris	Zig-zag, compressed, 2.5–15(–20) mm broad × 2–3 mm thick	Alternately distichously branched from face of main axes	Alternately distichously branched, complanate	Ovoid to compressed, smooth to torulose, 1.5–2.5 mm wide × 1 mm thick	Spherical to ovoid 2–7(–10) mm diameter
C. siliquosa	Straight, quadrangular to square in section, 2–5 mm broad and thick	Alternately distichously branched from the face of the main axes	Alternately distichously branched, becoming subdichotomous with rounded axils	Slightly to markedly compressed, 30–80 mm long ×1–2 mm broad, conceptacles unisexual and thalli dioecious	Absent

Table 4.2 (Continued)

Species	Primary axis	Primary branching	Branching of laterals	Receptacles	Vesicles
C. cuspidata	Fairly straight, compressed, 3–6 mm broad × 1–2 thick	Alternately distichously branched from the face of the main axes	Usually irregularly alternately (sometime sub-distichously) and closely branched.	Simple or branched, (10–)20–60(–80) mm long with prominent swollen conceptacles closely arranged in 2–3 rows in the basal half or more of receptacles, and here 1.5–4 mm broad	Absent
C. subfarcinata	Slightly flexuous, compressed, 2–7 mm broad × 1–2(–3) thick	Densely and distichously branched from the face of the main axes	Irregularly alternately branched on all sides	Often branched, (5–)10–30(–50) mm long × 1–2 mm thick at conceptacles, with prominent scattered conceptacles separated by sterile tissue with a sterile awn, ostiole apical on conceptacle swelling.	Present or absent, elongate ovoid to sub spherical, 2–04(–6) mm long × 2–3(–4) mm diameter
C. torulosa	Compressed, 3–10 mm broad × 2–5 mm thick, straight to slightly flexuous	Alternately densely branched from the face of the main axes	Densely, irregularly alternately subdistichously branched	Terete to slightly three sided, swollen with a blunt rounded apex, 10–50(–70) mm long × (1–)2–3(–4) mm diameter	Subspherical to ovoid, 3–8(–10) long × 3–6(–9) mm diameter

4.4 Results

Amplification and species sampling

The *cox*1 region was amplified from 108 specimens of 13 species of *Cystophora* and five specimens of putative outgroup genera (*Caulocystis*, *Carpoglossum* and *Landsburgia*) (Table 4.1). I was unable to amplify the *cox*1 region of *Cystophora expansa* (Areschoug) Womersley with any primer combination and suspect a primer mismatch. The GAZF2–fcox808R primer combination successfully amplified most *Cystophora* species, whereas the GAZF2–GAZR2 combination was successful when amplifying *Caulocystis*, *Carpoglossum*, *Landsburgia* and other outgroup species.

The ITS1–5.8S–ITS2 cistron was amplified from 47 specimens of *Cystophora* and from *Landsburgia quercifolia* (Hooker *f. et* Harvey) Harvey. Preliminary trees positioned *Landsburgia* as the genus most closely related to *Cystophora* and *L. quercifolia* was designated as an outgroup in subsequent analyses.

Molecular distance

A distance tree inferred from *cox*1 sequences showed clusters that were generally consistent with morphological species assignments. Sequences from four specimens of *Cystophora* were incongruent with morphological assignments (indicated by arrows in Fig. 4.2). DNA from these specimens was subsequently re-extracted and sequenced, with the same results. In two of these specimens (WELT A031539 and WELT A031523) morphological identification was unambiguous and this identification is retained. Two specimens (WELT A028492 and WELT A030930) were initially assigned to *C. scalaris* J. Agardh but grouped with *C. torulosa* (R. Brown) J. Agardh and *C. retroflexa* (Labillardière) J. Agardh respectively. These specimens were re-examined and as identity is ambiguous I designated them as *Cystophora sp.* cf. *scalaris.*

Uncorrected interspecific pairwise differences between *cox*1 sequences in *Cystophora* varied from 0 to 49 (0–8%). Intraspecific distances varied from 0 to 4 (0–0.65%). Kimura 2-parameter distances ranged from 0 to 8.6% between species and 0 to 0.7% within species (Table 4.2, Fig. 4.2). In general, species with wide geographic ranges (e.g., *C. retorta* (Mertens) J. Agardh, *C. retroflexa*) showed greater intraspecific variation than geographically restricted species (e.g., *C. monilifera* J. Agardh).

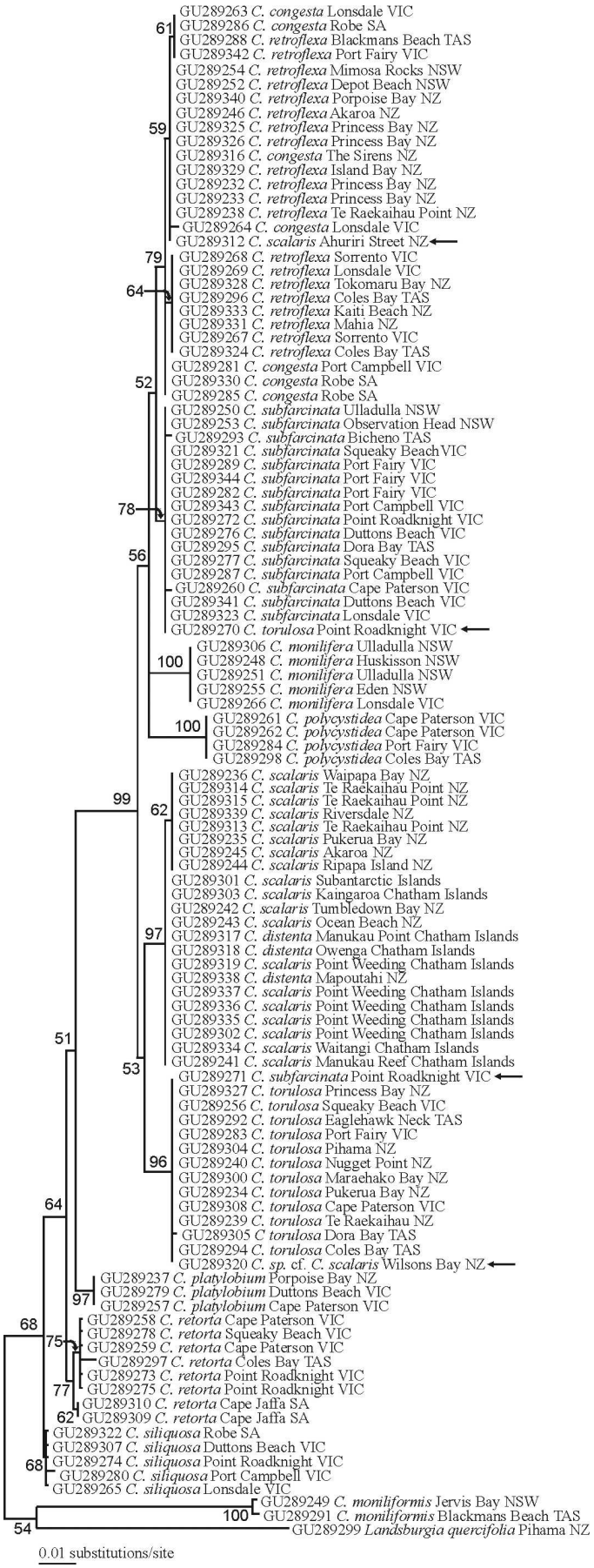
GU289263 C. congesta Lonsdale VIC
GU289286 C. congesta Robe SA
GU289288 C. retroflexa Blackmans Beach TAS
GU289342 C. retroflexa Port Fairy VIC
GU289254 C. retroflexa Mimosa Rocks NSW
GU289252 C. retroflexa Depot Beach NSW
GU289340 C. retroflexa Porpoise Bay NZ
GU289246 C. retroflexa Akaroa NZ
GU289325 C. retroflexa Princess Bay NZ
GU289326 C. retroflexa Princess Bay NZ
GU289316 C. congesta The Sirens NZ
GU289329 C. retroflexa Island Bay NZ
GU289232 C. retroflexa Princess Bay NZ
GU289233 C. retroflexa Princess Bay NZ
GU289238 C. retroflexa Te Raekaihau Point NZ
GU289264 C. congesta Lonsdale VIC
GU289312 C. scalaris Ahuriri Street NZ
GU289268 C. retroflexa Sorrento VIC
GU289269 C. retroflexa Lonsdale VIC
GU289328 C. retroflexa Tokomaru Bay NZ
GU289296 C. retroflexa Coles Bay TAS
GU289333 C. retroflexa Kaiti Beach NZ
GU289331 C. retroflexa Mahia NZ
GU289267 C. retroflexa Sorrento VIC
GU289324 C. retroflexa Coles Bay TAS
GU289281 C. congesta Port Campbell VIC
GU289330 C. congesta Robe SA
GU289285 C. congesta Robe SA
GU289250 C. subfarcinata Ulladulla NSW
GU289253 C. subfarcinata Observation Head NSW
GU289293 C. subfarcinata Bicheno TAS
GU289321 C. subfarcinata Squeaky BeachVIC
GU289289 C. subfarcinata Port Fairy VIC
GU289344 C. subfarcinata Port Fairy VIC
GU289282 C. subfarcinata Port Fairy VIC
GU289343 C. subfarcinata Port Campbell VIC
GU289272 C. subfarcinata Point Roadknight VIC
GU289276 C. subfarcinata Duttons Beach VIC
GU289295 C. subfarcinata Dora Bay TAS
GU289277 C. subfarcinata Squeaky Beach VIC
GU289287 C. subfarcinata Port Campbell VIC
GU289260 C. subfarcinata Cape Paterson VIC
GU289341 C. subfarcinata Duttons Beach VIC
GU289323 C. subfarcinata Lonsdale VIC
GU289270 C. torulosa Point Roadknight VIC
GU289306 C. monilifera Ulladulla NSW
GU289248 C. monilifera Huskisson NSW
GU289251 C. monilifera Ulladulla NSW
GU289255 C. monilifera Eden NSW
GU289266 C. monilifera Lonsdale VIC
GU289261 C. polycystidea Cape Paterson VIC
GU289262 C. polycystidea Cape Paterson VIC
GU289284 C. polycystidea Port Fairy VIC
GU289298 C. polycystidea Coles Bay TAS
GU289236 C. scalaris Waipapa Bay NZ
GU289314 C. scalaris Te Raekaihau Point NZ
GU289315 C. scalaris Te Raekaihau Point NZ
GU289339 C. scalaris Riversdale NZ
GU289313 C. scalaris Te Raekaihau Point NZ
GU289235 C. scalaris Pukerua Bay NZ
GU289245 C. scalaris Akaroa NZ
GU289244 C. scalaris Ripapa Island NZ
GU289301 C. scalaris Subantarctic Islands
GU289303 C. scalaris Kaingaroa Chatham Islands
GU289242 C. scalaris Tumbledown Bay NZ
GU289243 C. scalaris Ocean Beach NZ
GU289317 C. distenta Manukau Point Chatham Islands
GU289318 C. distenta Owenga Chatham Islands
GU289319 C. scalaris Point Weeding Chatham Islands
GU289338 C. distenta Mapoutahi NZ
GU289337 C. scalaris Point Weeding Chatham Islands
GU289336 C. scalaris Point Weeding Chatham Islands
GU289335 C. scalaris Point Weeding Chatham Islands
GU289302 C. scalaris Point Weeding Chatham Islands
GU289334 C. scalaris Waitangi Chatham Islands
GU289241 C. scalaris Manukau Reef Chatham Islands
GU289271 C. subfarcinata Point Roadknight VIC
GU289327 C. torulosa Princess Bay NZ
GU289256 C. torulosa Squeaky Beach VIC
GU289292 C. torulosa Eaglehawk Neck TAS
GU289283 C. torulosa Port Fairy VIC
GU289304 C. torulosa Pihama NZ
GU289240 C. torulosa Nugget Point NZ
GU289300 C. torulosa Maraehako Bay NZ
GU289234 C. torulosa Pukerua Bay NZ
GU289308 C. torulosa Cape Paterson VIC
GU289239 C. torulosa Te Raekaihau NZ
GU289305 C torulosa Dora Bay TAS
GU289294 C. torulosa Coles Bay TAS
GU289320 C. sp. cf. C. scalaris Wilsons Bay NZ
GU289237 C. platylobium Porpoise Bay NZ
GU289279 C. platylobium Duttons Beach VIC
GU289257 C. platylobium Cape Paterson VIC
GU289258 C. retorta Cape Paterson VIC
GU289278 C. retorta Squeaky Beach VIC
GU289259 C. retorta Cape Paterson VIC
GU289297 C. retorta Coles Bay TAS
GU289273 C. retorta Point Roadknight VIC
GU289275 C. retorta Point Roadknight VIC
GU289310 C. retorta Cape Jaffa SA
GU289309 C. retorta Cape Jaffa SA
GU289322 C. siliquosa Robe SA
GU289307 C. siliquosa Duttons Beach VIC
GU289274 C. siliquosa Point Roadknight VIC
GU289280 C. siliquosa Port Campbell VIC
GU289265 C. siliquosa Lonsdale VIC
GU289249 C. moniliformis Jervis Bay NSW
GU289291 C. moniliformis Blackmans Beach TAS
GU289299 Landsburgia quercifolia Pihama NZ
0.01 substitutions/site

Fig. 4.3. ML topology of *Cystophora* species inferred using combined ITS1–5.8S–ITS2 and *cox*1 sequences, support is shown at nodes as maximum parsimony/maximum likelihood/Bayesian posterior probabilities. Specimen labels show GenBank accession numbers for ITS sequences only.

Fig. 4.2. (Previous page). Distance tree inferred in PAUP* by neighbour-joining from 108 *cox*1 sequences of *Cystophora* and one outgroup. Confidence is shown at nodes based on 1000 bootstrap replicates. Arrows indicate specimens where morphology and sequence position were incongruent.

Table 4.3. Number of changes in *cox*1 sequences between species (above diagonal) and K2P distances (below diagonal)

	C. congesta	*C. monilifera*	*C. moniliformis*	*C. platylobium*	*C. polycystidea*	*C. retorta*	*C. retroflexa*	*C. scalaris*	*C. siliquosa*	*C. subfarcinata*	*C. tor*
C. congesta	0–2	9–11	47–48	16–18	11–13	16–20	0–3	8–11	18–21	3–6	10–13
C. monilifera	0.015–0.018	0 (0.00)	49	18	14	19–22	10–11	13–14	20–21	8–9	13–14
C. moniliformis	0.082–0.084	0.086	2 (0.003)	41–43	51	40–43	47–48	46	37–40	47–49	48–49
C. platylobium	0.027–0.030	0.030	0.071–0.075	0 (0.00)	19	6–9	17–18	16–17	8–9	15–16	15–16
C. polycystidea	0.018–0.022	0.023	0.090	0.032	0 (0.00)	20–23	12–13	15–16	23–24	10–11	13–14
C. retorta	0.029–0.034	0.032–0.037	0.069–0.075	0.010–0.015	0.035–0.039	0–4 (0.00–0.07)	17–20	16–19	7–10	16–20	16–20
C. retroflexa	0.00–0.005	0.017–0.018	0.082–0.084	0.027–0.030	0.020–0.22	0.028–0.032	0–3 (0.00–0.005)	8–10	19–21	4–6	11–13
C. scalaris/distenta	0.013–0.018	0.022–0.023	0.080	0.028–0.027	0.025–0.027	0.027–0.032	0.013–0.017	0–1 (0.00–0.002)	18–20	9–11	8–10
C. siliquosa	0.030–0.035	0.034–0.035	0.064–0.069	0.013–0.015	0.039–0.041	0.012–0.015	0.032–0.034	0.030–0.034	0–1 (0.00–0.002)	19–21	19–21
C. subfarcinata	0.002–0.010	0.013–0.015	0.082–0.086	0.025–0.027	0.017–0.018	0.027–0.034	0.007–0.010	0.015–0.018	0.032–0.035	0–2 (0.00–0.003)	9–11
C. torulosa	0.017–0.022	0.022–0.023	0.084–0.086	0.025–0.027	0.032–0.035	0.029–0.034	0.018–0.022	0.015–0.017	0.034–0.035	0.015–0.018	0–1 (0.002)

Distances between closely related species were often low, in the same order as intraspecific distances. In some cases these species were easily distinguished by morphology (e.g., *C. retorta* and *C. platylobium* (Mertens) J. Agardh), in other cases they were not (*C. siliquosa* J. Agardh and *C. retorta*, *C. retroflexa* and *C. subfarcinata* (Mertens) J. Agardh).

Phylogeny

A partition homogeneity test showed significant congruence between ITS and *cox*1 data sets (P = 0.999). Analyses of combined data produced trees with good support for most branches, but did not separate *C. congesta/retroflexa* and *C. scalaris/distenta*, and did not show support for the position of *C. platylobium* (Fig. 4.3).

Cystophora moniliformis (Esper) Womersley & Nizamuddin is sister to all other *Cystophora* species. The early branching position of *C. moniliformis* is consistent with the rather anomalous form of this species (a straight, dorsiventrally flattened main axis, with fine laterals developing from the sides of the main axes). The remaining species formed two groups, with *C. platylobium* sister to a group containing *C. retorta* and *C. siliquosa*, but with only weak support, and this group is sister to a larger group containing all other species. Species level clades were well supported within this latter group, but the higher level branching was only weakly supported (61–68%).

4.5 Discussion

Few studies have attempted *cox*1 DNA barcoding of brown algae. These studies have either not discriminated species (Lane *et al.* 2007), not sampled across species ranges, risking underestimating intraspecific divergence (Kucera & Saunders 2008), or have sampled a limited number of congeners (McDevit & Saunders 2009). While using DNA sequences to identify algae is an established technique (e.g., Sasaki *et al.* 2003; Lane & Saunders 2005; Fox & Swanson 2007), a prerequisite for extending this case-by-case molecular identification into a DNA barcoding system is to firmly establish the utility of one or more markers for delimiting a wide range of species. I discuss the utility of *cox*1 barcoding in *Cystophora* according to the criteria outlined in the introduction:

1. Does the marker resolve discrete clusters within a genus? Yes, a 615 bp fragment from the *cox*1 was sufficient to resolve clusters, albeit with little bootstrap support, and in some cases, with low molecular distances between clusters.

2. Are clusters concordant with recognised species boundaries? Most specimens collected in this study could be assigned to species using *cox*1 sequences alone. In two cases, closely related species could not be separated by *cox*1 or combined *cox*1–ITS (*C. distenta*-*C. scalaris*, *C. retroflexa*-*C. congesta*). In several cases, interspecific variation between morphologically distinct species was only slightly greater than intraspecific variation. This is unsatisfactory if distance methods alone are used to define species boundaries.

3. Is sampling sufficient to represent intraspecific variation across the range of each species? Sampling of species that occur in New Zealand was reasonably intensive and widespread, which strengthens confidence in *cox*1 as an estimator of species identity, estimates of intraspecific variation in Australian species is likely to be low because of limited sampling of Australian populations. Further sampling of these species might unearth novel haplotypes that are difficult to assign to species.

4. Is sampling of congeners sufficient to show resolution of closely related species? Some western Australian *Cystophora* species and some deepwater species were not sampled. I was unable to amplify *C. expansa* with any primer combination. *Cystophora expansa* is morphologically similar to *C. monilifera*, and I am unable to evaluate *cox*1 for discriminating these species. However species sampled included several pairs of sister species, an important consideration for evaluating a barcoding marker.

Distance methods

McDevit & Saunders (2009) suggested that the ten-fold threshold between intraspecific and interspecific variation proposed by Hebert *et al.* (2004) for separating species with *cox*1 could be applied to brown algae. In *Cystophora, C. moniliformis* was highly divergent from most other species, with pairwise distances between all other species exceeding the ten-fold threshold. Species in which low intraspecific variation was detected were also separated from most other congeners by this threshold. However, species with moderate intraspecific variation (*C. retorta, C retroflexa* and *C. subfarcinata*) often showed distances to closely related congeners of around twice intraspecific distances. A lower threshold must be applied if this method

is used to separate species in *Cystophora*. The appeal of distance thresholds is that species can be efficiently demarcated from large datasets using sequence data alone and automated using algorithms. It is generally recognised that a single threshold value cannot be applied to all groups of organisms (Vogler & Monaghan 2006), but establishing an operational threshold requires extensive sampling to accurately estimate intraspecific marker variation. Mitochondrial DNA variation in brown algae is often highly spatially structured (Hoarau *et al.* 2007; Fraser *et al.* 2009; Uwai *et al.* 2009; Chapter 2) so sampling has to be intensive within populations and, ideally, across the range of the species. Only Lane *et al*. (2007) have applied barcoding methods to an intensively sampled collection of brown algae and they failed to delineate species. Kucera & Saunders (2008) used multiple samples of *Fucus* species, but only sampled in Canada where their most clearly delineated species, *F. serratus*, is a recent introduction (Lyons & Scheibling 2009). While I sampled across the range of several *Cystophora* species, it is likely that intraspecific variation is underestimated.

Species level differences in intraspecific variation in *Cystophora* did not correlate with sampling effort. *Cox*1 variation was high in *C. subfarcinata* (n = 16) and *C. retroflexa* (n = 20) which were extensively sampled, but was also high in *C. retorta*, where sampling was limited (n = 8). Conversely low intraspecific variation was found in *C. scalaris* (n = 19) and *C. torulosa* (n = 12), but collections span mainland Australia, Tasmania and New Zealand. Intraspecific *cox*1 variation in brown algae appears to vary widely. Kucera & Saunders (2008) found low (0–0.3%) *cox*1 intraspecific variation in *Fucus*, albeit based on limited geographical sampling, whereas a worldwide study of *Durvillea antarctica* (Fraser *et al.* 2009) found relatively high *cox*1 diversity (uncorrected distances of up to 5.4%). Similar problems have been found when using *cox*1 to demarcate well sampled red algal species (Sherwood *et al.* 2008; Yang *et al.* 2008).

Geographically widespread sampling and the inclusion of sister species are expected to reduce variation thresholds between species, especially if the marker used is not evolving and sorting sufficiently quickly to demarcate species formed in recent radiations (Shaffer & Thomson 2007). McDevit & Saunders' (2009) survey of interspecies *cox*1 variation of brown algae did not include sister species or extensive intraspecies sampling (apart from *Fucus* sequences from Kucera & Saunders (2008)), and their support for a ten-times threshold in brown algae is likely to be an artefact of

low sampling of conspecifics and congenerics rather than an indicator of the efficacy of *cox*1 barcoding.

Character based DNA barcoding is an alternative to distance methods (DeSalle *et al.* 2005; Rach *et al.* 2008). This has the advantage of defining species by synapomorphies, rather than phenetic clustering, and is concordant with phylogenetic species concepts, but would also require wider geographical sampling of closely related species to ensure characters nominated are conserved in species.

Taxonomic oversplitting

Four *Cystophora* species were not delimited in any molecular analyses. No evidence was found for a distinct obligate epiphyte species equivalent to *C. cymodoceae*; no specimens could be unequivocally assigned to *C. cuspidata*, and specimens assigned to *C. congesta* and *C. distenta* grouped with other species in molecular analyses. Failure to separate species by molecular markers can be attributed to three processes: (1) insufficient time for stochastic lineage sorting processes (coalescence) to sort the marker to reciprocal monophyly; (2) ongoing gene flow by hybridisation; or (3) taxonomic over-splitting of species. In these cases taxonomic oversplitting (Dayrat 2005) is a probable cause.

Cystophora retroflexa (Labillardière) J. Agardh and *C. congesta* Womersley & Nizamuddin ex Womersley are morphologically similar and these two species were not separated in any molecular analysis (Figs. 4.2, 4.3). Rather, haplotypes and ribotypes from *C. congesta* and *C. retroflexa* were intermixed. Womersley (1964, 1987) separated these species by vegetative characters, but accepted that intergrades occurred. *Cystophora retroflexa* has longer (20–60 mm) receptacles and a more openly branched habit. *Cystophora congesta* has shorter (15–30 mm) receptacles and tufted laterals and ramuli, and usually ridges on the primary axes. Seven specimens were assigned to *C. congesta*, including three from the type locality (Robe, SA) but some species assigned to *C. retroflexa* (e.g., WELT A031533) tended toward a *C. congesta*-like morphology.

Cystophora scalaris and *C. distenta* did not separate in any molecular analysis. *Cystophora distenta* was established by J. Agardh based on material from the Chatham Islands (Agardh 1870). The species has been reported from Wellington, New Zealand southwards and the Chatham Islands (Lindauer *et al.* 1961). It is similar to *C. scalaris* but with longer, broader and more torulose receptacles, but dimensions

given by Womersley (1964) overlap (Table 4.2). Three specimens collected from the Chatham Islands and the South Island of New Zealand were considered sufficiently morphologically distinct to assign to *C. distenta*, other specimens showed intermediate morphologies and were assigned to *C. scalaris*.

I consider that morphological characters used to separate *C. distenta*/*C. scalaris* and *C. retroflexa*/*C. congesta* are continuous and in the absence of molecular divergence oversplitting is probable. These results should be regarded as provisional as other closely related species of brown algae cannot be separated using mitochondrial sequences, due to ongoing gene flow or incomplete lineage sorting, but can be resolved with microsatellite data (Bergström *et al.* 2005; Billard *et al.* 2005; Pereyra *et al.* 2009).

The southern Australian species *Cystophora subfarcinata* and *C. cuspidata* are separated according to the arrangement and development of conceptacles (Womersley 1987). *Cystophora cuspidata* has receptacles that are broader (1.5–4 mm) with the lower conceptacles less scattered and arranged in two or three rows. *Cystophora subfarcinata* has narrower ((5–)10–30(–50) mm long × 1–2 mm) receptacles with usually scattered conceptacles. These characters intergrade and some specimens (e.g., WELT A031517) could have been assigned to either species. Specimens resembling *C. cuspidata* did not cluster separately from *C. subfarcinata* in the molecular analyses. I agree with Womersley (1987) that *C. cuspidata* is probably only a form of *C. subfarcinata*, but I was unable to collect samples from the type locality of *C. cuspidata* (Encounter Bay, SA), and specimens sampled did not have conceptacles as prominent as those illustrated by Womersley 1987 (Fig. 149 H, p. 403). Therefore I do not reduce *C. cuspidata* to synonymy with *C. subfarcinata* but recommend further work on these species.

Revisions in species assignments

Some specimens were morphologically intermediate between *C. retroflexa* and *C. subfarcinata*, or between *C. retorta* and *C. siliquosa*, and preliminary morphological assignments were incongruent with *cox*1 sequence clusters. These specimens were re-examined and species assignments were revised using characters discussed below.

Cystophora retroflexa and *C. subfarcinata* can resemble one another when young, infertile or stunted, but fertile specimens could be distinguished using the shape of the conceptacle. In *C. subfarcinata* the conceptacle is spherical or

subspherical; in *Cystophora retroflexa* the conceptacles are elongate. Some specimens that were initially assigned to *C. retroflexa* clustered with *C. subfarcinata* in molecular analyses. Careful examination of conceptacles in these specimens allowed us to reassign them to *C. subfarcinata*, despite other characters being consistent with *C. retroflexa*.

Cystophora siliquosa and *C. retorta* share complanately branched laterals, rounded axils and relatively straight main axes. Presence of vesicles is a diagnostic character for *C. retorta*, but this is of limited value as vesicles are absent on some specimens (Womersley 1964, 1987). I could not distinguish species by the shape of the main axis, as suggested by Womersley (1964) (see Table 4.2), and found microscopic examination of conceptacles to determine monoecy/dioecy was required to confidently assign specimens to species.

In both these species pairs, low morphological divergence and plasticity made species assignments difficult. In these cases *cox*1 sequences provided a useful indication that more careful morphological examination was required.

Other markers

These results, together with similar low variation in previous studies (Lane *et al.* 2007; Kucera & Saunders 2008) suggests while *cox*1 has some utility for species identification in brown algae, it is too conserved to serve as a basis for a single marker barcoding system. Mitochondrial markers with sufficient resolution to discriminate closely related brown algae are rare, and low genetic divergence between recognised species of brown algae appears common (Van Oppen *et al.* 1993; Coyer *et al.* 2001, 2006; Stiger *et al.* 2000). A small proportion of errors (either failing to separate sister species, or recognising demes as species) might be acceptable in some applications of barcoding, where the trade-off is reduced time and cost (e.g., Moniz & Kaczmarska 2009), but if barcoding is to contribute usefully to brown algal taxonomy other candidate markers should be evaluated.

Screening the mitochondrial genome of *Saccharina* species (Yotsukura *et al.* 2009) found mitochondrial regions that are more variable than *cox*1 and these could be evaluated for species discrimination. In addition, the ITS1–5.8S–ITS2 cistron, or for ease of amplification, ITS2 alone, might be a useful addition to *cox*1 for species identification (see Coleman & Mai 1997 in Chlorophyta, Guillemin *et al.* 2008 and Hu *et al.* 2009 in Rhodophyta, Litaker *et al.* 2007 in dinoflagellates, Moniz &

Kaczmarska 2009, 2010 in diatoms). The use of ITS sequences for barcoding has been criticised because length differences between species create alignment problems (McDevit & Saunders 2009). However if barcoding is limited to species assignment (Ratnasingham & Hebert 2007) this is not problematic, as inability to produce an alignment can be regarded as strong evidence for separating species (Müller *et al.* 2009) and alignment methods that better handle indels (Steinke *et al.* 2005) or alignment-free methods of sequence comparison (Chu *et al.* 2006, 2009; Kuksa & Pavlovic 2009) can be used.

In conclusion, molecular data did not support the separate species status of *C. distenta* and *C. congesta*, and further work is required to confirm the separate status of *C. cuspidata* and *C. cymodoceae*. Other species were well supported by both morphological and molecular data. These results suggest that *cox*1 barcoding might have utility in brown algae where delimiting closely related or sister species is not an issue (for example many studies identifying invasive algae, or studies where generic identity of cryptic life stages is sufficient). Species identification by *cox*1 might also be useful in selected groups of brown algae or in restricted geographical areas, but reliance on this marker will require preliminary sampling across the area of interest with adequate sampling and corroboration by other data.

4.6 Acknowledgements

I thank the New Zealand Department of Conservation and Education NZ Study Abroad Scheme for funding assistance; Wendy Nelson, Tracy Farr, Kate Neill and the Department of Conservation Chatham Islands staff for assistance with collections and Alan Millar (National Herbarium of New South Wales) and Jenn Dalen (Museum of New Zealand Te Papa Tongarewa herbarium) for assistance with herbaria access.

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Chapter 5

Taxonomic oversplitting in the New Zealand members of the brown algal genus *Cystophora* (Phaeophyceae, Sargassaceae): Synonymisation of two species

5.1 Abstract

Six species of *Cystophora* are currently recognized in New Zealand waters. Molecular evidence supports four of these (*C. platylobium* (Mertens) J. Agardh, *C. retroflexa* (Labillardière) J. Agardh, *C. scalaris* J. Agardh and *C. torulosa* (R. Brown) J. Agardh), but data have failed to separate two currently accepted species from sister species: *Cystophora congesta* Womersley & Nizamuddin is a form of *C. retroflexa*, with short laterals and fascicled ramuli, and represents part of a range of morphological variation; *Cystophora distenta* J. Agardh is a form of *C. scalaris*, with wide, compressed receptacles, but receptacle dimensions do not separate species. I synonymise *C. congesta* with *C. retroflexa* and *C. distenta* with *C. scalaris* on the basis of available molecular and morphological data, and provide amended descriptions of these species and a key to New Zealand species of *Cystophora*.

5.2 Introduction

ALGAE OF THE GENUS *CYSTOPHORA* are a significant component of the New Zealand marine flora (Shears & Babcock 2007). *Cystophora* thalli are common in the shallow sub-tidal, rock pools and lower intertidal. They provide structure and shelter for other marine organisms (Taylor & Cole 1994, McDermott & Shima 2006), and are a significant contributor to primary productivity (Schiel 1990).

Six species of *Cystophora* are currently recognised in New Zealand (Womersley 1964, 1987, Adams 1994). *Cystophora torulosa* (R. Brown) J. Agardh and *C. retroflexa* (Labillardière) J. Agardh are widespread in New Zealand and southern Australia. *Cystophora platylobium* (Mertens) J. Agardh is found from Cook Strait southwards and in southern Australia. *Cystophora scalaris* J Agardh is found south of Cook Strait in New Zealand only. *Cystophora congesta* Womersley & Nizamuddin was described from Robe, South Australia, in 1964 and has been occasionally reported from New Zealand (Adams 1994, Nelson *et al.* 1992). Early collections from New Zealand include specimens determined as *C. dumosa* (Greville) J. Agardh, a name rejected as a *nomen confusum* by Womersley (1964), under the then current Montreal Code (Lanjouw *et al.* 1961) Art. 69 (although reported by Womersley as Art. 66). Womersley determined herbarium material used for the original description

(Sonder 1846) consisted of a mixture of species, and synonymised some of the material with *C. monilifera*. New Zealand specimens might be referable to *C. congesta* (Adams 1994). *Cystophora distenta* J. Agardh has been reported from the Chatham Islands (Agardh 1870, Lindauer *et al.* 1961) and other southern New Zealand locations (Nelson *et al*. 1991, 1992, Adams 1994). A detailed taxonomic history of *Cystophora* is provided by Womersley (1964, 1987).

Lindauer *et al.* (1961) also report doubtful records of four Australian species (including *Cystophora cephalornithos*, now *Caulocystis*) collected from New Zealand. These appear to be many mistakes in identification or incorrect recording of localities on early collections. Difficulties arise in species identification in *Cystophora* (Adams 1994). Lindauer *et al*. (1961) suggested two reasons for this: the state of dried herbarium material, which is often poorly preserved and incomplete, and because "within the New Zealand species a degree of hybridization occurs in certain areas where some of the species overlap" (p. 295). Lindauer *et al.* (1961) only consider *C. platylobium* as distinctive, as other species intergrade. Womersley (1964) also noted that the morphology of some specimens were intermediate between species. Further confusion has arisen from unclear synonymy and inadequate descriptions. Lindauer *et al.* (1961) describe specimens under the name *Cystophora dumosa.* Womersley (1964) rejected that name, and created a new species, *C. congesta,* containing many specimens previously assigned to *C. dumosa*. Adams' (1994, p. 141) illustration of *C. congesta* does not resemble Womersley's description, and keys in Lindauer *et al.* (1961) and Adams (1994) use different characters from Womersley (1964).

Cystophora congesta and *C. distenta* are rarely reported outside of the taxonomic literature. Surveys of the New Zealand coastlines by Morton & Miller (1968) and Shears & Babcock (2007) recorded *Cystophora platylobium*, *C. retroflexa*, *C. scalaris* and *C. torulosa*, but not *C. congesta* or *C. distenta*. A molecular study of the genus (Chapter 4) failed to separate *C. congesta* from *C. retroflexa* and *C. distenta* from *C. scalaris*. Here I evaluate the evidence for recognising six species of *Cystophora* in New Zealand, provide additional morphological and molecular data, and formally synonymise the species *C. congesta* and *C. distenta*.

Table 5.1. Voucher numbers of specimens used in analyses, collection locations, latitude and longitude. A. *Cystophora retroflexa* and outgroups with haplotypes and GenBank accession numbers of mitochondrial spacer sequences.

Herbarium voucher number[7]	**Species**	**Location**	**Latitude, Longitude**	**Haplotype (see Fig. 1) and GenBank Accession number**
WELT A031531	*C. retroflexa*	Depot Beach NSW	35°37′37.91″S, 150°19′37.27″E	C (JF309054)
WELT A030917	*C. retroflexa*	Island Bay, Wellington, NZ	41°20′51.22″S, 174°46′9.77″E	B (JF309069)
WELT A031510	*C. retroflexa*	Lonsdale VIC	38°17′5.30″S, 144°36′54.74″E	B (JF309057)
WELT A031532	*C. retroflexa*	Mimosa Rocks, Arragunnu NSW	36°35′2.07″S, 150° 3′15.18″E	C (JF309056)
WELT A031533	*C. retroflexa*	Point Lonsdale VIC	38°17′20.52″S, 144°36′4.42″E	D (JF309059)
WELT A031509	*C. retroflexa*	Port Campbell VIC	38°37′14.84″S, 142°59′32.69″E	C (JF309060)
WELT A030926	*C. retroflexa*	Princess Bay, Wellington NZ	41°20′43. 05″S, 174°47′14.08″E	B (JF309051)
WELT A030914	*C. retroflexa*	Princess Bay, Wellington NZ	41°20′43.05″S, 174°47′14.08″E	B (JF309066)
WELT A030913	*C. retroflexa*	Princess Bay, Wellington NZ	41°20′43.05″S, 174°47′14.08″E	B (JF309067)
WELT A031511	*C. retroflexa*	Robe SA	37° 9′23.33″S, 139°44′56.32″E	C (JF309061)
WELT A031508	*C. retroflexa*	Robe SA	37° 9′23.33″S, 139°44′56.32″E	A (JF309062)

[7] Field numbers are shown. These specimens have been deposited at WELT. Herbarium numbers will be added when the accession process is completed.

Table 5.1 (Continued)

Herbarium voucher number	**Species**	**Location**	**Latitude, Longitude**	**Haplotype (see Fig. 1) and GenBank Accession number**
WELT A031512	*C. retroflexa*	Robe SA	37° 9′23.33″S, 139°44′56.32″E	C (JF309070)
WELT A031530	*C. retroflexa*	Sorrento, VIC	38°20′5.35″S, 144°44′33.96″E	D (JF309058)
WELT A030927	*C. retroflexa*	The Sirens, Wellington NZ	41°20′56.91″S, 174°45′51.05″E	B (JF309063)
WELT A030932	*C. retroflexa*	Tokomaru Bay, Eastland, NZ	38° 6′35.83″S, 178°21′18.27″E	D (JF309068)
WELT A031528	*C. subfarcinata*	Lonsdale VIC	38°17′5.30″S, 144°36′54.74″E	F (JF309065)
WELT A031513	*C. subfarcinata*	Observation Head, Batemans Bay NSW	35°43′45.50″S, 150°12′30.26″E	E (JF309055)
WELT A031527	*C. subfarcinata*	Squeaky Beach, Wilsons Promontory VIC	39° 1′45.89″S, 146°18′24.24″E	F (JF309063)
WELT A031529	*C. subfarcinata*	Ulladulla, NSW	35°21′37.65″S, 150°29′5.55″E	E (JF309053)

Table 5.1. B. *Cystophora scalaris* samples with reference numbers to Fig. 5.1.

Herbarium voucher number	**Species**	**Location**	**Latitude, Longitude**	**Reference number (Fig. 5.1)**
WELT A030925	*C. scalaris*	Pukerua Bay NZ	41° 1′28.36″S, 174°54′5.54″E	1
WELT A030921	*C. scalaris*	Riversdale, Wairarapa NZ	41° 6′24.49″S, 176° 4′10.73″E	2
WELT A031338	*C. scalaris*	Evans Bay, Wellington, NZ	41°18′59.61″S, 174°48′23.25″E	3
WELT A031337	*C. scalaris*	Lyall Bay, Wellington, NZ	41°20′12.67″S, 174°47′35.14″E	4
WELT A030902	*C. scalaris*	Moa Point, Wellington, NZ	41°20′37.68″S, 174°48′31.54″E	5
WELT A030904	*C. scalaris*	Moa Point, Wellington, NZ	41°20′37.68″S, 174°48′31.54″E	6
WELT A030901	*C. scalaris*	Tarakena Bay, Wellington, NZ	41°20′39.95″S, 174°49′12.46″E	7
WELT A030903	*C. scalaris*	Princess Bay, Wellington NZ	41°20′43.05″S, 174°47′14.08″E	8
WELT A031341	*C. scalaris*	Te Raekaihau, Wellington NZ	41°20′50.92″S, 174°47′29.01″E	9
WELT A031344	*C. scalaris*	Te Raekaihau, Wellington NZ	41°20′50.92″S, 174°47′29.01″E	10
WELT A030905	*C. scalaris*	Te Raekaihau, Wellington NZ	41°20′50.92″S, 174°47′29.01″E	11
WELT A030923	*C. scalaris*	Te Raekaihau, Wellington NZ	41°20′50.92″S, 174°47′29.01″E	12
WELT A031336	*C. scalaris*	Red rocks, Wellington, NZ	41°20'54.91″S, 174°44′30.19″E	13

Table 5.1. B. (Continued)

WELT A031334	*C. scalaris*	Red rocks, Wellington, NZ	41°20′54.91″S, 174°44′30.19″E	14
WELT A031335	*C. scalaris*	Red rocks, Wellington, NZ	41°20′54.91″S, 174°44′30.19″E	15
WELT A031346	*C. scalaris*	Kaingaroa, Chatham Islands	43°43′51.20″S, 176°16′2.26″W	16
WELT A031345	*C. scalaris*	Point Weeding, Chatham Islands	43°56′32.08″S, 176°34′14.74″W	17
WELT A030909	*C. scalaris*	Point Weeding, Chatham Islands	43°56′32.08″S, 176°34′14.74″W	18
WELT A030908	*C. scalaris*	Point Weeding, Chatham Islands	43°56′32.08″S, 176°34′14.74″W	19
WELT A030910	*C. scalaris*	Point Weeding, Chatham Islands	43°56′32.08″S, 176°34′14.74″W	20
WELT A030911	*C. scalaris*	Waitangi, Chatham Islands, NZ	43°56′41.58″S, 176°33′41.78″W	21
WELT A030922	*C. scalaris*	Ocean Beach, Jackson Bay NZ	43°57′54.44″S, 168°36′21.70″E	22
WELT A031343	*C. scalaris*	Owenga, Chatham Islands	44° 2′0.30″S, 176°19′58.80″W	23
WELT A031340	*C. scalaris*	Manukau Reef, Chatham Islands	44° 2′4.90″S, 176°19′44.31″W	24
WELT A030933	*C. scalaris*	Mapoutahi, Otago Peninsula NZ	45°44′5.57″S, 170°37′4.53″E	25
WELT A030906	*C. scalaris*	Wellers Rock, Otago Harbour, NZ	45°47′51.97″S, 170°42'53.85″E	26

5.3 Materials and Methods

Cystophora specimens were collected from around New Zealand, including the Chatham Islands, and from southern Australia and Tasmania (Table 5.1). *A priori* species assignments were made on the basis of morphological characters from Womersley (1964, 1987). Thalli were pressed as herbaria specimens and one or a few ramuli were cleaned of epiphytes and the tissue rapidly dried and stored in silica gel for DNA extraction. In addition, specimens held in WELT were examined (Table 5.1).

Length, width (wider axis of compressed receptacle) and breadth (narrower axis of compressed receptacle) of receptacles of herbaria specimens of *C. scalaris* and *C. distenta* were measured with digital callipers. Ten randomly selected receptacles were measured from 26 specimens. In three specimens, selected as representing the range of receptacle size, measurements were taken of fresh receptacles. These receptacles were then air dried and remeasured, and a factor estimated by regression to determine a multiplier to convert measurements from herbarium specimens to the equivalent fresh dimensions. ANOVAs, implemented in SPSS16, were used to test for significant differences between mean dimensions. In addition to these specimens, herbaria specimens of all New Zealand species of *Cystophora* were examined from WELT and my collections and additional measurements were made to determine the range of character dimensions for revised descriptions.

DNA was extracted using a modified CTAB extraction (Zuccarello & Lokhorst 2005), with the addition of 1% polyvinylpolypyrrolidone to the extraction buffer. The 23S-tRNA-Lys mitochondrial spacer (Hoarau *et al.* 2007, Chapter 2) was amplified. Primers and PCR mixes and routines were described in Chapter 2. PCR products were cleaned with ExoSAP-IT (USB, Cleveland, Ohio) enzymes and sequenced commercially (Macrogen Inc., Seoul, Korea). A statistical parsimony network was inferred from mitochondrial spacer sequences from 20 specimens of *Cystophora* using TCS software (Clement *et al*. 2000), with linkages made at the 95% confidence level.

5.4 Results

Some specimens were difficult to assign to species using existing keys and morphological characters. Ambiguous specimens were assigned to the more common species. Consequently only seven specimens were assigned to *C. congesta* and four

specimens to *C. distenta* (Table 5.1). Measurements of receptacles of putative *Cystophora scalaris* and *C. distenta* specimens show a wide range of receptacle forms, often with high variation on a single thallus (Fig. 5.1). Specimens from highly exposed locations (e.g., specimens 10–13 in Fig. 5.1) show very reduced receptacle length. Neither length nor width:breadth ratios (a measure of compression of the receptacle) show clusters consistent with separate species (Fig. 5.1).

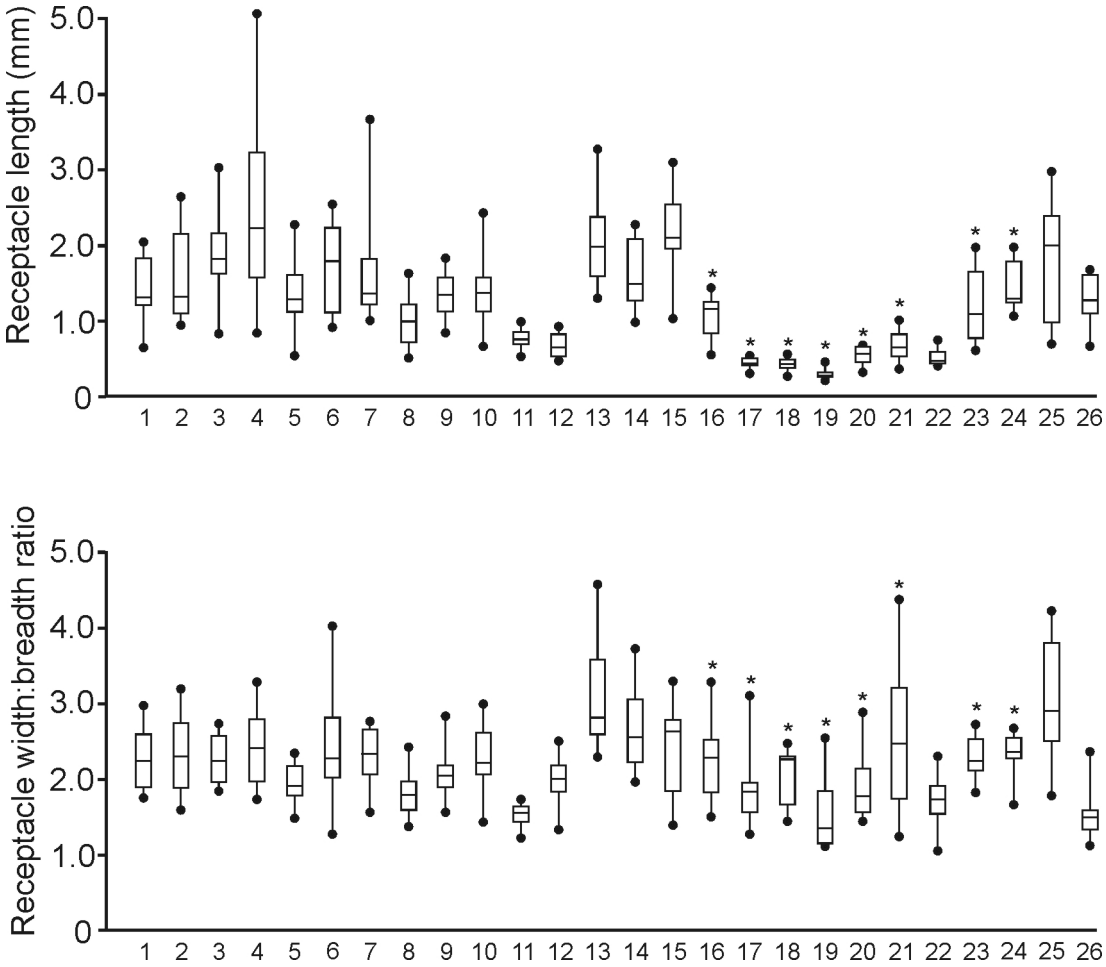


Fig. 5.1. Morphological variation from 26 specimens of *Cystophora scalaris.* Graphs show maximum, minimum median and 25% and 75% quartiles from 26 specimens. Data are arranged from northernmost (1) to southernmost (26) specimens, asterisks indicate specimens from the Chatham Islands. A. Receptacle length. B. Receptacle width: breadth ratios.

ANOVA analysis of log-transformed data showed significant ($P<0.01$) differences between means in the data, but post-hoc tests (Tukey's HSD pairwise and

range tests) showed significant differences only existed between outlier data. For example, mean length of receptacles from specimens 5 and 18 (in Fig. 5.1a) were significantly different from specimens 8–13, but differences between both groups and intermediate specimens was not significant ($P > 0.05$). Similarly, specimens with the greatest receptacle width:breadth ratios (specimens 5 and 16 in Fig. 5.1A) showed significant differences from those specimens with the lowest ratios (specimens 12, 23 and 26 in Fig. 5.1B), but none of these specimens showed significant differences from intermediate specimens.

Molecular data

Four mitochondrial spacer haplotypes were found in *C. retroflexa* and *C. congesta* (Fig. 5.2, A-D; Appendix 6). Two haplotypes associated with *C. subfarcinata* were included as outgroups (E, F). Four *C. retroflexa/C. congesta* haplotypes were separated by a single change in each case. Two haplotypes were sampled in specimens of both *C. congesta* and *C. retroflexa*. Only one specimen of *C. congesta* (WELT A031508 from Robe, South Australia) had a unique haplotype (Haplotype A in Fig. 5.2).

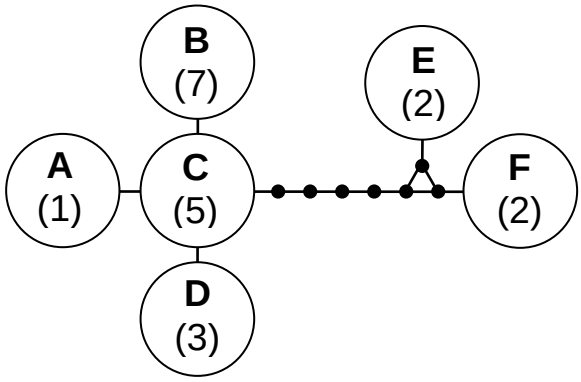


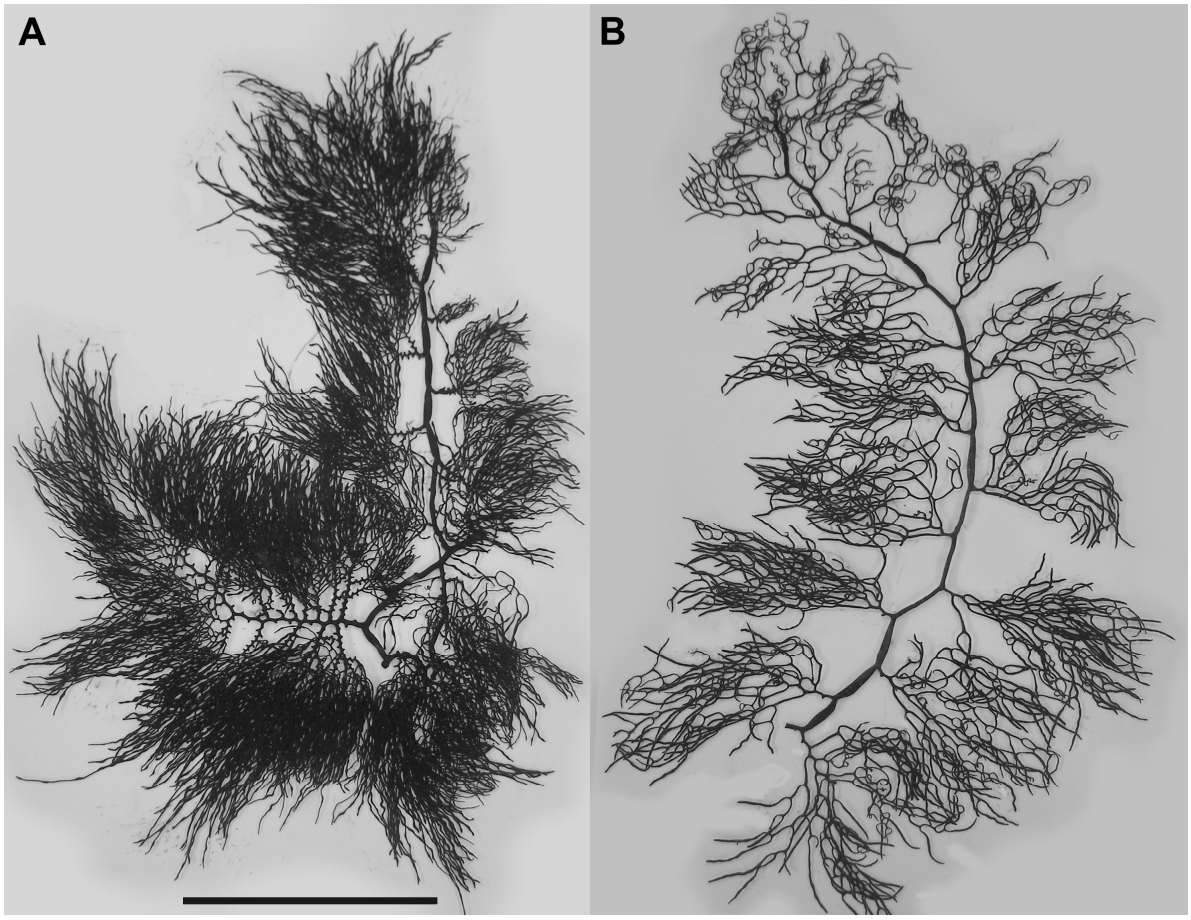
Fig. 5.2. Statistical parsimony network inferred from 20 mitochondrial spacer sequences of *Cystophora retroflexa* (A–D) and *C. subfarcinata* (E, F). Estimated at 95% confidence. Haplotypes A–C include both *C. retroflexa* and *C. congesta* morphotypes. Numbers are sample frequencies.

5.5 Discussion

Two species of *Cystophora* found in New Zealand do not present any taxonomic problems. *Cystophora platylobium* is usually found in relatively deep water, and collections are often based on drift specimens. Attached specimens were collected in southern Otago only (at 1–2 metres depth). For this reason, sampling of this species was limited, but available data suggests this species is ecologically, morphologically and genetically (Chapter 4) distinct from other species, and New Zealand specimens are genetically very similar to Australian specimens, with identical *cox*1 haplotypes (Chapter 4). *Cystophora torulosa* is also morphologically and ecologically and genetically distinct from other species (Chapter 4). No difficulty was found in assigning specimens to these species using existing keys and descriptions. These species will not be commented on further.

The remaining New Zealand species of *Cystophora* present some problems. Specimens of *Cystophora congesta* can be difficult to separate from *C. retroflexa*, and *C. scalaris* can be difficult to separate from *C. distenta*. Some confusion also appears to have arisen in distinguishing *C. scalaris* and *C. retroflexa* (Lindauer *et al*. 1961). These problems arise in part from inadequate descriptions and poor character choice in the keys provided in Lindauer *et al.* (1961) and Adams (1994), which rely on the relative zig-zaggedness of the main axes and laterals for species identification. This is confusing, as from my observations, the degree of zig-zagging in the primary and secondary axes is fairly plastic in several species of *Cystophora* and is probably related to the growth rate (Klemm & Hallam 1987). Womersley's (1964) keys separated these species by the arrangement of branching of the ramuli (complanate in *C. scalaris* and *C. distenta*, irregularly radial in *C. retroflexa* and *C. congesta*) and the size and form of the receptacles, characters which are reliable for separating *C. retroflexa* from *C. scalaris*, but still inadequate for separating both *C. retroflexa* from *C. congesta* and *C. scalaris* from *C. distenta*. Intermediate forms are common in both pairs of species and I consider the morphological characters used to split species are not discontinuous, but rather lie on a continuum of intra-specific variation. The evidence for maintaining *C. congesta* and *C. distenta* as separate species can be attributed to limited sampling, with specimens examined representing the extremes of a morphological continuum.

Fig. 5.3. Morphological variation in *Cystophora retorta*. A. congested form, zig-zag laterals [WELT A031546]; B. distended form, straight laterals [WELT A031547]. Scale bar = 100 mm.



5.5.1 *Cystophora congesta* and *C. retroflexa*

In Chapter 4 I could not separate *C. retroflexa* and *C. congesta* using ITS and *cox*1 sequences. The highly variable 23S-tRNA-Lys mitochondrial spacer (Fig. 5.2) also fails to separate these species. Both this study and Chapter 4 include *C. congesta* specimens from the type locality (Robe, South Australia). While sampling in this study is limited, the mitochondrial spacer used here is highly variable in most species of brown algae (Hoarau *et al.* 2007, Chapter 2), and haplotypes found in all but one specimens assigned to *C. congesta* were also found in typical specimens of *C. retroflexa*. In addition, these species share *cox*1 haplotypes and ITS ribotypes (Chapter 4). Together, this is strong evidence against genetic differentiation.

According to Womersley (1964: 87) "*C. congesta* is most closely related to *C. retroflexa*, differing in the much denser laterals and secondary axes, shorter receptacles and thicker, more rigid, primary axis. Occasional intergrades between these species occur." Womersley then separated the species entirely on these vegetative characters, all of which are variable in other species of *Cystophora*. For example, specimens of *C. retorta* can have a thick primary axis and zig-zag laterals bearing fascicled ramuli and receptacles, and other specimen have a thin primary axis, with straight and sparsely branched laterals (Fig. 5.3). All morphological types share the scattered bisexual conceptacles that distinguish this species from its near relatives (Womersley 1964).

Some specimens could be easily assigned to *C. retroflexa* or *C. congesta* using Womersley's criteria, but many specimens were intermediate and could be assigned to either species (Fig. 5.4). The plasticity of these characters in other species of *Cystophora*, for example in *C. siliquosa* and *C. subfarcinata* (see dimensions in Womersley 1964), the presence of intergrades and the absence of genetic differentiation suggest these characters are inadequate for inferring distinct species.

The weight of the evidence is that *C. congesta* should be regarded as either an ecotype or a developmental form of *C. retroflexa*. I consider *C. congesta* Womersley & Nizamuddin as synonymous with *C. retroflexa* (Labillardière) J. Agardh, the latter name having priority.

Fig. 5.4. Morphological variation in *Cystophora retroflexa.* **A.** Distended thallus [WELT A031534]. **B.** Intermediate thallus [WELT A031533]. **C.** Congested thallus [WELT A031508]. Scale bar = 100 mm.

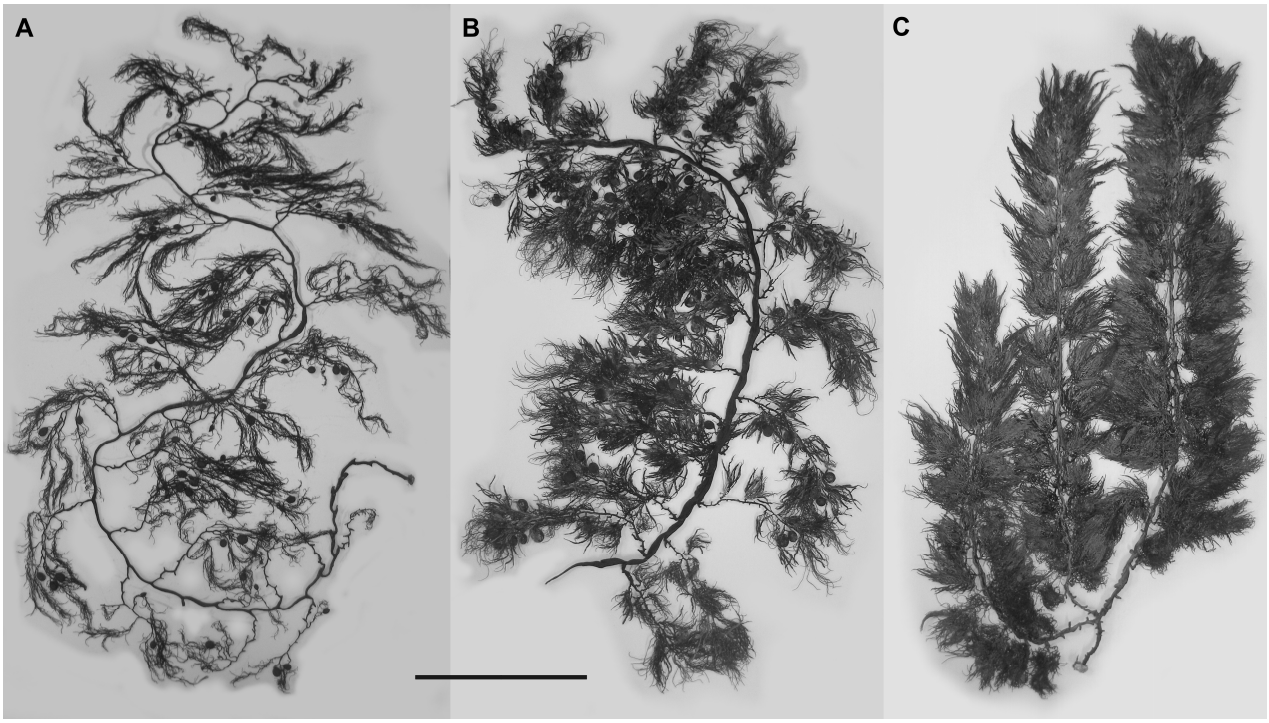
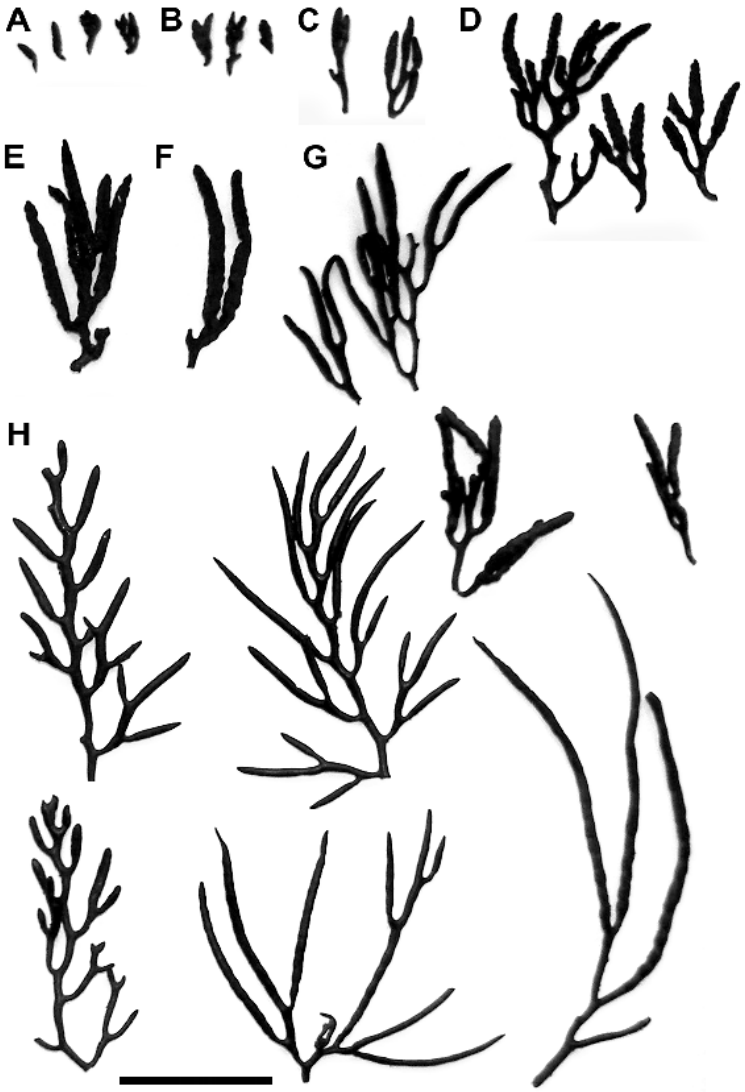


Fig. 5.5. Morphological variation in receptacles of *Cystophora scalaris* A. WELT A031345A; B. WELT A031345B; C. WELT A030922; D. WELT A031346; E. WELT A031340; F. WELT A031343; G. WELT A031341; H. Examples of receptacles from a single thallus, WELT A031337). Scale bar = 20 mm.



5.5.2 Amended description

Cystophora retroflexa (Labillardière) J. Agardh *Species Genera et Ordines Algarum* 1848: 242. Womersley *Aus. J. Bot.* 1964: 89, Figs. 31, 32, pl. 10, Fig. 2. *Marine Flora of Southern Australia 3* 1987: 396, Figs. 132 I, 146B, 149B, C.
Cystophora congesta Womersley & Nizamuddin ex Womersley *Aus. J. Bot.* 1964: 86, Fig. 30, pl. 9 Fig. 2. Womersley *Marine Flora of Southern Australia 3* 1987: 394. Figs. 146A, 149A.

Figs. 4, 5.

Thallus brown, 0.3–1.5 (–2) m high, with the primary axes bearing alternate laterals and secondary axes. *Holdfast* discoid–conical, 3–15 mm diameter, with a short, terete stipe; epilithic. *Primary axes* compressed, 3–12 mm broad below and 1–4(–5) mm thick, 2–6(–10) mm broad above, narrow edged, fairly straight to zig-zag and scalariform, alternately distichously branched from the face of the axes, lower parts often denuded with prominent residues, each branch position giving rise to 1(-3) determinate laterals and 1(–2) indeterminate laterals; lower laterals usually retroflex, often with broad basal wings. *Laterals* determinate or indeterminate, determinate laterals arising from the edge of an outgrowth of the main axes, 20–100 mm long, with usually irregular radial, sometimes subtristichous or subdistichous branching, bearing ramuli, sometimes developing into receptacles, lower determinate laterals often with enlarged and flattened ramuli. Indeterminate laterals arising from the centre of outgrowths from the axes, 20–300 mm long, initially terete, becoming flattened, alternately distichously branched, becoming slightly to strongly zig-zag; bearing determinate secondary laterals and/or secondary indeterminate laterals. *Ramuli* 20–40(–50) mm long, 0.4–2(–3) mm broad. Ramuli on lower laterals sometimes enlarged and flattened. *Vesicles* ovoid, becoming subspherical when old, replacing basal (or lower 2) ramuli of laterals, stalked, mutic, 4–12(–15) mm long and 3–10(–12) mm broad.
Reproduction thalli monecious. Receptacles usually simple, occasionally branched, compressed, margins undulate to torulose when fresh, often strongly torulose when dried, 15–50 (–60) mm long and 1–2 (–4) mm broad, attenuate at apex usually with sterile terminal awn, awn rarely with scattered conceptacles, with 2 rows of conceptacles along the margins; conceptacles bisexual, ostioles along the margin.

Type locality “Cape van Diemen” (southern Tasmania).
Ho1otype FI (Herbarium Webbianum).
Distribution Australia: Womersley (1964) reports the species from Bondi (Sydney), NSW. I found specimens from Kiama, NSW southwards. I have not ascertained the western limit of *C. retroflexa* in Australia. Womersley (1987) reports the western limit as “from Cottesloe to Nannarup (Albany), W.A., and from Kangaroo I.”. Tasmania, New Zealand, Chatham Is., Auckland Is.

5.5.3 *Cystophora distenta* and *C. scalaris*

J. G. Agardh (1870) described *C. scalaris* and *C. distenta* from the Chatham Islands, but regarded them as contracted and distended forms of the same species, stating: “C. scalarem & C. distentam esse ejusdem specie formas – unam contractam, alteram distentam” (Agardh 1870). [“*C. scalaris* and *C. distenta* are forms of the same species – one contracted and the other distended.”]. De Toni (1895: 141) also stated that *C. distenta* was “Anne forma laxa *C. scalaris* J. Ag.?.”

Treatment as separate species seems to begin with Laing (1899) who reported both forms as species without comment, and continued to do so in further publications (Laing 1926). Lindauer (1947) followed Laing in listing these as separate species (using the generic name *Blossevillea* (following Gardner 1913). Lindauer (1947) gave the distribution of *C. distenta* (as *Blossevillea distenta*) as Chatham Islands and Stewart Island, but noted that in Stewart Island “the habit of the plant is less typical” (p. 560). Lindauer *et al.* (1961) reported the two entities as separate species, as did Womersley (1964, 1987), Nelson *et al.* (1991, 1992) and Adams (1994).

Womersley (1964: 91) described *C. distenta* and *C. scalaris* as separate species, “differing in the more compressed and much longer receptacles, straighter and less robust axis, and looser branching ” [in *C. distenta*]. This is unhelpful as the dimensions of the axis and looseness of branching are variable in many species of *Cystophora* (for example, in *C. retorta*, Fig. 5.3), and receptacle dimensions provided by Womersley (1964) and Lindauer *et al.* (1961) for these species overlap. Receptacles are very variable in length in *C. scalaris* (Figs. 5.1 and 5.5), and from my observations compression appears to be related to the reproductive state of the

receptacle, rather than any underlying genetic difference. Receptacle dimensions are highly variable, even on a single thallus (Figs. 5.1 and 5.5).

Cystophora distenta was first described from the Chatham Islands by J. Agardh (1870). De Toni listed its distribution as the Chatham Islands and Bluff. Lindauer *et al*. (1961) extended its distribution to include Stewart Island and Frenchman's Pass (presumably French Pass in the Marlborough Sounds), and also noted that specimens were found in drift at Tauranga. Later specimens were reported from Cape Palliser on the southern North Island (Adams 1994) and Durville Island, on the northern tip of the South Island (Nelson *et al.*1992). The species appears to be distributed on the mainland south of Cook Strait and on Stewart Island and the Chatham Islands. This distribution is the same as that of *C. scalaris* (Lindauer *et al.* 1961, Adams 1994).

As I am unable to separate *C. scalaris* and *C. distenta* using morphological or molecular data, I consider Agardh's (1870) assessment to be correct and regard *C. distenta* as a form of *C. scalaris.* The latter name is in more common usage (e.g., Morton & Miller 1968, Shears & Babcock 2007).

5.5.4 Amended species description

Cystophora scalaris J. Agardh ***Öfversigt af Kongl. Vetenskaps-Academiens Förhandlingar*** 1870: 442. Womersley *Aus. J. Bot.* 1964: 82, Figs. 22, 23, pl. 7, fig. 2.

Figs. 5.5, 5.6.

Thallus brown, (0.1–)0.3–1 m long, with the primary axes bearing alternate, openly to densely branched laterals and secondary axes. *Holdfast* discoid-conical, 5–20 mm diameter, with a short, terete stipe; epilithic. *Primary axes* strongly flattened, 3–15 (–20) mm broad and 2–3 mm thick, strongly zigzag and scalariform to fairly straight, lower parts often denuded with scalariform residues, alternately distichously branched from the face of the axis, with each branch position producing 1(–2) indeterminate secondary axes from the centre of the face and/or (0–1) –2 determinate laterals from the edge of the scales, laterals 10–30 (–50) mm apart; secondary and tertiary axes similar, 20–80 cm long, initially terete, becoming flattened, strongly zig-zag and scalariform to fairly straight; lower parts of primary, secondary, and older tertiary axes usually denuded, with very prominent, close-set scalariform residues, and

markedly broader basal wings on the primary (and often secondary) axis at branch positions. *Laterals* 20–80 (–100) mm long, complanately branched, often bifurcate at their base, then alternately distichous with ramuli also alternately distichous, with acute to rounded axils; ultimate ramuli terete to compressed, 5–10 (–30) mm long, 1–3(–4) mm broad. Ramuli of lower laterals on main axis often enlarged and flattened. Vesicles sparse to numerous, replacing lower 1 to 2 or more ramuli of the lateral of each bifurcation, sub-spherical to slightly ovoid, stalked, mutic, 2–8 (–12) mm diameter.

Reproduction. Thalli monoecious, receptacles developed from ultimate ramuli, simple or occasionally branched, ovoid to compressed in section, 10–50 mm long, (0.5–)1–3 mm broad and 1–1.5 mm thick, elongate, margins undulate to strongly torulose. Conceptacles bisexual, ostioles in two rows along the margins of the receptacle, rarely in three rows.

Type locality Chatham Islands.

Lectotype LD (Herbarium Agardh, No. 1113).

Distribution New Zealand, from Wellington southwards; Chatham Islands, Auckland Islands.

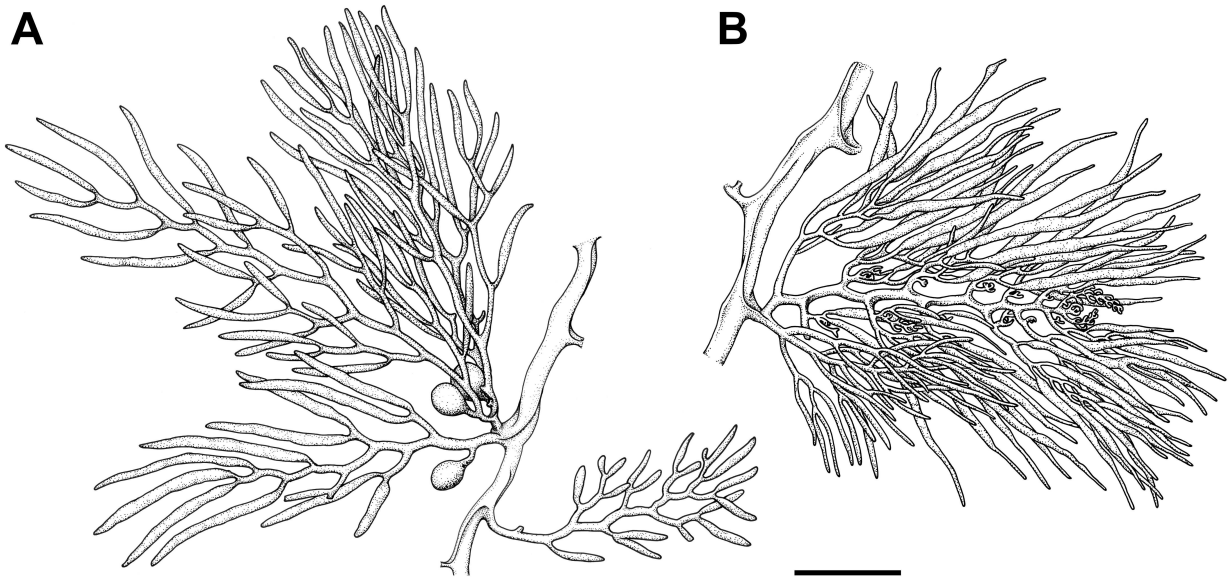


Fig. 5.6. Organisation of ramuli and receptacles in *Cystophora*. Both specimens show indeterminate (upper) and determinate (lower) laterals. **A.** Complanate organisation in *C. scalaris*. **B.** Radial organisation in *C. retroflexa*. Scale bar = 20 mm.

5.6 Conclusion

Four species of *Cystophora* are present in New Zealand. These can be diagnosed with molecular and morphological characters. These species can be separated by the form and arrangement of receptacles (Table 5.2).

Table 5.2. Key to the New Zealand species of *Cystophora*

1. Branching from the edge of the main axes, receptacles leaf-like……...*C..platylobium*
1. Branching from the face of the axes, receptacles terete or flattened……..……...2

2. Receptacles swollen, smooth, terete with rounded apex, densely clustered on laterals; lower intertidal or shallow sub-tidal.……..……..*C. torulosa*
2. Receptacles elongate, slightly to strongly flattened, apex blunt, pointed or with sterile awn, densely to openly clustered on laterals, sub-tidal…….……..3

3. Receptacles and ramuli arranged radially, usually narrow with long terminal awn …….....*C. retroflexa*

3. Receptacles and ramuli distichous, forming complanate lateral branches; Receptacles slightly to strongly flattened, with blunt to acute tips…….*C. scalaris*

5.7 Acknowledgements

I thank the New Zealand Department of Conservation and Education NZ Study Abroad Scheme for funding assistance; Wendy Nelson, Tracy Farr, Kate Neill and the Department of Conservation Chatham Islands staff for assistance with collections and Alan Millar (National Herbarium of New South Wales) and Jenn Dalen (Museum of New Zealand Te Papa Tongarewa herbarium) for assistance with herbaria access.

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Chapter 6

Species delimitation in brown algae – insights and applications to New Zealand brown algae.

6. 1 Abstract

Species delimitation in brown algae has traditionally proceeded by inferring genetic discontinuities from morphological characters, with additional data from culturing, crossing experiments, and studies of natural hybridisation. The development of molecular methods has provided an additional source of data, but few studies have proceeded to a phylogenetic concept of species. Rather, taxonomists have delimited species using a subjective assessment of available data. I review approaches to species delimitation in brown algae with reference to findings from previous chapters. Recent phylogeographic studies have produced a complex picture of species' history, with extensive changes in distribution, isolation and secondary contact, and a variety of speciation scenarios. I argue that this complexity means no single approach can delimit all species. Rather, an explicitly historical species concept is needed, with the history of species included in species descriptions.

6.2 Introduction

THE PREVIOUS CHAPTERS have investigated genetic diversity of *Carpophyllum* and *Cystophora* at different scales. Characterising genetic diversity is an essential task for biologists seeking to understand evolution, and different approaches have emerged to deal with the multiple scales of diversity. Phylogeneticists focus on historical relationships between species. Taxonomists use various data to infer discontinuities in genetic variation in order to delimit and describe species. Population geneticists study allele frequencies within and between populations. This thesis began with a detailed mitochondrial DNA phylogeography of *C. maschalocarpum* (Chapter 2), then an investigation into the relationship between two species of *Carpophyllum* (Chapter 3). I produced a broad phylogeny of *Cystophora* (Chapter 4), and dealt with the taxonomic separation of two pairs of species (Chapter 5). Here I review this work with reference to approaches to species delimitation in brown algae.

6.3 Species delimitation

Species are basic units of ecological studies and biodiversity assessments, and are a fundamental point of reference for nearly all biological science. Despite this, consistent delimitation of species continues to be problematic and sometimes contentious (Dayrat 2005, Will *et al.* 2005, De Queiroz 2007). The availability of genetic data and dissatisfaction with traditional methods (Tautz *et al.* 2003, Godfray *et al.* 2007) has prompted several attempts at developing objective methods of species delimitation (Sites & Marshall 2004, Shaffer & Thomson 2007, Pons *et al.* 2009, Wiens 2007). These include phylogenetic (tree-based) methods (Brower 1999, Templeton 2001, Wiens & Penkrot 2002) and population genetic methods, either estimating gene flow (Porter 1990) or genetic distance between populations (Good & Wake 1992, Highton 1990, 1995). Other approaches include DNA barcoding (Hebert *et al.* 2003a, Hebert & Gregory 2005) or deviation from expectations of a coalescence model (Pons *et al*. 2006).

How to delineate species is the central task of taxonomy. A second task, providing tools (names, descriptions, keys) for accurate species identification, is dependent on accurate species delineation. As the purpose of taxonomy changed from classification – the convenient ordering of life to meet the needs of biologists – to an attempt to explain biological reality, the focus has changed from phenetics to phylogenetics. Most taxonomists now accept a phylogenetic approach (Quicke 1993, Dayrat 2005). This theoretical position has been reinforced by the availability of molecular data that is easily analysed by phylogenetic methods. Taxonomy has become a study of history, specifically, the history of speciation. Reading history directly is difficult for most taxonomists, but is especially difficult for phycologists dealing with brown algae as the fossil record of brown algae is negligible (Silberfield *et al.* 2010), and well-preserved specimens amenable to ancient DNA work are almost unknown[8]. Therefore the history of speciation in brown algae must be inferred from extant data. Molecular data from brown algae has revealed hybridisation, cryptic speciation, intra-specific variation and incongruities between morphological and molecular data, increasing the complexity of defining species boundaries. An additional problem is applying

[8] Dillehay *et al.* (2008) recovered well preserved 14 000-year-old fragments of seaweed from archaeological sites in South America so the possibility of ancient DNA work exists.

nomenclatural rules, which have a typological basis, to species delineated using molecular methods (Dayrat 2005). In the brown algae, species boundaries have often been described or suggested from molecular data, but authors have refrained from making appropriate nomenclatural changes.

Like other organisms, brown algal species have traditionally been delimited using morphological characters. Cryptic speciation, morphological plasticity, and complex life histories have often confounded morphological prescriptions, which have not always been congruent with other sources of information (e.g., molecular data, culturing, and natural and artificial crossing experiments). In addition, theoretical progress in formulating species concepts and methods of species delimitation has produced a more complex picture of species (Hey 2006, De Queiroz 2007, Mallet 2008). These approaches are discussed below.

6.4 Morphological approaches

Morphological approaches have been the mainstay of brown algal taxonomy (e.g., Agardh 1848, De Toni 1895, Setchell & Gardner 1925, Abbott & Hollenberg 1976, Fletcher 1987, Womersley 1987), and species delimitation using morphological characters has often been robust, despite rearrangements of higher taxonomic groups. Problems of morphological approaches often invoked (e.g., McDevit & Saunders 2009) include a limited number of characters, plasticity (Burrows & Lodge 1951, Mathieson *et al.* 1981, Henkel *et al.* 2007, Demes *et al*. 2009) and convergence (Harvey & Goff 2006, Jones *et al.* 2010, Tronholm *et al.* 2010a). Despite this, morphological characters continue to be given considerable weight, although very recent studies have prioritised molecular data (Macaya & Zuccarello 2010, Fraser *et al.* 2010b). Morphological methods are generally descriptive, although numerical taxonomic methods have been used in the brown algae (Russell & Fletcher 1975, Rice & Chapman 1985, Pérez-Ruzafa *et al.* 1993), but have largely been superseded by molecular data, with morphological characters mapped onto trees (e.g., De Clerck *et al.* 2006, Silberfield *et al*. 2010). Morphometric methods have also been used (e.g., Widdowson 1971, Rice 1989). Morphometrics are useful when used to determine diagnostic characters in

conjunction with molecular markers or culturing (Kraan *et al.* 2001, Roberson & Coyer 2004, Tronholm *et al.* 2010a).

In some genera, morphological plasticity has led to a plethora of synonyms and sub-species names. For example, there are 1046 names for species and varieties of *Fucus* and 869 names for *Sargassum* in Algaebase (Guiry & Guiry 2011). In *Cystophora*, morphological methods have resulted in oversplitting of some species and I have synonymised *C. scalaris/C. distenta* and *C. retroflexa/congesta.* Oversplitting probably results from limited sampling and a typological approach, which fail to capture a continuum of morphological variation. *A priori* concepts of species might result in conscious or unconscious collection bias for "typical" or "healthy" specimens, reinforcing selective sampling.

6.5 Culture experiments

Heteromorphic life histories and environmental plasticity complicates morphological studies. Culture studies can overcome some of these difficulties. Culture experiments have been used to test morphological characters. This is especially informative in species with heteromorphic life histories (e.g., Wynne 1969, Clayton 1976, 1978, Kogame 1996, 1997a, 1997b, Kogame & Yamagishi 1997) and multiple pathways in morphological development.

6.6 Crossing experiments and reproductive isolation

Complete reproductive isolation is often viewed as the ultimate step in speciation and the most fundamental test of species delimitation. Reproductive isolation can be tested directly, at least in species that are amenable to growth in culture, but is often inferred from morphological or phylogenetic data.

The Biological Species Concept (BSC, Mayr 1942, 1996) bases species delimitation on reproductive isolation, although most proponents allow some level of hybridization or gene flow (O'Brien & Mayr 1991, Coyne & Orr 2004). The BSC has a compelling logic, but becomes problematic where hybridization is

widespread[9], such as in *Fucus* and *Carpophyllum*, and can be ahistorical (Velasco 2008). Reproductive isolation is usually inferred, generally from morphological discontinuities, and often with subjective values placed on different characters by taxonomists (Mayr 1996). Reproductive isolation can be tested directly using crossing experiments.

Crossing experiments have been used fairly extensively in a few genera of brown algae, especially *Ectocarpus* and some Laminariales. Often there has been a reluctance to formally apply results of crossing experiments to name new species or synonymise existing species, even where reproductive compatibility or isolation is clearly shown. For example, Müller (1988) declined to separate Chilean and Mediterranean isolates of *Ectocarpus siliculosus*, even after crosses showed hybrid sporophytes could not reproduce sexually and therefore could be considered separate species under the Biological Species Concept. In spite of supporting information (differences in development and morphology), Müller declined to separate these species "for practical reasons," apparently because morphological diagnosis would be demanding. Conversely, Lewis *et al.* (1986) and Lewis & Neushul (1994) produced viable and fertile sporophytes from crosses of geographically separated *Macrocystis* specimens, then considered as separate species. However the authors considered that these should be maintained as separate species on the basis of morphological differences. In these cases the morphological species concept appears to have been given priority.

The complexity of crossing data was shown in *Alaria* (Kraan & Guiry 1998, 2000, Kraan *et al.* 2001), where crosses between some isolates of the same morphologically-determined species show limited viability, whereas distinct species can be fully interfertile. This is congruent with molecular data which shows greater genetic variation within *A. esculenta* compared to variation between other recognised *Alaria* species. Kraan & Guiry (2000) reject the morphological and biological species concepts for *Alaria*, and consider only a phylogenetic species concept is appropriate, but refrained from splitting reproductively isolated lineages into species.

[9] O'Brien & Mayr (1991) accept hybridisation between biological species, as long as this does not "disintegrate the genetic integrity of the species". It is not clear what this means for closely related species where gene flow from hybridisation is difficult to distinguish from incomplete lineage sorting.

Druehl *et al.* (2005) addressed some of the technical difficulties in conducting crossing experiments. Development of sporophytes in negative controls showed several Laminarean species are able to reproduce asexually via parthenogenesis or apogamy. Five species combinations produced putative hybrids in culture, but biparental inheritance of ITS DNA was confirmed in only one combination, raising doubt that progeny are hybrids. Druehl *et al.* (2005) recommend using molecular data (e.g., Coyer *et al*. 2002) to test hybrid status of cultured specimens. Parthenogenesis has also been reported in Ectocarpales (Bothwell *et al.* 2010), Chordariales (Peters 1987) and Fucales (Clayton *et al.* 1998) and might be common in brown algae. In addition to these problems, crossing experiments are limited to species that are amenable to growth in culture. My attempts to grow *Carpophyllum* in culture failed, and this difficulty has been reported by other workers (Dromgoole 1973, D. Schiel pers. comm.). This precludes performing artificial crosses, and investigating the fertility of hybrids. *Carpophyllum* hybrids form receptacles and zygotes, but their subsequent viability is unknown. One approach to these difficulties, and to determine whether potential for interspecific fertility demonstrated in culture is realised in nature, is to study natural hybrids.

6.7 Hybridisation as natural crossing experiments

Hybridisation appears to be fairly common in brown algae (Lewis 1996), and hybridisation can be regarded as a natural crossing experiment (Hewitt 1988), with assessments of the fitness and fertility of hybrids pertinent to the understanding of species limits (Arnold & Hodges 1995, Riesberg 1995).

Several problems arise in determining the prevalence of hybridisation and the importance of hybridisation to species boundaries. First, there are technical issues with identifying hybrids. Morphological intermediacy does not necessarily identify hybrids (Wilson 1992, Riesberg 1995) and Coyer *et al.* (2006b) showed morphologically similar salt marsh forms of *Fucus* could be interspecific hybrids or polyploid *F. vesiculosus*. A response to this problem is to identify hybrids by molecular markers, but Lane & Saunders (2005) showed that the presence of cryptic, epiphytic, gametophyte stages of Laminarialean species on sporophytes of related species could contaminate DNA samples, mimicking the signature of

hybrids. Wallace *et al.* (2004) identified *Fucus* hybrids by microsatellite admixture, but Engel *et al*. (2005) suggested this inference was confounded by inadequate characterisation of genetic variation in parent populations.

Secondly, hybridisation might be a consequence of taxonomic inflation rather than biologically reality, where progeny of oversplit species are considered hybrids. *Macrocystis* species were considered to produce hybrids in culture (Lewis *et al.* 1986, Lewis & Neushul 1994), but recent studies have synonymised species (Westermeier *et al.* 2007, Demes *et al.* 2009) and all forms of *Macrocystis* are now considered conspecific (Macaya & Zuccarello 2010).

Lastly, population genetic studies of hybrids are usually limited to one or a few populations (e.g., Wallace *et al*. 2004, Coyer *et al*. 2006b, 2006c, 2007). Different population of hybrids with the same parent species might be under different selective regimes and have different evolutionary trajectories (Butlin *et al.* 2008). Local hybrid zones might not be representative of patterns of hybridisation across species' ranges.

In vitro hybridisation has been widely reported in Laminariales (Liptack & Druehl 2000, Kraan & Guiry 2000), but natural hybrids might be rare (Druehl *et al*. 2005), although an extensive molecular survey of North Pacific *Alaria* species found evidence for widespread hybridisation (Lane *et al.* 2007). In this study there was no congruity between *cox*1 clusters and morphology and no thresholds in genetic distance between groups. ITS sequences had many polymorphic sites and showed a continuum of variation rather than clustering as species level groups. Lane *et al*. (2007) suggest extensive hybridisation occurred after secondary contact between previously allopatric species, and ITS data indicates recombination following a breakdown of species barriers.

Extensive hybridisation raises the question of how species are maintained. In *Fucus*, where hybridisation is best studied (Scott & Hardy 1994, Coyer *et al.* 2002, Wallace *et al.* 2004, Billard *et al.* 2005, Coyer *et al.* 2006b, 2006c, Mathieson *et al.* 2006, Coyer *et al.* 2007, 2010), selfing acts to restrict introgression in some hermaphrodite species (Engel *et al*. 2005, Perrin *et al.* 2007). Selfing is correlated with height in the intertidal, with the inference that species in the upper intertidal have longer periods of emersion, and less time to disperse gametes, and gametes will often be lost from stands, and selfing is advantageous

under these conditions. Selection against hybrids should reinforce evolution of selfing or of mating barriers.

It is not clear how often species are lost through hybridization. This is unlikely to be obvious in the fossil record, even of species that leave good fossils, such as vertebrates or foraminiferans, as fusion of species may be difficult to distinguish from extinction of one lineage. In a changing environment (climate change, tectonics) a degree of allopatric divergence followed by secondary contact and fusion might be common (Butlin *et al.* 2008). Major changes in species ranges (Hoarau *et al*. 2007, Maggs *et al*. 2008, Fraser *et al*. 2009b) and demography (Graham *et al*. 2010) suggest a highly dynamic history of species distributions over the last 20 000 years. This in turn suggests a dynamic pattern of allopatry and sympatry (Coyer *et al.* 2010), where lineages might often diverge for a period, before reticulating following secondary contact.

In *Carpophyllum* hybridisation appears to be following different patterns in different areas. In Northland there is evidence for hybridisation between *C. angustifolium* and *C. maschalocarpum* that confounds phylogenetic methods. In the Bay of Plenty *C. angustifolium* and *C. maschalocarpum* lineages are distinguishable with nuclear markers, but *C. maschalocarpum* mitochondrial markers appear to have introgressed into populations of *C. angustifolium*. In *Carpophyllum*, all species are dioecious and sub-tidal. Strong selection might be responsible for the persistence of separate morphological species in areas of sympatry. In particular, *C. angustifolium* might be adapted for high wave exposure, with a strong terete stipe and a low drag morphology (Hodge 2009). I hypothesise that periodic extreme weather events would remove hybrids and *C. maschalocarpum* from exposed habitats, maintaining species boundaries. Further experimental work to determine the impacts of wave exposure (Thomsen & Wernberg 2005) on this system would be valuable. Hybrids between *C. maschalocarpum* and *C. flexuosum*, and between *C. maschalocarpum* and *C. plumosum*, have been reported (Dromgoole 1973, Lindauer *et al.* 1961) but seem more localised or morphologically cryptic than the widespread hybridisation found between *C. maschalocarpum* and *C. angustifolium*.

In *Cystophora* most species are hermaphrodite, and hybrids should be rare. Further investigation of the sister species *C. retorta* (hermaphrodite) and *C.*

siliquosa (dioecious) might show similar patterns of hybridisation and introgression to those found in *Fucus*. *Cystophora subfarcinata* and *C. retroflexa* are morphologically similar, often sympatric and both species are morphologically plastic. This might hide hybridisation between these species and further investigation might also reveal past or current hybridisation. Two specimens resembling *C. scalaris* were found north of the usual range of this species, one from Wilson Bay, Coromandel, the other from Ahuriri Street, Napier. Mitochondrial DNA sequences from these specimens grouped with *C. torulosa* and *C. retroflexa* (data not shown). Further data might confirm that these are *C. torulosa* × *C. retroflexa* hybrids.

Generally, the high frequency of natural hybridisation and absence of reproductive isolation in recognised species suggests reproductive isolation occurs at a late stage of speciation in brown algae, and is often preceded by ecological and morphological differentiation.

6.8 Molecular data

Early molecular studies focussed on characterising classes among the heterokontophyta (Bhattacharya *et al.* 1992, Daugbjerg & Andersen 1997a, b), or resolving ordinal or familial level phylogenetic relationships within the Phaeophyceae (Tan & Druehl 1993, 1994, Saunders & Druehl 1992, 1993, Rousseau *et al.* 1997, Peters & Clayton 1998, Siemer *et al.* 1998, Boo *et al.* 1999, Rousseau & de Reviers 1999a, 1999b, Serrão *et al.* 1999, Kawai & Sasaki 2000, Rousseau *et al.* 2000). Intra-familial relationships were investigated in *Ectocarpus* and *Kukuckia* (Stache-Crain *et al.* 1997), and *Laminariocolax* (Burhardt & Peters 1998), *Pelvetia* and *Pelvetiopsis* (Lee *et al.* 1998, 1999), and *Undaria* (Yoon & Boo 1999). This early work was reviewed by Druehl & Saunders (1992) and de Reviers & Rousseau (1999). It resulted in substantial reorganisation of the orders and families within the Phaeophyceae.

Phylogenetic resolution of the brown algal lineage has increased dramatically over the last decade. Taxon representation has increased considerably (Kawai *et al.* 2001, Rousseau *et al.* 2001, Draisma *et al.* 2002, Lee & Bae 2002, Cho *et al.* 2006, De Clerck *et al.* 2006), as have the number of molecular markers used (Kim *et al.* 2002, 2003, Cho *et al.* 2004, Lane *et al.* 2006,

Phillips *et al.* 2008, Kogishi *et al.* 2010). Recent studies have combined markers from nuclear, mitochondrial and chloroplast genomes (Bittner *et al.* 2008, Fraser *et al*. 2010b, Tronholm *et al.* 2010b, Silberfield *et al.* 2010).

A second focus of molecular studies has been resolving the systematic position of "difficult" or anomalous species, including *Notheia anomala* (Saunders & Kraft 1995), *Myagropsis myagroides* (Horiguchi & Yoshida 1998), *Halosiphon tomentosus* (Peters 1998), *Laminarionema elsbetiae* (Peters & Burkhardt 1998), *Desmarestia chordalis* (Peters *et al.* 2000), *Caepidium antarcticum* (Peters & Ramírez 2001), *Microzonia velutina* (Burrowes *et al.* 2003), *Stschapovia flagellaris* (Kawai & Sasaki 2004), *Phaeostrophion irregulare* (Kawai *et al.* 2005), *Petrospongium rugosum* (Cho & Boo 2006), *Discosporangium mesartrocarpum* (Kawai *et al.* 2007), and *Herpodiscus durvillaeae* (Heesch *et al.* 2008).

While not aimed at species differentiation, this phylogenetic work did offer relevant insights. In the Scytosiphonaceae, Kogame *et al.* (1999) found molecular differentiation was better correlated with the morphology of the sporophyte microthallus than with the morphology of the upright stage, which appears to be more morphologically plastic. Low variation in markers was shown in ITS sequences from *Desmarestia* (van Oppen *et al.* 1993), *Fucus* (Leclerc *et al.* 1998) and *Sargassum* (Stiger *et al.* 2000) and in mitochondrial markers (Coyer *et al.* 2006a); this suggests these markers are conserved or speciation is recent. The notion that morphological complexity is only associated with the most derived groups of brown algae was finally dispensed with (Rousseau & de Reviers 1999a, Draisma *et al.* 2001, Phillips *et al.* 2008).

Numerous studies have proposed new species supported by phylogenetic data, including *Asterocladon lobatum* (Müller *et al.* 1998), *Sargassum boreale* (Yoshida *et al.* 2000), *Chorda rigida* (Kawai *et al.* 2001), *Neoleptonema yongpilii* (Lee *et al.* 2002), *Newhousia imbricata* (Kraft *et al.* 2004), *Asterocladon interjectum* (Uwai *et al.* 2005), *Cladosiphon umezakii* (Ajisaka *et al.* 2007), *Aureophycus aleuticus* (Kawai *et al.* 2008), *Fucus radicans* (Bergström *et al.* 2005), *Dictyota cyanoloma* and *D. cymatophila* (Tronholm *et al.* 2010a, 2010b), and four *Padina* species (Ni-Ni-Win *et al.* 2010). Particularly revealing of the power of molecular techniques is the reinstatement of *Ectocarpus crouaniorum*, a third European species in this well-studied genus (Peters *et al.* 2010). In most

cases, phylogenetic data was used in support of morphological diagnoses, and to place new species in higher taxonomic groups. Recently, Tronholm *et al*. (2010a) separated the "pseudo-cryptic" *Dictyota cymatophila* from *D. dichotoma*, primarily by phylogenetic data. Phenological and morphometric data also separated species, but showed some overlap, and it is unlikely two species would have been diagnosed without phylogenetic data.

In the last decade an increasing number of studies have addressed the phylogeography of single species or closely related species over all or most of their range (Coyer *et al.* 2003, Cho *et al*. 2005, Harvey & Goff 2006, Hoarau *et al*. 2007, Lane *et al*. 2007, Fraser *et al.* 2009a, 2009b, 2009c, Pereyra *et al.* 2009, Tellier *et al*. 2009, Uwai *et al.* 2009, Coyer *et al*. 2010, Peters *et al.* 2010, Fraser *et al*. 2010, Kogishi *et al*. 2010, Neiva *et al.* 2010, Olsen *et al*. 2010, Cheang *et al.* 2010a, 2010b). These studies have sufficient detail to explore the history of species themselves (rather than a broad history of relationships between species). In many cases (e.g., Tellier *et al*. 2009, Coyer *et al*. 2010), multiple lineages have been found within current species, and some authors have proposed splitting species (Fraser *et al.* 2010b, Martin & Zuccarello in preparation).

These data should allow species delimitation to proceed from a morphological concept of species to a phylogenetic concept. In general this hasn't happened, as there has been a separation of phylogenetic analyses and taxonomic treatment of species. For example, Stache-Crain *et al.* (1997), in an early phylogenetic study that included worldwide sampling, found *Ectocarpus siliculosus* formed several clades which they suggested might have species status, but declined to create new species due to lack of morphological differentiation. Cho *et al.* (2005) found two species of *Colpomenia peregrina*, separated by phylogeographic and ecological data, and Cho *et al.* (2007) found phylogenetic evidence for two species of *Scytosiphon lomentaria*, but neither study established new species. Only a few authors have explicitly addressed phylogenetic species concepts. Harvey & Goff (2006) rejected the morphological species concept as misleading in the case of *Halidrys* and *Cystoseira* species from the Pacific Coast of North America, showing Pacific species had a common origin, separate from Atlantic members of these genera. Pacific species had independently converged to morphologies similar to Atlantic species. These morphologies were diverse, despite low genetic variation between Pacific species. The authors concluded that

revision using a phylogenetic species concept was needed. Fraser *et al.* (2009a) applied a phylogenetic species concept to *Durvillaea antarctica* and suggested that a morphologically variant form (the 'cape' morphology, where the thallus forms a broad sheet, rather than dividing into thongs) can be separated by morphological and molecular data and should be regarded as a separate species. This highlights the problems with incipient species. The 'cape' form forms a monophyletic group in *cox*1 trees, but this renders *D. antarctica* polyphyletic, as the 'thong form' Chilean lineage is sister to the "cape" clade, while the New Zealand and southern ocean lineage is derived (Fraser *et al.* 2009b). There is no evidence for morphological separation of the Chilean and New Zealand clades, but a pure phylogenetic species concept would support this (Fraser *et al.* 2010b). Either several lineages must be separated solely on the basis of molecular data or *D. antarctica* should be regarded as a meta-species (*sensu* de Queiroz & Donoghue 1988), encompassing several distinct mitochondrial lineages but without ascribing species status to these lineages. Conversely, *D. chathamensis* is morphologically distinct from the allopatric *D. antarctica* lineage, but is not differentiated by most markers (only by *rbc*L, Fraser *et al.* 2010b). In these examples, the phylogenetic species concept has been invoked to support *suggestions* for species delimitation, and in support of other data, but no author has split species of brown algae under a purely phylogenetic species concept, although this has been done with green algae (Leliaert *et al.* 2009) and microalgae (Lilly *et al*. 2007, Coffroth & Santos 2005). Instead, phycologists have delimited species by considering the weight of available evidence. Recently, phylogenetic data has been given greater weight, with the synonymisation of some morphologically variable species (Macaya & Zuccarello 2010).

In part, the reluctance to apply a pure phylogenetic species concept relates to the difficulty in assigning hierarchical levels to phylogenetic lineages and the increasing power of molecular data to diagnose lineage differentiation (Avise & Wollenberg 2007). Phylogenies are limited in being based on a small sample of genetic information from a small sample of specimens, and generally produce coarse-scale lineages showing simplified relationships between species. Complex histories, with lineages within species lineages, as well as reticulation, raise a major question for taxonomic work: to what degree are speciation processes active within most lineages? At one extreme, lineages might undergo brief periods

of speciation, separated by long periods of stasis (with cohesive, well differentiated species), at the other extreme, lineages might be continually sub-dividing, with morphological differentiation, reproductive isolation and other properties exhibiting themselves at different levels throughout a lineages history.

Recent studies in North Atlantic *Fucus* species demonstrate the difficulty of applying methods of species delimitation that assume simple speciation histories and test properties of species that are confounded by hybridisation and intraspecific morphological variation (Coyer *et al.* 2006a). Over 125 intraspecific taxa have been described in *Fucus* (Coyer *et al.* 2010), based on morphology. Various numbers of species can be recognised, depending on the degree of lineage separation and which property of a species-lineage is tested for (De Queiroz 2007). Phylogenetic approaches using ITS (Serrão *et al.* 1999) and mitochondrial markers (Coyer *et al.* 2006a) discerned two major clades, one containing *F. vesiculosus, F. spiralis, F. radicans, F. ceranoides* and *F. virsoides*, but were unable to separate these species. Cluster analyses using microsatellites separated *F. vesiculosus* from *F. spiralis* (Engel *et al.* 2005) and also separated *F. ceranoides* (Billard *et al.* 2005). Bergström *et al.* (2005) delimited a third species *Fucus radicans*, which is estimated to have diverged from *F. vesiculosus* as little as 400 years ago (Pereyra *et al.* 2009), driven by adaption to a low salinity habitat (the Baltic Sea), and this delimitation is supported by functional divergence in heat shock response (Lago-Lestón *et al.* 2010). Billard *et al.* (2010) then divided *F. spiralis* into two clusters, spatially segregated into a high shore and low shore varieties. Despite ecological, molecular and morphological differences, they did not interpret these as species, partly because there is less differentiation between the forms than between *F. spiralis* and *F. vesiculosus*. Most recently, Coyer *et al.* (2010) found that the southern part of the *F. spiralis* range forms a third cluster. This southern cluster hybridised with *F. vesiculosus* at some point in the past, producing the low shore cluster. Also, while a nuclear marker shows reciprocal monophyly between *F. ceranoides* and *F. vesiculosus*, the entire northern range of *F. ceranoides* is introgressed by mitochondrial DNA from *F. vesiculosus* (Neiva *et al.* 2010). Coyer *et al.* (2010) propose a “sliding window” concept of speciation for *Fucus*, where different species are delineated within a continuum of species separation.

These studies, and in-depth sampling of other species (Lane *et al.* 2007, Olsen *et al.* 2010) show and increasing ability to infer more detailed histories of species, and make the application of an informed historical species concept plausible in brown algae.

6.9 Species delimitation in *Carpophyllum*

Four species are currently recognised in *Carpophyllum* (Adams 1994, Lindauer *et al.* 1961), and my molecular data support continued recognition of these species. All species are morphologically and ecologically distinct, but genetic distances between markers are low. *C. maschalocarpum* and *C. angustifolium* can be separated by mitochondrial spacer data (Chapter 2) but with some reservations: (1) there is evidence for mitochondrial introgression in some populations (Chapter 3); (2) genetic distances within species are greater than distance between species; and (3) one cluster, associated with specimens from North Cape and Wekarua, is not clearly associated with either species (Chapter 2). Some genetic data was collected on *Carpophyllum plumosum* and *C. flexuosum* but was not included in the previous chapters. These data support the continued recognition of these species. For example I found unique ITS2 sequences for *C. plumosum* and *C. flexuosum* (but only one mutational step removed from each other and from *C. maschalocarpum/C. angustifolium* sequences), and mitochondrial spacer sequences that are genetically distant from other species.

The phylogenetic relationship between species is not completely clear. Markers such as ITS and *rbc*LS spacer show low variation (*rbc*LS spacer sequences are identical in *C. maschalocarpum* and *C. angustifolium*, with three changes separating these from *C. flexuosum* and six changes separating these from *C. plumosum*). *Carpophyllum angustifolium* and *C. maschalocarpum* are sister species according to all markers tested (the above and *rbc*L, *cox*3, *rps*14-*atp*8), and *C. plumosum* appears to be the earliest branching species. The history of *Carpophyllum* species is only partly elucidated by these data. Significant changes in the distribution of *C. maschalocarpum* appear to have taken place since the LGM, especially the colonisation of the southern North Island and South Island. The centre of genetic diversity of *C. angustifolium* appears to be Northland, but data are limited, whereas *C. maschalocarpum* is most genetically diverse in the

Bay of Plenty. Possibly these lineages separated in allopatry during periods of high sea level during the Pliocene (when the northern North Island was separated into islands), and are now hybridising following secondary contact. An alternative explanation is sympatric speciation from a common ancestor driven by strong selective pressure for different habitats, with *C. angustifolium* occupying exposed areas, *C. flexuosum* sheltered areas and *C. maschalocarpum* occupying intermediate environments. Further molecular data is needed to complete this picture, especially from *C. angustifolium*, as is ecophysiological data.

6.10 Species delimitation in *Cystophora*

Cystophora species have been delimited on the basis of morphological data. In New Zealand species I consider that some species have been over-split and I synonymise two pairs of names. I also suspect the Australian species *C. cuspidata* should be synonymised with *C. subfarcinata*, but sampling is inadequate to establish this. I was unable to find *C. cymodocea*, despite an extensive search of the type locality (Duttons Beach, Portland, Victoria) and suspect this is a distended form of *C. retroflexa* growing epiphytically. In all these cases, oversplitting, or potential oversplitting, appears to have arisen from limited characterisation of the range of morphological expression in species. Further molecular work can address some of these questions, and more work is needed, especially to to clarify species boundaries in Australian species, and to further understand the history of the evolution and dispersal of *Cystophora* species.

6.11 Dispersal and history

Dispersal ability is expected to correlate strongly with gene flow, and be a major factor in algal speciation. Species with limited dispersal potential, with immotile gametes and/or negatively-buoyant thalli, should show limited gene flow between populations, and stronger signatures of genetic structure and founder effects (van den Hoek 1987). Genetic structure is strong in many species of brown algae (Chapter 2, Williams & DiFiori 1996, Kusmo & Druehl 2000, Cho *et al*. 2005, 2007, Hoarau *et al.* 2007, Fraser *et al.* 2009b, Cheang *et al.* 2010), but the relationship between genetic structure and dispersal is complex. *Carpophyllum*

maschalocarpum has positively buoyant thalli and is frequently found in drift. Some well separated populations – such as mainland New Zealand and the Chatham Islands, share mitochondrial haplotypes, whereas other relatively close populations are apparently isolated (Chapter 2). In *Cystophora torulosa, C. platylobium* and *C. retroflexa*, *cox*1 haplotypes and ITS ribotypes are shared between New Zealand and Australia, as were haplotypes of a rapidly evolving mitochondrial spacer in *C. retroflexa*, suggesting multiple trans-Tasman dispersal (Chapter 4). In *Macrocystis pyrifera*–a species that has been shown to remain fertile after extended periods afloat (Macaya *et al*. 2005, Hernández-Carmona *et al.* 2006)–Macaya & Zuccarello (2010) found one *cox*1 haplotype that is distributed throughout the southern hemisphere, but several other *cox*1 haplotypes were sampled in single populations only. *Durvillaea* is especially interesting, having large buoyant thalli that appear capable of long-distance dispersal through the southern oceans resulting in genetically homogenous populations (Fraser *et al.* 2009b), but has limited north-south dispersal resulting in well differentiated lineages in lower latitudes. Also curious is that one lineage of *Durvillaea* (the "cape form") is found from New Zealand's sub-Antarctic islands and along the eastern coasts of New Zealand's South Island, while other lineages are latitudinally restricted. The cape form has no obvious differences in dispersal potential. These buoyant species contrast with *Lessonia variegata* and *L. nigrescens*, species with heavy, negatively buoyant thalli, which show strong genetic differentiation between regions of New Zealand (Martin & Zuccarello in review) and Chile (Tellier *et al.* 2009), respectively.

These studies suggest brown algae with buoyant thalli have increased potential for long distance transport, but also that this potential is not always realised. Partly this results from geography. Dispersal is rapid along pathways such as the southern ocean west wind drift system (Waters 2008), and perhaps along the East Auckland Current (Chapter 2, although data from other species is needed to confirm this), but might be restricted elsewhere (Fraser *et al.* 2009b, 2010b, Chapter 2). But biological interactions such as density blocking (suggested by Fraser *et al.* 2009b, 2010b) might be important. Several areas remain to be addressed. One is the pathways taken by drifting algae. Data from *Carpophyllum* suggest relatively high gene flow between the Chatham Islands and the North Island. Data from drift devices show current transport between the mainland and

the Chatham Islands requires 30 to 50 days (Chiswell 2009), but windblown floating material might travel considerably faster, especially during extreme weather events, for example, material from a boat wrecked in Cook Strait has been recorded arriving at the Chatham Islands three weeks later (Young 1929). Direct measurements of seaweed transport rates (Harrold & Lisina 1989, Hawes 2008) should elucidate drift rates.

Several lines of evidence suggest *Cystophora* evolved in Australia and species dispersed to New Zealand relatively recently: (1) Species diversity in *Cystophora* is greatest in southern Australia, and the earliest diverging species (*C. moniliformis*) is endemic to southern Australia; (2) Several species are endemic to Australia, whereas only one species (*C. scalaris*) is endemic to New Zealand (Womersley 1964); and (3) two mitochondrial spacer haplotypes found in New Zealand specimens are shared with Australian specimens (Chapter 4). Sharing of mitochondrial spacer haplotypes suggests very recent dispersal, given the strong structuring shown in this marker in *Carpophyllum*. *Cystophora* is thus part of what Parsons (1985) termed the Australasian element of the New Zealand seaweed flora. All New Zealand species are vesiculate, whereas several Australian species (e.g., *C. moniliformis, C. siliquosa, C. xiphocarpa*) lack vesicles, or rarely develop vesicles (Womersley 1964). It seems likely that vesicles are associated with long distance dispersal, although several vesiculate Australian species are absent from New Zealand, despite tolerating similar temperature regimes (Adey & Steneck 2001). Whether successful trans-Tasman dispersal is a matter of chance or if there are other ecophysiological factors involved in dispersal or establishment is not known.

6.12 Genetic distance – molecular data without history

Genetic distance is a straightforward method of delineating species that uses phenetic, rather than historical criteria. Various methods of delineating species using genetic distance have been proposed (Sites & Marshall 2004), usually requiring population level data. These include comparing geographical distance with genetic distance (Good & Wake 1992), with significant deviations from isolation-by-distance suggesting more than one species. Highton (1989) suggests testing for a significant bimodal distribution of Nei’s D parameter for population

differentiation, with a threshold suggested above which separate peaks represent multiple species. These methods have not been applied to brown algae. Both methods are essentially ahistorical, and might be confounded by strong structuring of populations, for example, the mtDNA spacer dataset for *Carpophyllum maschalocarpum* shows a bimodal distribution of Nei's D, this is a consequence of demographic expansion of the southern lineage, but there is no supporting evidence that this represents a speciation process. Similarly, the existence of particular dispersal pathways in *C. maschalocarpum* confounds the assumption of a linear relationship between geographical and genetic distance.

Using genetic distance might overcome the problem created by modern molecular data's power to diagnose extremely fine lineages, which could result in oversplitting of species under the phylogenetic species concept (Avise & Wollenberg 1997). But, there are problems that genetic distance might not be correlated with reproductive isolation (Ferguson 2002, Gourbière & Mallet 2009, Presgraves 2010). Speciation times appear very variable. Knowlton *et al.* (1993) suggested that many marine organisms had developed reproductive barriers since the closing of the Central American isthmus, c. 3 million years ago. Conversely, *Fucus radicans* might have attained some reproductive isolation in 400 years (Pereyra *et al.* 2009). In many cases, especially in higher latitudes, substantial demographic and geophysical changes (temperature, sea level changes) have occurred in the last 3 million years (Hewitt 2004, Maggs *et al.* 2008), creating complex patterns of isolation and contact (Coyer *et al*. 2010) that confound inferences based on genetic distance.

A recent development in distance methods is the emergence of DNA barcoding. Strictly, barcoding is a method of specimen identification, but the leading proponent of DNA barcoding, Paul Hebert, has described DNA barcoding as a method of species delimitation (Hebert & Gregory 2005) and assessing the barcoding method requires assessing the ability of mitochondrial data to delimit species.

Molecular tools have long been used for species identification in brown algae. Early studies used allozymes (Benzie *et al.* 2000, Hull *et al.* 2001), RAPDs (Ho *et al*. 1995) or sequence data (Kogame & Masuda 2001). Molecular identification continues to be a useful tool, especially with cryptic forms (Fox &

Swanson 2007), morphologically plastic (Kucera & Saunders 2008, Endo *et al*. 2009) or convergent species (Camus *et al.* 2005, Jones *et al.* 2010).

DNA barcoding distinguishes species using genetic distance between phenetic clusters inferred from short (c. 600 bp) mitochondrial DNA sequences. DNA barcoding has a compelling simplicity: a threshold between intraspecific and interspecific genetic variation is defined (usually ten-times, Golding *et al.* 2009) and specimens exceeding this threshold are considered to be separate species. Species can then be identified by simple sequence comparisons against a universal database.

Barcoding requires that genetic variation in the selected gene region can be adequately characterised. Intraspecific variation requires extensive sampling to understand the genetic structure – and therefore the species history. For example, extensive global sampling of *Durvillaea antarctica* by Fraser *et al.* (2010b) showed extensive *cox*1 variation that was highly geographically structured, with sufficient lineage delineation to propose a species complex. One *cox*1 haplotype was extensively distributed – around most of the southern oceans. In this situation, even widespread sampling, by the standards of barcoding studies, might return a single haplotype, even if samples were drawn from eight different countries. Such a result might suggest identification of *D. antarctica* by *cox*1 barcoding was straightforward, but this sampling would not enable accurate identification of further specimens from northern localities.

Similarly in Chapter 2, one mitochondrial spacer haplotype is present over a wide part of the range of *Carpophyllum maschalocarpum*. This marker that is probably more variable than regions used for barcoding but it illustrates the point. A collection of 10 specimens might detect only a single haplotype. With slightly wider sampling one might get two haplotypes, only two steps removed from each other and conclude that intraspecific variation is low. With more sampling one might find a third haplotype, four steps removed from the others, or even further removed if you sample one of these haplotypes which appear to have entered the *C. maschalocarpum* mitochondrial genome following introgressive hybridisation with *C. angustifolium*, and assume cryptic speciation was taking place. Accurate characterisation of genetic variation requires sampling from the Bay of Plenty, where most of the variation is found.

Proponents of barcoding appeal to the speed and low cost of assembling sets of “barcodes” that can then be used as vouchers for subsequent species identification. According to proponents, barcoding will facilitate documenting as many species as possible before they go extinct (Baker *et al.* 2009), allow fast organisation of molecular data into putative species groups before formal description (Litaker *et al*. 2007, Borisenko *et al*. 2009), eliminate the need for the complex taxonomic training that is currently required for species description and identification (Sass *et al.* 2007), allow identification of incomplete specimens, (Schindel & Miller 2005), and provide a tool for non-specialists, for example in water quality assessments (Moniz & Kaczmarska 2009), identifying invasive species (Saunders 2009), correct identification of difficult and cryptic species (Starr *et al.* 2009), greater discrimination of cryptic species (Sherwood *et al.* 2008) and standardisation of molecular data (McDevit & Saunders 2010). The appeal of many of these rationales is low cost, rather than scientific rigour. This might be reasonable in some applications where a number of misidentifications are acceptable, especially where error rates can be estimated and reported, but is of questionable scientific value.

Only a few studies have attempted to validate the use of *cox*1 for barcoding brown algae. Lane *et al.* (2007) attempted a *cox*1 barcoding study of *Alaria* species from the north-east Pacific. Genetic variation was relatively low and there was no clear threshold between inter- and intra-clade variation. In addition, clusters of *cox*1 mitotypes were not congruent with *a priori* morphological species assignments, or with ITS clades. ITS variation was relatively high, and did not resolve species level groups. This result was interpreted as showing widespread introgression following secondary contact by species that evolved in allopatry, with introgression and ITS recombination.

Kucera & Saunders (2008) used *cox*1 to delineate North American samples of *Fucus*. Mitotypes separated into three clades. *Fucus serratus* from Canada were well demarcated from other *Fucus* species but this species is a recent (1887) introduction to North America and this deme would be expected to show low variation. *Fucus spiralis* and *F. vesiculosus* were not separated, which is not surprising given the low variation and extensive hybridisation between these species. *Fucus distichus* and north Pacific specimens identified in the fields as *F. spiralis* were also not separated, but the authors consider *F. spiralis* in this clade

to result from incorrect morphological assignment of specimens of *F. distichus* that has undergone morphological convergence to an *F. spiralis* form. The authors then made an *a posterioi* reassignment of these specimens to *F. distichus*, with hybridisation ruled out due to the absence of polymorphic sites in ITS sequences. This was not accompanied by any anatomical evidence, and it seems possible that these are *F. spiralis* with introgressed *F. distichus* mitochondria and ITS which has homogenised to a *F. distichus*-ribotype following an earlier hybridisation event. Widespread mitochondrial introgression has been reported in European *F. serratus* (Coyer *et al*. 2010) and *F. ceranoides* (Neiva *et al* 2010). This study was reported as demonstrating the efficacy of *cox*1 barcoding in brown algae, but it is unclear what hypothesis was being tested here, as genetic distance between clusters was low or absent, morphology and molecular delimitation was not congruent, and sampling was limited to North American demes of *Fucus* species which have their centre of diversity in Europe (Coyer *et al*. 2003, Hoarau *et al*. 2007, Coyer *et al*. 2006a, 2010).

Other studies of brown algae have shown broad variation in *cox*1 sequences across orders (McDevit & Saunders 2009), but did not address species delimitation, which requires inclusion of sister species, and addressed biogeographical structure of populations with extensive sampling (McDevit & Saunders 2010). In general these studies have shown mean genetic distances between *cox*1 sequences of sister species of brown algae are often less than the 10X threshold proposed for barcoding animals. Presumably a high threshold is to compensate for low sampling and distance methods' limitations in capturing evolutionary processes. In *Cystophora* intraspecific variation was of a similar order to interspecific variation between some sister species. Recognition of these species is supported by morphology and ITS sequences, and further sampling is likely to increase intraspecific variation. An alternative to genetic distances is character based barcoding methods, where a designated number of nucleotide positions or combination of positions is used to characterise species (DeSalle 2006, Rach *et al*. 2008). This requires extensive sampling to ensure proposed characters are fixed (Wiens & Servedio 2010).

Cytoplasmic introgression is an obvious problem for mitochondrial sequence based barcoding, and has been found in *Fucus* (Neiva *et al.* 2010, Coyer *et al.* 2010), *Alaria* (Lane *et al.* 2007) and *Carpophyllum angustifolium* (Chapter

4). Comparison of cytoplasmic markers with single copy nuclear markers (Neiva *et al*. 2010) should show introgression, but so far these markers have not been widely available for brown algae. Future work should show whether these are unusual cases, or whether cytoplasmic introgression is widespread in the brown algae.

Two developments suggest future barcoding studies will be better integrated with other approaches. One is multi-locus barcoding (Kress *et al.* 2009) which allows barcoding data to be analysed using phylogenetic methods. Kress *et al.* (2009) also focussed on a single locality (Barro Colorado Island, Panama), where species diversity is well characterised and sampling intra-species genetic diversity should be relatively straightforward. Other barcoding studies have employed additional markers to overcome uncertainty (Lane *et al.* 2007, McDevit & Saunders 2010). A second is development is more widespread sampling, allowing barcoding data to be used for mitochondrial phylogeographic studies (McDevit & Saunders 2010). These trends might overcome the limitations of low sampling and single markers proposed in the original barcoding concept (Hebert *et al*. 2003a, 2003b).

6.13 Model based methods

Microsatellites analysis has been used to separate closely related brown algal species. Most studies use a Bayesian procedure (STRUCTURE) that simultaneously generates clusters and assigns specimens to clusters based on a model that assumes Hardy-Weinberg equilibrium and linkage equilibrium within populations (Pritchard *et al.* 2000). This approach has been used to separate species of *Fucus* (Coyer *et al*. 2003, Billard *et al*. 2005, Engel *et al.* 2005, Tatarenkov *et al.* 2007) where it has successfully discriminated species where there is low phylogenetic divergence and gene flow between species. This is useful but does not elucidate species histories, as there are difficulties in using microsatellites for phylogenetic inference (Noor *et al.* 2001, Buschiazzo & Gemmell 2006).

Knowles & Carstens (2007) suggested using coalescence to estimate the probabilities of gene trees under a particular history to evaluate the possibility of lineage splitting (i.e. speciation). This models species histories probabilistically

rather than inferring species history from indications of reciprocal monophyly in sampled genes. A method that combines population biology and phylogenetic approaches was developed by Pons *et al*. (2006). This method attempts to determine a threshold between inter-species level (phylogenetic) lineage branching and intraspecies (tokogenetic) branching, using a "Generalised Yule Mixed Coalescent model." A maximum likelihood tree is inferred from sequence data, the ages of nodes are estimated, and a lineage-through-time plot constructed. A change in branching rates in this plot should correspond to a change from phylogenetic processes to coalescent processes. This method has not been used in brown algae. Leliaert *et al.* (2009) applied the method to green algae (*Boodlea*), but found estimated confidence intervals were very broad, allowing a wide range of species divisions. A preliminary attempt at applying this method to *cox*1 data from *Cystophora*, with changes in branching rates estimated visually from the lineage-through-time plot, was not congruent with expected species boundaries, and different species boundaries were indicated with small changes in parameters.

6.14 Synthesising species concepts

Eukaryote algae have not featured greatly in the development of species concepts. Birds seem somewhat overrepresented, probably because of the influence of Ernst Mayr; terrestrial plants usually get a mention (hybridisation), as do prokaryotes (asexuality). None-the-less, debates over species concepts have influenced phycologists and are pertinent to any discussion of species delimitation.

At the end of the 1990s various attempts were made to synthesise species concepts (Avise & Wollenberg 1997, Mayden 1997, de Queiroz 1998). These attempts were anticipated by Hull (1965) who proposed understanding species as a cluster concept. Avise & Wollenberg (1997) suggested that there is no difficulty in combining the biological (BSC) and phylogenetic species concepts (PSC), as long as diagnosis of monophyly uses multiple lines of evidence. This "multi-locus PSC" would minimise conflicts between gene trees and species trees, and limit diagnosis of sub-species lineages. Mayden (1997) argues for a hierarchy of species concepts, with the highest order species concept not operable, but based on theory, but supported by various lower order concepts that are, to varying degrees, operable. This allows a unification of species concepts, with the

relationships between them clearly defined. De Queiroz (1998, 2007) suggests separating the definition of species from operational methods used to delimit species, as these are different philosophical concepts, albeit ones that can inform one another. In De Queiroz's scheme (2007), species are metapopulation lineages that acquire different properties (monophyly, reproductive isolation, etc.) at different times during the process of speciation. Species are delimited by testing for these various properties, but not all properties will coincide at one moment in the history of speciation, so no single property is required (or expected) in every species. Successful delimitation of species, therefore, will depend on the property tested and the history of lineage separation.

Piglucci (2003) develops a similar argument to De Queiroz, but one that is not based on history. They argue that the species problem is essentially a philosophical question that needs empirical data to answer it (rather than a scientific question with philosophical aspects). The question can be resolved by viewing species as a cluster concept *sensu* Wittgenstein, where there are shared properties between all species, none of which are necessary or sufficient alone.

These concepts are compatible with Coyer *et al*.'s (2010) sliding window concept of species for *Fucus*. They also suggest a weight of evidence approach to species delimitation, rather than a Popperian hypothesis testing approach. This is similar the actual practice by taxonomists dealing with brown algae. With the availability of various lines of evidence, it is becoming rare for a single property of a species to be privileged above all others. Rather, various properties are considered and weighed before a decision is made.

Uncertainty is likely to remain a problem for species delimitation. Contemporary data will rarely – if ever – support a unique hypothesis about historical events. The complexity of biological processes, the use of proxies (morphology, genetic distance) for speciation, inference from limited data and researchers' biases (splitters and lumpers) suggests species delimitation will remain an exercise in approximation, subject to revision.

I suggest no method is likely to encompass the diversity of evolutionary processes and historical scenarios found in speciation, and conclude that: (1) a weight of evidence approach should be taken in delimiting species, with no single method being privileged (this essentially continues the *status quo* in brown algal taxonomy) and; (2) different sets of species' properties will be found in different

species. These properties are best encapsulated by including species' history in species descriptions.

Contingency plays a large role in speciation processes, and the delimitation of species is an exploration of biological history. There are expected to be major differences in the speciation histories of different lineages, for example the fast speciation in *Fucus radicans* where reinforcement (selection for characters limiting hybridisation) might be expected to play a role, or the slow drift to reproductive isolation in allopatric species. Incorporating species histories into the description of species would overcome the lingering influence of the typological approach, where species are described by a name and morphological diagnosis. Explicitly including history as part of species descriptions would emphasise that recognition that a lineage has species status can reflect different speciation processes.

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Chapter 7
Appendices

7.1 Appendix 1. Alignment of mitochondrial spacer sequences from *Carpophyllum maschalocarpum.* A single specimen is shown for each haplotype/site. Specimen names are truncated as: FieldnumberSitenameHaplotype.

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B560Opap01 GTAAAGTTTTCAGTGGGGGTGAAATTTAATCACTTTGATAGGCGGGGGGTGTAAGAGCTGTGAGGTTTTTAGCTGACC
B213Kiri02 GTAAAGTTTTCAGTGGGGGTGAAATTTAATCACTTTGATAGGCGGGGGGTGTAAGAGCTGTGAGGTTTTTAGCTGACC
B051Dors03 GTAAAGTTTTCAGTGGGGGTGAAATTTAATCACTTTGATAGGCGGGGGGTGTAAGAGCTGTGAGGTTTTTAGCTGACC
A737Kiri04 GTaAaGTTTTCAGTGGGGGTGAAATTTAATCACTTTGATAGGCGGGGGGTGTAAGAGCTGTGAGGTTTTTAGCTGACC
A738Kiri05 GTAAAGTTTTCAGTGGGGGTGAAATTTAATCACTTTGATAGGCGGGGGGTGTAAGAGCTGTGAGGTTTTTAGCTGACC
B307Whal06 GTAAAGTTTTCAGTGGGGGTGAAATTTAATCACTTTGATAGGCGGGGGGTGTAAGAGCTGTGAGGTTTTTAGCTGACC
B256Piha07 GTAAAGTTTTCAGTGGGGGTGAAATTTAATCACTTTGATAGGCGGGGGGTGTAAGAGCTGTGAGGTTTTTAGCTGACC
B590Ahur08 GTAAAGTTTTCAGTGGGGGTGAAATTTAATCACTTTGATAGGCGGGGGGTGTAAGAGCTGTGAGGTTTTTAGCTGACC
B228Wait09 GTAAAGTTTTCAGTGGGGGTGAAATTTAATCACTTTGATAGGCGGGGGGTGTAAGAGCTGTGAGGTTTTTAGCTGACC
B220Wait10 GTAAAGTTTTCAGTGGGGGTGAAATTTAATCACTTTGATAGGCGGGGGGTGTAAGAGCTGTGAGGTTTTTAGCTGACC
A679Waik11 GTaAAGTTTTCAGTGGGGGTGAAATTTAATCACTTTGATAGGCGGGGGGTGTAAGAGCTGTGAGGTTTTTAGCTGACC
B255Rive12 GTAAAGTTTTCAGTGGGGGTGAAATTTAATCACTTTGATAGGCGGGGGGTGTAAGAGCTGTGAGGTTTTTAGCTGACC
A865Leig13 GTAAAGTTTTCAGTGGGGGTGAAATTTAATCACTTTGATAGGCGGGGGGTGTAAGAGCTGTGAGGTTTTTAGCTGACC
B392Math14 GTAAAGTTTTCAGTGGGGGTGAAATTTAATCACTTTGATAGGCGGGGGGTGTAAGAGCTGTGAGGTTTTTAGCTGACC
A847Mara14 GTAAAGTTTTCAGTGGGGGTGAAATTTAATCACTTTGATAGGCGGGGGGTGTAAGAGCTGTGAGGTTTTTAGCTGACC
A809Hybr15 GTAAAGTTTTCAGTGGGGGTGAAATTTAATCACTTTGATAGGCGGGGGGTGTAAGAGCTGTGAGGtTTTTAGCTGACC
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B289Dors24 GTAAAGTTTTCAGTGGGGGTGAAATTTAATCACTTTGATAGGCGGGGGGTGTAAGAGCTGTGAGGTTTTTAGCTGACC
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A834Mara38 GTAAAGTTTTCAGTGGGGGTGAAATTTAATCACTTTGATAGGCGGGGGGTGTAAGAGCTGTGAGGTTTTTAGCTGACC
B223Wait39 GTAAAGTTTTCAGTGGGGGTGAAATTTAATCACTTTGATAGGCGGgGGgTGTAAGAGCTGTGAGGTTTTTAGCTGACC
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Appendix 1. (Continued)

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Appendix 1. (Continued)

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7.2 Appendix 2. Alignment of ITS2 sequences from *Carpophyllum.* Specimen names are truncated as: FieldNumberSpecies [Cangu=*C. angustifolium;* Cmasc=*C. maschalocarpum;* Chybr= *Carpophyllum* hybrid].

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Appendix 2. (Continued)

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Appendix 2. (Continued)

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Appendix 2. (Continued)

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A922Chybr TGGGCAGTCTCGAGAATACCGGAGAGAGACCGGTGATAATGACGATGCCATACCCCCGAT
A916Chybr TGGGCAGTCTCGAGAATACCGGAGAGAGACCGGTGATAATGACGATGCCATACCCCCGAT
A895Chybr TGGGCAGTCTCGAGAATACCGGAGAGAGACCGGTGATAATGACGATGCCATACCCCCGAT
A909Chybr TGGGCAGTCTCGAGAATACCGGAGAGAGACCGGTGATAATGACGATGCCATACCCcCGAT
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A796Cmasc TGGGCAGTCTCGAGAATACCGGAGAGAGACCGGTGATAATGACGATGCCATACCCCCGAT
A836Cmasc TGGGCAGTCTCGAGAATACCGGAGAGAGACCGGTGATAATGACGATGCCATACCCCCGAT
A766Cmasc TGGGCAGTCTCGAGAATACCGGAGAGAGACCGGTGATAATGACGATGCCATACCCCCGAT
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A597Cmasc TGGGCAGTCTCGAGAATACCGGAGAGAGACCGGTGATAATGACGATGCCATACCCCCGAT
A509Cmasc TGGGCAGTCTCGAGAATACCGGAGAGAGACCGGTGATAATGACGATGCCATACCCCCGAT
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A342Cmasc TGGGCAGTCTCGAGAATACCGGAGAGAGACCGGTGATAATGACGATGCCATACCCCCGAT
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A111Cmasc TGGGCAGTCTCGAGAATACCGGAGAGAGACCGGTGATAATGACGATGCCATACCCCCGAT
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Appendix 2. (Continued)

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A389Cmasc TGGGCAGTCTCGAGAATACCGGAGAGAGACCGGTGATAATGACGATGCCATACCCCCGAT
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A575Cmasc TGGGCAGTCTCGAGAATACCGGAGAGAGACCGGTGATAATGACGATGCCATACCCCCGAT
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7.3 Appendix 3. Alignment of cloned ITS2 sequences from *Carpophyllum*. Each alignment shows 8–12 sequences recovered from cloned DNA from one specimen.

7.3.1 Sequences from A347, Waterfall Reef, Leigh.

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A347_3O ATCATCGAAACTTCGAACGCATCTTGCGCTCCCGGGATATGCCTGGGAGCATGCTTGTCGGGGAGGAGGAGGCGAAAAAT
A347_3C ATCATCGAAACTTCGAACGCATCTTGCGCTCCCGGGATATGCCTGGGAGCATGCTTGTCGGGGAGGAGGAGGCGAAAAAT
A347_3D ATCATCGAAACTTCGAACGCATCTTGCGCTCCCGGGATATGCCTGGGAGCATGCTTGTCGGGGAGGAGGAGGCGAAAAAT
A347_3G ATCATCGAAACTTCGAACGCATCTTGCGCTCCCGGGATATGCCTGGGAGCATGCTTGTCGGGGAGGAGGAGGCGAAAAAT
A347_3H ATCATCGAAACTTCGAACGCATCTTGCGCTCCCGGGATATGCCTGGGAGCATGCTTGTCGGGGAGGAGGAGGCGAAAAAT
A347_3P ATCATCGAAACTTCGAACGCATCTTGCGCTCCCGGGATATGCCTGGGAGCATGCTTGTCGGGGAGGAGGAGGCGAAAAAT
A347_3K ATCATCGAAACTTCGAACGCATCTTGCGCTCCCGGGATATGCCTGGGAGCATGCTTGTCGGGGAGGAGGAGGCGAAAAAT
A347_3L ATCATCGAAACTTCGAACGCATCTTGCGCTCCCGGGATATGCCTGGGAGCATGCTTGTCGGGGAGGAGGAGGCGAAAAAT
A347_3a ATCATCGAAACTTCGAACGCATCTTGCGCTCCCGGGATATGCCTGGGAGCATGCTTGTCGGGGAGGAGGAGGCGAAAAAT
A347_3I ATCATCGAAACTTCGAACGCATCTTGCGCTCCCGGGATATGCCTGGGAGCATGCTTGTCGGGGAGGAGGAGGCGAAAAAT

A347_3O CGCCCACAGCTTCGGGTTCGATCTCGATCTCGAGGCGGTGGAGCGGACTCTGAGTGTTCCGGGGGGCAGTGGTGCCGTGT
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A347_3D CGCCCACAGCTTCGGGTTCGATCTCGATCTCGAGGCGGTGGAGCGGACTCTGAGTGTTCCGGGGGGCAGTGGTGCCGTGT
A347_3G CGCCCACAGCTTCGGGTTCGATCTCGATCTCGAGGCGGTGGAGCGGACTCTGAGTGTTCCGGGGGGCAGTGGTGCCGTGT
A347_3H CGCCCACAGCTTCGGGTTCGATCTCGATCTCGAGGCGGTGGAGCGGACTCTGAGTGTTCCGGGGGGCAGTGGTGCCGTGT
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A347_3K CGCCCACAGCTTCGGGTTCGATCTCGATCTCGAGGCGGTGGAGCGGACTCTGAGTGTTCCGGGGGGCAGTGGTGCGGTGT
A347_3L CGCCCACAGCTTCGGGTTCGATCTCGATCCCGAGGCGGTGGAGCGGACTCTGAGTGTTCCGGGGGGCAGTGGTGCGGTGT
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A347_3O GTATTTTCACGTACATACTGCCAGCTCGTCCCCTGAGTTCACCGAAGCCTAGAGAGGAAACCGACCGTCCGGGCATTTAT
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A347_3D GTATTTTCACGTACATACTGCCAGCTCGTCCCCTGAGTTCACCGAAGCCTAGAGAGGAAACCGACCGTCCGGGCATTTAT
A347_3G GTATTTTCACGTACATACTGCCAGCTCGTCCCCTGAGTTCACCGAAGCCTAGAGAGGAAACCGACCGTCCGGGCATTTAT
A347_3H GTATTTTCACGTACATACTGCCAGCTCGTCCCCTGAGTTCACCGAAGCCTAGAGAGGAAACCGACCGTCCGGGCATTTAT
A347_3P GTATTTTCACGTACATACTGCCAGCTCGTCCCCTGAGTTCACCGAAGCCTAGAGAG-AAACCGACCGTCCGGGCATTTAT
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A347_3O TCTCT?TGCGGCGCTTAAGGCAGGTTCACTTTGCGTCCTCCAGAAGATCCGTTGTTGACGGCGCCCCCTTTCGTGGGGCG
A347_3C TCTCTTTGCGGCGCTTAAGGCAGGTTCACTTTGCGTCCTCCAGAAGATCCGTTGTTGACGGCGCCCCCTTTCGTGGGGCG
A347_3D TCTCTTTGCGGCGCTTAAGGCAGGTTCACTTTGCGTCCTCCAGAAGATCCGTTGTTGACGGCGCCCCCTTTCGTGGGGCG
A347_3G TCTCTTTGCGGCGCTTAAGGCAGGTTCACTTTGCGTCCTCCAGAAGATCCGTTGTTGACGGCGCCCCCTTTCGTGGGGCG
A347_3H TCTCTTTGCGGCGCTTAAGGCAGGTTCACTTTGCGTCCTCCAGAAGATCCGTTGTTGACGGCGCCCCCTTTCGTGGGGCG
A347_3P TCTCTTTGCGGCGCTTAAGGCAGGTTCACTTTGCGTCCTCCAGAAGATCCGTTGTTGACGGCGCCCCCTTTCGTGGGGCG
A347_3K TCTCTTTGCGGCGCTTAAGGCAGGTTCACTTTGCGTCCTCCAGAAGATCCGTTGTTGACGGCGCCCCCTTTCGTGGGGCG
A347_3L TCTCTTTGCGGCGCTTAAGGCAGGTTCACTTTGCGTCCTCCAGAAGATCCGTTGTTGACGGCGCCCCCTTTCGTGGGGCG
A347_3a TCTCTTTGCGGCGCTTAAAGCAGGTTCACTTTGCGTCCTCCAGAAGATCCGTTGTTGACGGCGCCCCCTTTCGTGGGGCG
A347_3I TCTCTTTGCGGCGCTTAAGGCAGGTTCACTTTGCGTCCTCCAGAAGATCCGTTGTTGACGGCGCTCCCTTTCGTGGGGCG

A347_3O GGGACACGACGGGTTGCTGGGGATGAGCGCGGGTGGCCTTGCTGCGTCGCGCTGGAGGCCCGTGGACGGTGGGCAGTCTC
A347_3C GGGACACGACGGGTTGCTGGGGATGAGCGCGGGTGGCCTTGCTGCGTCGCGCTGGAGGCCCGTGGACGGTGGGCAGTCTC
A347_3D GGGACACGACGGGTTGCTGGGGATGAGCGCGGGTGGCCTTGCTGCGTCGCGCTGGAGGCCCGTGGACGGTGGGCAGTCTC
A347_3G GGGACACGACGGGTTGCTGGGGATGAGCGCGGGTGGCCTTGCTGCGTCGCGCTGGAGGCCCGTGGACGGTGGGCAGTCTC
A347_3H GGGACACGACGGGTTGCTGGGGATGAGCGCGGGTGGCCTTGCTGCGTCGCGCTGGAGGCCCGTGGACGGTGGGCAGTCTC
A347_3P GGGACACGGCGGGTTGCTGGGGATGAGCGCGGGTGGCCTTGCTGCGTCGCGCTGGAGGCCCGTGGGCGGTGGGCAGTCTC
A347_3K GGGACACGACGGGTTGCCGGGGATGAGCGCGGGTGGCCTTGCTGCGTCGCGCTGGAGGCCCGTGGACGGTGGGCAGTCTC
A347_3L GGGACACGACGGGTTGCTGGGGATGAGCGCGGGTGGCCTTGCTGCGTCGCGCTGGAGGCCCGTGGACGGTGGGCAGTCTC
A347_3a GGGACACGACAGGTTGCTGGGGATGAGCGCGGGTGGCCTTGCTGCGTCGCGCTGGAGGCCCGTGGACGGTGGGCAGTCTC
A347_3I GGGACACGACGGGTTGCTGGGGATGAGCGCGGGTGGCCTTGCTGCGTCGCGCTGGAGGCCCGTGGACGGTGGGCAGTCTC

A347_3O GAGAATACCGGAGAGAGACCGGTGATAATGACGATGCCATACCCCCGAT
A347_3C GAGAATACCGGAGAGAGACCGGTGATAATGACGATGCCATACCCCCGAT
A347_3D GAGAATACCGGAGAGAGACCGGTGATAATGACGATGCCATACCCCCGAT
A347_3G GAGAATACCGGAGAGAGACCGGTGATAATGACGATGCCATACCCCCGAT
A347_3H GAGAATACCGGAGAGAGACCGGTGATAATGACGATGCCATACCCCCGAT
A347_3P GAGAATACCGGAGAGAGACCGGTGATAATGACGATGCCATACCCCCGAT
A347_3K GAGAATACCGGAGAGAGACCGGTGATAATGACGATGCCATACCCCCGAT
A347_3L GAGAATACCGGAGAGAGACCGGTGATAATGACGATGCCATACCCCCGAT
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A347_3I GAGAATACCGGAGAGAGACCGGTGATAACGACGATGCCATACCCCCGAT
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7.3.2 Sequences from A810, Maraehako Bay

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A810_4K ATCATCGAAACTTCGAACGCATCTTGCGCTCCCGGGATATGCCTGGGAGCATGCTTGTCGGGGAGGAGGAGGCGAAAA
A810_4G ATCATCGAAACTTCGAACGCATCTTGCGCTCCCGGGATATGCCTGGGAGCATGCTTGTCGGGGAGGAGGAGGCGAAAA
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Appendix 3. (Continued)

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A810_4M ATCATCGAAACTTCGAACGCATCTTGCGCTCCCGGGATATGCCTGGGAGCATGCTTGTCGGGGAGGAGGAGGCGAAAA
A810_4B ATCATCGAAACTTCGAACGCATCTTGCGCTCCCGGGATATGCCTGGGAGCATGCTTGTCGGGGAGGAGGAGGCGAAAA
A810_4J ATCATCGAAACTTCGAACGCATCTTGCGCTCCCGGGATATGCCTGGGAGCATGCTTGTCGGGGAGGAGGAGGCGAAAA
A810_4T ATCATCGAAACTTCGAACGCATCTTGCGCTCCCGGGATATGCCTGGGAGCATGCTTGTCGGGGAGGAGGAGGCGAAAA
A810_4D ATCATCGAAACTTCGAACGCATCTTGCGCTCCCGGGATATGCCTGGGAGCATGCTTGTCGGGGAGGAGGAGGCGAAAA
A810_4N ATCATCGAAACTTCGAACGCATCTTGCGCTCCCGGGATATGCCTGGGAGCATGCTTGTCGGGGAGGAGGAGGCGAAAA

A810_4R ATCGCCCACAGCTTCGGGTTCGATCTCGATCTCGAGGCGGTGGAGCGGACTCTGAGTGTTCCGGGGGGCAGTGGTGCC
A810_4O ATCGCCCACAGCTTCGGGTTCGATCTCGATCTCGAGGCGGTGGAGCGGACTCTGAGTGTTCCGGGGGGCAGTGGTGCC
A810_4K ATCGCCCACAGCTTCGGGTTCGATCTCGATCTCGAGGCGGTGGAGCGGACTCTGAGTGTTCCGGGGGGCAGTGGTGCC
A810_4G ATCGCCCACAGCTTCGGGTTCGATCTCGATCTCGAGGCGGTGGAGCGGACTCTGAGTGTTCCGGGGGGCAGTGGTGCC
A810_4H ATCGCCCACAGCTTCGGGTTCGATCTCGATCTCGAGGCGGTGGAGCGGACTCTGAGTGTTCCGGGGGGCAGTGGTGCC
A810_4A ATCGCCCACAGCTTCGGGTTCGATCTCGATCTCGAGGCGGTGGAGCGGACTCTGAGTGTTCCGGGGGGCAGTGGTGCC
A810_4M ATCGCCCACAGCTTCGGGTTCGATCTCGATTTCGAGGCGGTGGAGCGGACTCTGAGTGTTCCGGGGGGCAGTGGTGCC
A810_4B ATCGCCCACAGCTTCGGGTTCGATCTCGATTTCGAGGCGGTGGAGCGGACTCTGAGTGTTCCGGGGGGCAGTGGTGCC
A810_4J ATCGCCCACAGCTTCGGGTTCGATCTCGATCTCGAGGCGGTGGAGCGGACTCTGAGTGTTCCGGGGGGCAGTGGTGCG
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A810_4D ATCGCCCACAGCTTCGGGTTCGATCTCGATCTCGAGGCGGTGGAGCGGACTCTGAGTGTTCCGGGGGGCAGTGGTGCG
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A810_4B CATTTATTCTCTTTGCGGCGCTTAAGGCAGGTTCACTTTGCGTCCTCCAGAAGATCCGTTGTTGACGGCGCCCCCTTT
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A810_4N CATTTATTCTCTTTGCGGCGCTTAAGGCAGGTTCACTTTGCGTCCTCCAGAAGATCCGTTGTTGACGGCGCCCCCTTT

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A810_4O CGTGGGGCGGGGACACGACGGGTTGCTGGGGATGAGCGCGGGTGGCCTTGCTGCGTCGCGCCGGAGGCCAGTGGACGG
A810_4K CGTGGGGCGGGGACACGACGGGTCGCTGGGGATGAGCGCGGGTGGCCTTGCTGCGTCGCGCCGGAGGCCAGTGGACGG
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7.3.3 Sequences from A980, Otanga

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A980_7A ATCATCGAAACTTCGAACGCATCTTGCGCTCCCGGGATATGCCTGGGAGCATGCTTGTCGGGGAGGAGGAGGCGAAAA
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A980_7G GTGTGTATTTTCACGTACATACTGCCAGCTCGTCCCCTGAGTTCACCGAAGCCTAGAGAG-GAAACCGACCGTCCGGG
A980_7H GTGTGTATTTTCACGTACATACTGCCAGCTCGTCCCCTGAGTTCACCGAAGCCTAGAGAG-GAAACCGACCGTCCGGG
A980_7I GTGTGTATTTTCACGTACATACTGCCAGCTCGTCCCCTGAGTTCACCGAAGCCTAGGGAG-GAAACCGACCGTCCGGG
A980_7K GTGTGTATTTTCACGTACATACTGCCAGCTCGTCCCCTGAGTTCACCGAAGCCTAGAGAG-GAAACCGACCGTCCGGG
A980_7L GTGTGTATTTTCACGTACATACTGCCAGCTCGTCCCCTGAGTTCACCGAAGCCTAGAGAG-GAAACCGACCGTCCGGG

A980_7G CATTTATTCTCTTTGCGGCGCTTAAGGCAGGTTCACTTTGCGTCCTCCAGAAGATCCGTTGTTGACGGCGCCCCCTTT
A980_7B CATTTATTCTCTTTGCGGCGCTTAAGGCAGGTTCACTTTGCGTCCTCCAGAAGATCCGTTGTTGACGGCGCCCCCTTT
A980_7A CATTTATTCTCTTTGCGGCGCTTAAGGCAGGTTCACTTTGCGTCCTCCAGAAGATCCGTTGTTGACGGCGCCCCCTTT
A980_7C CATTTATTCTCTTTGCGGCGCTTAAGGCAGGTTCACTTTGCGTCCTCCAGAAGATCCGTTGTTGACGGCGCCCCCTTT
A980_7D CATTTATTCTCTTTGCGGCGCTTAAGGCAGGTTCACTTTGCGTCCTCCAGAAGATCCGTTGTTGACGGCGCCCCCTTT
A980_7E CATTTATTCTCTTTGCGGCGCTTAAGGCAGGTTCACTTTGCGTCCTCCAGAAGATCCGTTGTTGACGGCGCCCCCTTT
A980_7G CATTTATTCTCTTTGCGGCGCTTAAGGCAGGTTCACTTTGCGTCCTCCAGAAGATCCGTTGTTGACGGCGCCCCCTTT
A980_7H CATTTATTCTCTTTGCGGCGCTTAAGGCAGGTTCACTTTGCGTCCTCCAGAAGATCCGTTGTTGACGGCGCCCCCTTT
A980_7I CATTTATTCTCTTTGCGGCGCTTAAGGCAGGTTCACTTTGCGTCCTCCAGAAGATCCGTTGTTGACGGCGCCCCCTTT
A980_7K CATTTATTCTCTTTGCGGCGCTTAAGGCAGGTTCACTTTGCGTCCTCCAGAAGATCCGTTGTTGACGGCGCCCCCTTT
A980_7L CATTTATTCTCTTTGCGGCGCTTAAGGCAGGTTCACTTTGCGTCCTCCAGAAGATCCGTTGTTGACGGCGCCCCCTTT

A980_7G CGTGGGGCGGGGACACGACGGGTTGCTGGGGATGAGCGCGGGTGGCCTTGCTGCGTCGCGCTGGAGGCCCGTGGACGG
A980_7B CGTGGGGCGGGGACACGACGGGTTGCTGGGGATGAGCGCGGGTGGCCTTGCTGCGTCGCGCTGGAGGCCCGTGGACGG
A980_7A CGTGGGGCGGGGACACGACGGGTTGCTGGGGATGAGCGCGGGTGGCCTTGCTGCGTCGCGCCGGAGGCCAGTGGACGG
A980_7C CGTGGGGCGGGGACACGACGGGTTGCTGGGGATGAGCGCGGGTGGCCTTGCTGCGTCGCGCCGGAGGCCAGTGGACGG
A980_7D CGTGGGGCGGGGACACGGCGGGTTGCTGGGGATGAGCGCGGGTGGCCTTGCTGCGTCGCGCCGGAGGCCAGTGGACGG
A980_7E CGTGGGGCGGGGACACGACGGGTTGCTGGGGATGAGCGCGGGTGGCCTTGCTGCGTCGCGCCGGAGGCCAGTGGACGG
A980_7G CGTGGGGCGGGGACACGACGGGTTGCTGGGGATGAGCGCGGGTGGCCTTGCTGCGTCGCGCCGGAGGCCAGTGGACGG
A980_7H CGTGGGGCGGGGACACGACGGGTTGCTGGGGTTGAGCGCGGGTGGCCTTGCTGCGTCGCGCCGGAGGCCAGTGGACGG
A980_7I CGTGGGGCGGGGACACGACGGGTTGCTGGGGATGAGCGCGGGTGGCCTTGCTGCGTCGCGCCGGAGGCCAGTGGACGG
A980_7K CGTGGGGCGGGGACACGACGGGTTGCTGGGGATGAGCGCGGGTGGCCTTGCTGCGTCGCGCCGGAGGCCAGTGGACGG
A980_7L CGTGGGGCGGGGACACGACGGGTTGCTGGGGATGAGCGCGGGTGGCCTTGCTGCGTCGCGCCGGAGGCCAGTGGACGG

A980_7G TGGGCAGTCTCGAGAATACCGGAGAGAGACCGGTGATAATGACGATGCCATACCCCCGAT
A980_7B TGGGCAGTCTCGAGAATACCGGAGAGAGACCGGTGATAATGACGATGCCATACCCCCGAT
A980_7A TGGGCAGTCTCGAGAATACCGGAGAGAGACCGGTGATAATGACGATGCCATACCCCCGAT
A980_7C TGGGCAGTCTCGAGAATACCGGAGAGAGACCGGTGATAATGACGATGCCATACCCCCGAT
A980_7D TGGGCAGTCTCGAGAATACCGGAGAGAGACCGGTGATAATGACGATGCCATACCCCCGAT
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A980_7K TGGGCAGTCTCGAGAATACCGGAGAGAGACCGGTGATAATGACGATGCCATACCCCCGAT
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7.3.4 Sequences from A868, Waterfall Reef, Leigh

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A868_5N ATCATCGAAACTTCGAACGCATCTTGCGCTCCCGGGATATGCCTGGGAGCATGCTTGTCGGGGAGGAGGAGGCGAAAA
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A868_5G ATCATCGAAACTTCGAACGCATCTTGCGCTCCCGGGATATGCCTGGGAGCATGCTTGTCGGGGAGGAGGAGGCGAAAA
A868_5L ATCATCGAAACTTCGAACGCATCTTGCGCTCCCGGGATATGCCTGGGAGCATGCTTGTCGGGGAGGAGGAGGCGAAAA
A868_5B ATCATCGAAACTTCGAACGCATCTTGCGCTCCCGGGATATGCCTGGGAGCATGCTTGTCGGGGAGGAGGAGGCGAAAA
A868_5D ATCATCGAAACTTCGAACGCATCTTGCGCTCCCGGGATATGCCTGGGAGCATGCTTGTCGGGGAGGAGGAGGCGAAAA
A868_5H ATCATCGAAACTTCGAACGCATCTTGCGCTCCCGGGATATGCCTGGGAGCATGCTTGTCGGGGAGGAGGAGGCGAAAA
A868_5K ATCATCGAAACTTCGAACGCATCTTGCGCTCCCGGGATATGCCTGGGAGCATGCTTGTCGGGGAGGAGGAGGCGAAAA

A868_5N ATCGCCCACAGCTTCGGGTTCGATCTCGATCTCGAGGCGGTGGAGCGGACTCTGAGTGTTCCGGGGGGCAGTGGTGCG
A868_5R ATCGCCCACAGCTTCGGGTTCGATCTCGATCTCGAGGCGGTGGAGCGGACTCTGAGTGTTCCGGGGGGCAGTGGTGCG
A868_5G ATCGCCCACAGCTTCGGGTTCGATCTCGATCTCGAGGCGGTGGAGCGGACTCTGAGTGTTCCGGGAGGCAGTGGTGCG
A868_5L ATCGCCCACAGCTTCGGGTTCGATCTCGATCTCGAGGCGGTGGAGCGGACTCTGAGTGTTCCGGGGGGCAGTGGTGCG
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A868_5H ATCGCCCACAGCTTCGGGTTCGATCTCGATCTCGAGGCGGTGGAGCGGACTCTGAGTGTTCCGGGGGGCAGTGGTGGG
A868_5K ATCGCCCACAGCTTCGGGTTCGATCTCGATCTCGAGGCGGTGGAGCGGACTCTGAGTGTTCCGGGGGGCAGTGGTGGG

A868_5N GTGTGTATTTTCACGTACATACTGCCAGCTCGTCCCCTGAGTTCACCGAAGCCTAGAGAGAGAAACCGACCGTCCGGG
A868_5R GTGTGTATTTTCACGTACATACTGCCAGCTCGTCCCCTGAGTTCACCGAAGCCTAGAGAGAGAAACCGACCGTCCGGG
A868_5G GTGTGTATTTTCACGTACATACTGCCAGCTCGTCCCCTGAGTTCACCGAAGCCTAGAGAGAGAAACCGACCGTCCGGG
A868_5L GTGTGTATTTTCACGTACATACTGCCAGCTCGTCCCCTGAGTTCACCGAAGCCTAGAGAGAGAAACCGACCGTCCGGG
A868_5B GTGTGTATTTTCACGTACATGCTGCCAGCTCGTCCCCTGAGTTCACCGAAGCCTAGAGAG--AAACCGACCGTCCGGG
A868_5D GTGTGTATTTTCACGTACATGCTGCCAGCTCGTCCCCTGAGTTCACCGAAGCCTAGAGAG--AAACCGACCGTCCGGG
A868_5H GTGTGTATTTTCACGTACATGCTGCCAGCTCGTCCCCTGAGTTCACCGAAGCCTAGAGAG--AAACCGACCGTCCGGG
A868_5K GTGTGTATTTTCACGTACATGCTGCCAGCTCGTCCCCTGAGTTCACCGAAGCCTAGAGAG--AAACCGACCGTCCGGG

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A868_5G CATTTATTCTCTTTGCGGCGCTTAAGGCAGGTTCACTTTGCGTCCTCCAGAAGATCCGTTGTTGACGGCGCCCCCTTT
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A868_5B CATTTATTCTCTTTGCGGCGCTTAAGGCAGGTTCACTTTGCGTCCTCCAGAAGATCCGTTGTTGACGGCGCCCCCTTT
A868_5D CATTTATTCTCTTTGCGGCGCTTAAGGCAGGTTCACTTTGCGTCCTCCAGAAGATCCGTTGTTGACGGCGCCCCCTTT
A868_5H CATTTATTCTCTTTGCGGCGCTTAAGGCAGGTTCACTTTGCGTCCTCCAGAAGATCCGTTGTTGACGGCGCCCCCTTT
A868_5K CATTTATTCTCTTTGCGGCGCTTAAGGCAGGTTCACTTTGCGTCCTCCAGAAGATCCGTTGTTGACGGCGCCCCCTTT

A868_5N CGTGGGGCGGGGACACGACGGGTTGCTGGGGATGAGCGCGGGTGGCCTTGCTGCGTCGCGCTGGAGGCCCGTGGACGG
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A868_5G CGTGGGGCGGGGACACGACGGGTTGCTGGGGATGAGCGCGGGTGGCCTTGCTGCGTCGCGCTGGAGGCCCGTGGACGG
A868_5L CGTGGGGCGGGGACACGACGGGTTGCTGGGGATGAGCGCGGGTGGCCTTGCTGCGTCGCGCTGGAGGCCCGTGGACGG
A868_5B CGTGGGGCGGGGACACGACGGGTTGCTGGGGATGAGCGCGGGTGGCCTTGCTGCGTCGCGCTGGAGGCCCGTGGACGG
A868_5D CGTGGGGCGGGGACACGACGGGTTGCTGGGGATGAGCGCGGGTGGCCTTGCTGCGTCGCGCCGGAGGCCCGTGGACGG
A868_5H CGTGGGGCGGGGACACGACGGGTTGCTGGGGATGAGCGCGGGTGGCCTTGCTGCGTCGCGCCGGAGGCCCGTGGACGG
A868_5K CGTGGGGCGGGGACACGACGGGTTGCTGGGGATGAGCGCGGGTGGCCTTGCTGCGTCGCGCCGGAGGCCCGTGGACGG

A868_5N TGGGCAGTCTCGAGAATACCGGAGAGAGACCGGTGATAATGACGATGCCATACCCCCGAT
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A868_5G TGGGCAGTCTCGAGAATACCGGAGAGAGACCGGTGATAATGACGATGCCATACCCCCGAT
A868_5L TGGGCAGTCTCGAGAATACCGGAGAGAGACCGGTGATAATGACGATGCCATACCCCCGAT
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A868_5D TGGGCAGTCTCGAGAATACCGGAGAGAGACCGGTGATAATGACGATGCCATACCCCCGAT
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A868_5K TGGGCAGTCTCGAGAATACCGGAGAGAGACCGGTGATAATGACGATGCCATACCCCCGAT
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7.4 Appendix 4. Alignment of *cox*1 sequences from *Cystophora*. Specimen names are truncated to FieldnumberSpecies.

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B601Ccephalorni GGTGTCTTGGGTACAGCTATGTCTGTTCTTATCAGGTTGCAGCTTGGTAGCccaGGAAATATGTTTTTAGGTGGCAAT
BO91Ccephalorni GGTGTCTTGGGTACAGCTATGTCTGTTCTTATCAGGTTGCAGCTTGGTAGCCCAGGAAATATGTTTTTAGGTGGCAAT
B327Cconfluens  GGTGTTTTAGGTACAATGATGTCTGTTATTATTAGGTTGCAGCTTGCTAGTCCTGGCAATATGTTTTTGGGTGGCAAT
B162Cconfluens  GGTGTTTTAGGTACAATGATGTCTGTTATTATTAGGTTGCAGCTTGCTAGTCCTGGCAATATGTTTTTGGGTGGCAAT
B174Lquercifoli GGTGTTTTGGGTACAGTAATGTCTGTTCTTATTAGGTTACAGCTTGCAAGCCCGGGTAATATGTTTTTGGGAGGCAAT
B318Cmonilifera GGTATTTTGGGTACAGCAATGTCTGTTCTTATTAGGTTGCAGCTTGCAAGCCCCGGCAATATGTTTTTGAGTGGCAAT
B092Cmonilifera GGTATTTTGGGTACAGCAATGTCTGTTCTTATTAGGTTGCAGCTTGCAAGCCCCGGCAATATGTTTTTGAGTGGCAAT
B095Cmonilifera GGTATTTTGGGTACAGCAATGTCTGTTCTTATTAGGTTGCAGCTTGCAAGCCCCGGCAATATGTTTTTGAGTGGCAAT
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B100Csubfarcina GGTATTTTGGGTACAGCAATGTCTGTTCTTATTAGGTTGCAGCTTGCAAGCCCCGGCAATATGTTTTTGAGTGGCAAT
B101Cretroflexa GGTATTTTGGGTACAGCAATGTCTGTTCTTATTAGGTTGCAGCTTGCAAGCCCCGGCAATATGTTTTTGAGCGGCAAT
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B166Csubfarcina GGTATTTTGGGTACAGCAATGTCTGTTCTTATTAGGTTGCAGCTTGCAAGCCCCGGCAATATGTTTTTGAGTGGCAAT
B108Cretorta    GGTATTTTGGGTACAGCAATGTCTGTTCTTATTAGGTTGCAGCTTGCAAGCCCCGGCAATATGTTTTTGGGTGGCAAT
B617Cretroflexa GGTATTTTGGGTACAGCAATGTCTGTTCTTATTAGGTTGCAGCTTGCAAGCCCCGGCAATATGTTTTTGAGCGGCAAT
B430Cretroflexa GGTATTTTGGGTACAGCAATGTCTGTTCTTATTAGGTTGCAGCTTGCAAGCCCCGGCAATATGTTTTTGAGCGGCAAT
A615Cretroflexa GGTATTTTGGGTACAGCAATGTCTGTTCTTATTAGGTTGCAGCTTGCAAGCCCCGGCAATATGTTTTTGAGCGGCAAT
B137Cretorta    GGTATTTTGGGTACAGCAATGTCTGTTCTTATTAGGTTGCAGCTTGCAAGCCCCGGCAATATGTTTTTGGGTGGCAAT
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B426Cretroflexa GGTATTTTGGGTACAGCAATGTCTGTTCTTATTAGGTTGCAGCTTGCAAGCCCCGGCAATATGTTTTTGAGCGGCAAT
B335Ccongesta   GGTATTTTGGGTACAGCAATGTCTGTTCTTATTAGGTTGCAGCTTGCAAGCCCCGGCAATATGTTTTTGAGCGGCAAT
B436Cretroflexa GGTATTTTGGGTACAGCAATGTCTGTTCTTATTAGGTTGCAGCTTGCAAGCCCCGGCAATATGTTTTTGAGCGGCAAT
A154Cscalaris   GGTATTTTGGGTACAGCAATGTCTGTTCTTATTAGGTTGCAGCTTGCAAGCCCCGGCAATATGTTTTTGAGTGGCAAT
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B412Csubfarcina GGTATTTTGGGTACAGCAATGTCTGTTCTTATTAGGTTGCAGCTTGCAAGCCCCGGCAATATGTTTTTGAGTGGCAAT
B121Cretroflexa GGTATTTTGGGTACAGCAATGTCTGTTCTTATTAGGTTGCAGCTTGCAAGCCCCGGCAATATGTTTTTGAGCGGCAAT
B109Cretorta    GGTATTTTGGGTACAGCAATGTCTGTTCTTATTAGGTTGCAGCTTGCAAGCCCCGGCAATATGTTTTTGGGTGGCAAT
B170Cretorta    GGTATTTTGGGTACAGCAATGTCTGTTATTATTAGGTTGCAGCTTGCAAGCCCCGGCAATATGTTTTTGGGTGGCAAT
B415Csiliquosa  GGTATTTTGGGTACAGCAATGTCTGTTCTTATTAGGTTGCAGCTTGCAAGCCCCGGCAATATGTTTTTGGGTGGCAAT
B111Cpolycystid GGTATTTTGGGTACAGCAATGTCTGTTCTTATTAGATTGCAGCTTGCAAGCCCTGGCAATATGTTTTTGAGTGGCAAT
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B115Ccongesta   GGTATTTTGGGTACAGCAATGTCTGTTCTTATTAGGTTGCAGCTTGCAAGCCCCGGCAATATGTTTTTGAGCGGCAAT
B152Ccongesta   GGTATTTTGGGTACAGCAATGTCTGTTCTTATTAGGTTGCAGCTTGCAAGCCCCGGCAATATGTTTTTGAGCGGCAAT
B455Ccongesta   GGTATTTTGGGTACAGCAATGTCTGTTCTTATTAGGTTGCAGCTTGCAAGCCCCGGCAATATGTTTTTGAGCGGCAAT
B129Cretorta    GGTATTTTGGGTACAGCAATGTCTGTTCTTATTAGGTTGCAGCTTGCAAGCCCCGGCAATATGTTTTTGGGTGGCAAT
B131Cretorta    GGTATTTTGGGTACAGCAATGTCTGTTCTTATTAGGTTGCAGCTTGCAAGCCCCGGCAATATGTTTTTGGGTGGCAAT
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B626Cretroflexa GGTATTTTGGGTACAGCAATGTCTGTTCTTATTAGGTTGCAGCTTGCAAGCCCCGGCAATATGTTTTTGAGTGGCAAT
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B165Ctorulosa   GGTATTTTGGGTACAGCAATGTCTGTTCTTATTAGGTTGCAGCTTGCAAGCCCCGGCAATATGTTTTTGAGTGGTAAT
B125Ctorulosa   GGTATTTTGGGTACAGCAATGTCTGTTCTTATTAGGTTGCAGCTTGCAAGCCCCGGCAATATGTTTTTGAGTGGCAAT
B147Ctorulosa   GGTATTTTGGGTACAGCAATGTCTGTTCTTATTAGGTTGCAGCTTGCAAGCCCCGGCAATATGTTTTTGAGTGGTAAT
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Appendix 4. (Continued)

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B605Cscalaris  GGTATTTTGGGTACAGCAATGTCTGTTCTTATTAGGTTGCAGCTTGCAAGCCCCGGCAATATGTTTTTGAGTGGCAAT
B332Cscalaris  GGTATTTTGGGTACAGCAATGTCTGTTCTTATTAGGTTGCAGCTTGCAAGCCCCGGCAATATGTTTTTGAGTGGCAAT
A099Cscalaris  GGTATTTTGGGTACAGCAATGTCTGTTCTTATTAGGTTGCAGCTTGCAAGCCCCGGCAATATGTTTTTGAGTGGCAAT
A614Cscalaris  GGTATTTTGGGTACAGCAATGTCTGTTCTTATTAGGTTGCAGCTTGCAAGCCCCGGCAATATGTTTTTGAGTGGCAAT
A596Cscalaris  GGTATTTTGGGTACAGCAATGTCTGTTCTTATTAGGTTGCAGCTTGCAAGCCCCGGCAATATGTTTTTGAGTGGCAAT
A292Cscalaris  GGTATTTTGGGTACAGCAATGTCTGTTCTTATTAGGTTGCAGCTTGCAAGCCCCGGCAATATGTTTTTGAGTGGCAAT
B351CspWilson  gGTATTTTGGGTACAGCAATGTCTGTTCTTATTAGGTTGCAGCTTGCAAGCCCCGGCAATATGTTTTTGAGTGGTAAT
B319Csiliquosa GGTATTTTGGGTACAGCAATGTCTGTTCTTATTAGGTTGCAGCTTGCAAGCCCCGGCAATATGTTTTTGGGTGGCAAT
B130Csiliquosa GGTATTTTGGGTACAGCAATGTCTGTTCTTATTAGGTTGCAGCTTGCAAGCCCCGGCAATATGTTTTTGGGTGGCAAT
B143Csiliquosa GGTATTTTGGGTACAGCAATGTCTGTTCTTATTAGGTTGCAGCTTGCAAGCCCCGGCAATATGTTTTTGGGTGGCAAT
B118Csiliquosa GGTATTTTGGGTACAGCAATGTCTGTTCTTATTAGGTTGCAGCTTGCAAGCCCCGGCAATATGTTTTTGGGTGGCAAT
B325Cretorta   GGTATTTTGGGTACAGCAATGTCTGTTCTTATTAGGTTGCAGCTTGCAAGCCCCGGCAATATGTTTTTGGGTGGCAAT
B324Cretorta   GGTATTTTGGGTACAGCAATGTCTGTTCTTATTAGGTTGCAGCTTGCAAGCCCCGGCAATATGTTTTTGGGTGGCAAT

B601Ccephalorni TATCAGTTGTATAATGTTATTGTAACAGCTCATGCTTTTTTGATGATTTTCTTTATGGTTATGCCTGTTCTGATTGGT
BO91Ccephalorni TATCAGTTGTATAATGTTATTGTAACGGCTCATGCTTTTTTGATGATTTTCTTTATGGTTATGCCTGTTCTGATTGGT
B327Carpoconflu TACCAGTTGTATAATGTTATTGTAACGGCTCACGCTTTTTTGATGATTTTTTTTATGGTGATGCCTGTCCTTATTGGC
B162Carpoconflu TACCAGTTGTATAATGTTATTGTAACGGCTCACGCTTTTTTGATGATTTTTTTTATGGTCATGCCTGTCCTTATTGGC
B174Lquercifoli TACCAGTTGTATAATGTTATTGTAACGGCTCACGCCTTTTTGATGATTTTTTTTATGGTTATGCCTGTCCTTATTGGC
B318Cmonilifera TACCAGTTGTATAATGTTATCGTAACGGCTCATGCTTTTTTGATGATTTTCTTTATGGTTATGCCTGTGCTTATCGGC
B092Cmonilifera TACCAGTTGTATAATGTTATCGTAACGGCTCATGCTTTTTTGATGATTTTCTTTATGGTTATGCCTGTGCTTATCGGC
B095Cmonilifera TACCAGTTGTATAATGTTATCGTAACGGCTCATGCTTTTTTGATGATTTTCTTTATGGTTATGCCTGTGCTTATCGGC
B102Cmonilifera TACCAGTTGTATAATGTTATCGTAACGGCTCATGCTTTTTTGATGATTTTCTTTATGGTTATGCCTGTGCTTATCGGC
B120Cmonilifera TACCAGTTGTATAATGTTATCGTAACGGCTCATGCTTTTTTGATGATTTTCTTTATGGTTATGCCTGTGCTTATCGGC
B093Cmoniliform TATCAGTTGTATAATGTCATTGTAACGGCTCACGCTTTTTTGATGATTTTCTTTATGGTTATGCCTGTCCTTATTGGC
B163Cmoniliform TATCAGTTGTATAATGTCATTGTAACGGCTCACGCTTTTTTGATGATTTTCTTTATGGTTATGCCTGTCCTTATTGGC
B094Csubfarcina TACCAGTTGTATAATGTTATTGTAACGGCTCATGCTTTTTTGATGATTTTCTTTATGGTTATGCCTGTGCTTATCGGC
B100Csubfarcina TACCAGTTGTATAATGTTATTGTAACGGCTCATGCTTTTTTGATGATTTTCTTTATGGTTATGCCTGTGCTTATCGGC
B101Cretroflexa TACCAGTTGTATAATGTTATTGTAACGGCTCATGCTTTTTTGATGATTTTCTTTATGGTTATGCCTGTGCTTATCGGC
B099Cretroflexa TACCAGTTGTATAATGTTATTGTAACGGCTCATGCTTTTTTGATGATTTTCTTTATGGTTATGCCTGTGCTTATCGGC
B123Cretroflexa TACCAGTTGTATAATGTTATTGTAACGGCTCATGCTTTTTTGATGATTTTCTTTATGGTTATGCCTGTGCTTATCGGC
B124Cretroflexa TACCAGTTGTATAATGTTATTGTAACGGCTCATGCTTTTTTGATGATTTTCTTTATGGTTATGCCTGTGCTTATCGGC
B166Csubfarcina TACCAGTTGTATAATGTTATTGTAACGGCTCATGCTTTTTTGATGATTTTCTTTATGGTTATGCCTGTGCTTATCGGC
B108Cretorta    TATCAGTTGTATAATGTTATTGTAACGGCTCACGCTTTTTTGATGATTTTCTTTATGGTTATGCCTGTGCTTATCGGC
B617Cretroflexa TACCAGTTGTATAATGTTATTGTAACGGCTCATGCTTTTTTGATGATTTTCTTTATGGTTATGCCTGTGCTTATCGGC
B430Cretroflexa TACCAGTTGTATAATGTTATTGTAACGGCTCATGCTTTTTTGATGATTTTCTTTATGGTTATGCCTGTGCTTATCGGC
A615Cretroflexa TACCAGTTGTATAATGTTATTGTAACGGCTCATGCTTTTTTGATGATTTTCTTTATGGTTATGCCTGTGCTTATCGGC
B137Cretorta    TATCAGTTGTATAATGTTATTGTAACGGCTCACGCTTTTTTGATGATTTTCTTTATGGTTATGCCTGTGCTTATCGGC
B169Cretroflexa TACCAGTTGTATAATGTTATTGTAACGGCTCATGCTTTTTTGATGATTTTCTTTATGGTTATGCCTGTGCTTATCGGC
B425Cretroflexa TACCAGTTGTATAATGTTATTGTAACGGCTCATGCTTTTTTGATGATTTTCTTTATGGTTATGCCTGTGCTTATCGGC
B426Cretroflexa TACCAGTTGTATAATGTTATTGTAACGGCTCATGCTTTTTTGATGATTTTCTTTATGGTTATGCCTGTGCTTATCGGC
B335Cconcesta   TACCAGTTGTATAATGTTATTGTAACGGCTCATGCTTTTTTGATGATTTTCTTTATGGTTATGCCTGTGCTTATCGGC
B436Cretroflexa TACCAGTTGTATAATGTTATTGTAACGGCTCATGCTTTTTTGATGATTTTCTTTATGGTTATGCCTGTGCTTATCGGC
A154Cscalaris   TACCAGTTGTATAATGTTATTGTAACGGCTCATGCTTTTTTGATGATTTTCTTTATGGTTATGCCTGTGCTTATCGGC
A080Cretroflexa TACCAGTTGTATAATGTTATTGTAACGGCTCATGCTTTTTTGATGATTTTCTTTATGGTTATGCCTGTGCTTATCGGC
B602Cretroflexa TACCAGTTGTATAATGTTATTGTAACGGCTCATGCTTTTTTGATGATTTTCTTTATGGTTATGCCTGTGCTTATCGGC
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B556Cretroflexa TACCAGTTGTATAATGTTATTGTAACGGCTCATGCTTTTTTGATGATTTTCTTTATGGTTATGCCTGTGCTTATCGGC
A196Cretroflexa TACCAGTTGTATAATGTTATTGTAACGGCTCATGCTTTTTTGATGATTTTCTTTATGGTTATGCCTGTGCTTATCGGC
B412Csubfarcina TACCAGTTGTATAATGTTATTGTAACGGCTCATGCTTTTTTGATGATTTTCTTTATGGTTATGCCTGTGCTTATCGGC
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Appendix 4. (Continued)

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B170Cretorta  TATCAGTTGTATAATGTTATTGTAACGGCTCACGCTTTTTTGATGATTTTCTTTATGGTTATGCCTGTGCTTATCGGC
B415Cretorta  TACCAGTTGTATAATGTCATTGTAACGGCTCACGCTTTTTTGATGATTTTCTTTATGGTTATGCCTGTGCTTATCGGC
B111Cpolycyst TACCAGTTATATAATGTTATTGTAACGGCTCATGCTTTTTTGATGATTTTCTTTATGGTTATGCCTGTGCTTATCGGC
B112Cpolycyst TACCAGTTATATAATGTTATTGTAACGGCTCATGCTTTTTTGATGATTTTCTTTATGGTTATGCCTGTGCTTATCGGC
B148Cpolycyst TACCAGTTATATAATGTTATTGTAACGGCTCATGCTTTTTTGATGATTTTCTTTATGGTTATGCCTGTGCTTATCGGC
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A168Cplatylob TACCAGTTGTATAATGTTATTGTAACGGCTCATGCTTTTTTGATGATTTTCTTTATGGTTATGCCTGTGCTTATCGGC
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B104Cplatylob TACCAGTTGTATAATGTTATTGTAACGGCTCATGCTTTTTTGATGATTTTCTTTATGGTTATGCCTGTGCTTATCGGC
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B152Ccongesta TACCAGTTGTATAATGTTATTGTAACGGCTCATGCTTTTTTGATGATTTTCTTTATGGTTATGCCTGTGCTTATCGGC
B455Ccongesta TACCAGTTGTATAATGTTATTGTAACGGCTCATGCTTTTTTGATGATTTTCTTTATGGTTATGCCTGTGCTTATCGGC
B129Cretorta  TATCAGTTGTATAATGTTATTGTAACGGCTCACGCTTTTTTGATGATTTTCTTTATGGTTATGCCTGTGCTTATCGGC
B131Cretorta  TATCAGTTGTATAATGTTATTGTAACGGCTCACGCTTTTTTGATGATTTTCTTTATGGTTATGCCTGTGCTTATCGGC
B161Ccuspidat TACCAGTTGTATAATGTTATTGTAACGGCTCATGCTTTTTTGATGATTTTCTTTATGGTTATGCCTGTGCTTATCGGC
B628Ccuspidat TACCAGTTGTATAATGTTATTGTAACGGCTCATGCTTTTTTGATGATTTTCTTTATGGTTATGCCTGTGCTTATCGGC
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B128Csubfarcc TACCAGTTGTATAATGTTATTGTAACGGCTCATGCTTTTTTGATGATTTTCTTTATGGTTATGCCTGTGCTTATCGGC
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B316Ctorulosa TACCAGTTGTATAATGTTATTGTAACGGCTCATGCTTTTTTGATGATTTTCTTTATGGTTATGCCTGTGCTTATCGGC
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B351CspWilson TACCAGTTGTATAATGTTATTGTAACGGCTCATGCTTTTTTGATGATTTTCTTTATGGTTATGCCTGTGCTTATCGGC
B319Csiliquos TACCAGTTGTATAATGTCATTGTAACGGCTCACGCTTTTTTGATGATTTTCTTTATGGTTATGCCTGTGCTTATCGGC
B130Csiliquos TACCAGTTGTATAATGTCATTGTAACGGCTCACGCTTTTTTGATGATTTTCTTTATGGTTATGCCTGTGCTTATCGGC
B143Csiliquos TACCAGTTGTATAATGTCATTGTAACGGCTCACGCTTTTTTGATGATTTTCTTTATGGTTATGCCTGTGCTTATCGGC
B118Csiliquos TACCAGTTGTATAATGTCATTGTAACGGCTCACGCTTTTTTGATGATTTTCTTTATGGTTATGCCTGTGCTTATCGGC
B325Cretorta  TATCAGTTGTATAATGTTATTGTAACGGCTCACGCTTTTTTGATGATTTTCTTTATGGTTATGCCTGTGCTTATCGGC
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Appendix 4. (Continued)

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B324Cretorta  TATCAGTTGTATAATGTTATTGTAACGGCTCACGCTTTTTTGATGATTTTCTTTATGGTTATGCCTGTGCTTATCGGC

B601Ccephalorni GGTTTCGGTAATTGGTTTGTTCCTTTAATGATAGGCGCTCCGGATATGGCGTTTCCCCGTATGAACAATATAAGTTTT
BO91Ccephalorni GGTTTCGGTAATTGGTTTGTTCCTTTAATGATAGGCGCTCCGGATATGGCGTTTCCCCGTATGAACAATATAAGTTTT
B327Carpoconflu GGATTTGGTAATTGGTTTGTTCCTTTAATGATAGGTGCTCCCGATATGGCGTTTCCTCGTATGAATAATATTAGTTTT
B162Carpoconflu GGATTTGGTAATTGGTTTGTTCCTTTAATGATAGGTGCTCCCGATATGGCGTTTCCTCGTATGAATAATATTAGTTTT
B174Lquercifoli GGGTTTGGTAATTGGTTTGTTCCTTTAATGATAGGTGCTCCAGATATGGCGTTTCCGCGTATGAACAACATAAGTTTT
B318Cmonilifera GGATTTGGTAATTGGTTTGTTCCTTTAATGATAGGTGCTCCAGATATGGCGTTTCCTCGCATGAATAATATAAGTTTT
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B095Cmonilifera GGATTTGGTAATTGGTTTGTTCCTTTAATGATAGGTGCTCCAGATATGGCGTTTCCTCGCATGAATAATATAAGTTTT
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B100Csubfarcina GGATTTGGTAATTGGTTTGTTCCTTTAATGATAGGTGCTCCAGATATGGCGTTTCCtCGCAtGAATAAtAtAAGTTTT
B101Cretroflexa GGATTTGGTAATTGGTTTGTTCCTTTAATGATAGGTGCTCCAGATATGGCGTTTCCTCGCATGAATAATATAAGTTTT
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B617Cretroflexa GGATTTGGTAATTGGTTTGTTCCTTTAATGATAGGTGCTCCAGATATGGCGTTTCCTCGCATGAATAATATAAGTTTT
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B335Ccongesta   GGATTTGGTAATTGGTTTGTTCCTTTAATGATAGGTGCTCCAGATATGGCGTTTCCTCGCATGAATAATATAAGTTTT
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B412Csubfarcina GGATTTGGTAATTGGTTTGTTCCTTTAATGATAGGTGCTCCAGATATGGCGTTTCCTCGCATGAATAATATAAGTTTT
B121Cretroflexa GGATTTGGTAATTGGTTTGTTCCTTTAATGATAGGTGCTCCAGATATGGCGTTTCCTCGCATGAATAATATAAGTTTT
B109Cretorta    GGATTTGGTAATTGGTTTGTTCCTTTAATGATAGGTGCTCCAGATATGGCGTTTCCTCGCATGAATAATATAAGTTTT
B170Cretorta    GGATTTGGTAATTGGTTTGTTCCTTTAATGATAGGTGCTCCAGATATGGCGTTTCCTCGCATGAATAATATAAGTTTT
B415Csiliquosa  GGATTTGGTAATTGGTTTGTTCCTTTAATGATAGGTGCTCCAGATATGGCGTTTCCTCGCATGAATAATATAAGTTTT
B111Cpolycystid GGATTTGGTAATTGGTTTGTTCCTTTAATGATAGGTGCTCCGGATATGGCGTTTCCTCGCATGAATAATATAAGTTTT
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B626Cretroflexa GGATTTGGTAATTGGTTTGTTCCTTTAATGATAGGTGCTCCAGATATGGCGTTTCCTCGCATGAATAATATAAGTTTT
B146Csubfarcina GGATTTGGTAATTGGTTTGTTCCTTTAATGATAGGTGCTCCAGATATGGCGTTTCCTCGCATGAATAATATAAGTTTT
B146Csubfarcina GGATTTGGTAATTGGTTTGTTCCTTTAATGATAGGTGCTCCAGATATGGCGTTTCCTCGCATGAATAATATAAGTTTT
B128Csubfarcina GGATTTGGTAATTGGTTTGTTCCTTTAATGATAGGTGCTCCAGATATGGCGTTTCCTCGCATGAATAATATAAGTTTT
B127Csubfarccus GGATTTGGTAATTGGTTTGTTCCTTTAATGATAGGTGCTCCGGATATGGCGTTTCCTCGCATGAATAATATAAGTTTT
B159Cretroflexa GGATTTGGTAATTGGTTTGTTCCTTTAATGATAGGTGCTCCAGATATGGCATTTCCTCGCATGAATAATATAAGTTTT
B134Csubfarcina GGATTTGGTAATTGGTTTGTTCCTTTAATGATAGGTGCTCCAGATATGGCGTTTCCTCGCATGAATAATATAAGTTTT
B168Csubfarcina GGATTTGGTAATTGGTTTGTTCCTTTAATGATAGGTGCTCCAGATATGGCGTTTCCTCGCATGAATAATATAAGTTTT
B136Csubfarcina GGATTTGGTAATTGGTTTGTTCCTTTAATGATAGGTGCTCCAGATATGGCGTTTCCTCGCATGAATAATATAAGTTTT
B626Cretroflexa GGATTTGGTAATTGGTTTGTTCCTTTAATGATAGGTGCTCCAGATATGGCATTTCCTCGCATGAATAATATAAGTTTT
B153Csubfarcina GGATTTGGTAATTGGTTTGTTCCTTTAATGATAGGTGCTCCAGATATGGCGTTTCCTCGCATGAATAATATAAGTTTT
B110Csubfarcina GGATTTGGTAATTGGTTTGTTCCTTTAATGATAGGTGCTCCAGATATGGCGTTTCCTCGCATGAATAATATAAGTTTT
B417Cretroflexa GGATTTGGTAATTGGTTTGTTCCTTTAATGATAGGTGCTCCAGATATGGCGTTTCCTCGCATGAATAATATAAGTTTT
B624Csubfarcina GGATTTGGTAATTGGTTTGTTCCTTTAATGATAGGTGCTCCAGATATGGCGTTTCCTCGCATGAATAATATAAGTTTT
B428Ctorulosa   GGATTTGGTAATTGGTTTGTTCCTTTAATGATAGGTGCTCCGGATATGGCGTTTCCTCGCATGAATAATATAAGTTTT
B103Ctorulosa   GGATTTGGTAATTGGTTTGTTCCTTTAATGATAGGTGCTCCGGATATGGCGTTTCCTCGCATGAATAATATAAGTTTT
B165Ctorulosa   GGATTTGGTAATTGGTTTGTTCCTTTAATGATAGGTGCTCCGGATATGGCGTTTCCTCGCATGAATAATATAAGTTTT
B125Ctorulosa   GGATTTGGTAATTGGTTTGTTCCTTTAATGATAGGTGCTCCAGATATGGCGTTTCCTCGCATGAATAATATAAGTTTT
B147Ctorulosa   GGATTTGGTAATTGGTTTGTTCCTTTAATGATAGGTGCTCCGGATATGGCGTTTCCTCGCATGAATAATATAAGTTTT
B179Ctorulosa   GGATTTGGTAATTGGTTTGTTCCTTTAATGATAGGTGCTCCGGATATGGCGTTTCCTCGCATGAATAATATAAGTTTT
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Appendix 4. (Continued)

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A093Ctorulosa   GGATTTGGTAATTGGTTTGTTCCTTTAATGATAGGTGCTCCGGATATGGCGTTTCCTCGCATGAATAATATAAGTTTT
B320Ctorulosa   GGATTTGGTAATTGGTTTGTTCCTTTAATGATAGGTGCTCCGGATATGGCGTTTCCTCGCATGAATAATATAAGTTTT
A198Ctorulosa   GGATTTGGTAATTGGTTTGTTCCTTTAATGATAGGTGCTCCGGATATGGCGTTTCCTCGCATGAATAATATAAGTTTT
B316Ctorulosa   GGATTTGGTAATTGGTTTGTTCCTTTAATGATAGGTGCTCCGGATATGGCGTTTCCTCGCATGAATAATATAAGTTTT
B167Ctorulosa   GGATTTGGTAATTGGTTTGTTCCTTTAATGATAGGTGCTCCGGATATGGCGTTTCCTCGCATGAATAATATAAGTTTT
B145Ccongesta   GGATTTGGTAATTGGTTTGTTCCTTTAATGATAGGTGCTCCAGATATGGCGTTTCCTCGCATGAATAATATAAGTTTT
B149Ccongesta   GGATTTGGTAATTGGTTTGTTCCTTTAATGATAGGTGCTCCAGATATGGCGTTTCCTCGCATGAATAATATAAGTTTT
B416Ccongesta   GGATTTGGTAATTGGTTTGTTCCTTTAATGATAGGTGCTCCAGATATGGCGTTTCCTCGCATGAATAATATAAGTTTT
B176Cscalaris   GGATTTGGTAATTGGTTTGTTCCTTTAATGATAGGTGCTCCAGATATGGCATTTCCTCGCATGAATAATATAAGTTTT
B178Cscalaris   GGATTTGGTAATTGGTTTGTTCCTTTAATGATAGGTGCTCCAGATATGGCATTTCCTCGCATGAATAATATAAGTTTT
A318Cscalaris   GGATTTGGTAATTGGTTTGTTCCTTTAATGATAGGTGCTCCAGATATGGCATTTCCTCGCATGAATAATATAAGTTTT
A469Cscalaris   GGATTTGGTAATTGGTTTGTTCCTTTAATGATAGGTGCTCCAGATATGGCATTTCCTCGCATGAATAATATAAGTTTT
B330Cscalaris   GGATTTGGTAATTGGTTTGTTCCTTTAATGATAGGTGCTCCAGATATGGCGTTTCCTCGCATGAATAATATAAGTTTT
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B337Cscalaris   GGATTTGGTAATTGGTTTGTTCCTTTAATGATAGGTGCTCCAGATATGGCATTTCCTCGCATGAATAATATAAGTTTT
B338Cscalaris   GGATTTGGTAATTGGTTTGTTCCTTTAATGATAGGTGCTCCAGATATGGCATTTCCTCGCATGAATAATATAAGTTTT
B611Cscalaris   GGATTTGGTAATTGGTTTGTTCCTTTAATGATAGGTGCTCCAGATATGGCATTTCCTCGCATGAATAATATAAGTTTT
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B607Cscalaris   GGATTTGGTAATTGGTTTGTTCCTTTAATGATAGGTGCTCCAGATATGGCATTTCCTCGCATGAATAATATAAGTTTT
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B177Cscalaris   GGATTTGGTAATTGGTTTGTTCCTTTAATGATAGGTGCTCCAGATATGGCATTTCCTCGCATGAATAATATAAGTTTT
B605Cscalaris   GGATTTGGTAATTGGTTTGTTCCTTTAATGATAGGTGCTCCAGATATGGCATTTCCTCGCATGAATAATATAAGTTTT
B332Cscalaris   GGATTTGGTAATTGGTTTGTTCCTTTAATGATAGGTGCTCCAGATATGGCATTTCCTCGCATGAATAATATAAGTTTT
A099Cscalaris   GGATTTGGTAATTGGTTTGTTCCTTTAATGATAGGTGCTCCAGATATGGCATTTCCTCGCATGAATAATATAAGTTTT
A614Cscalaris   GGATTTGGTAATTGGTTTGTTCCTTTAATGATAGGTGCTCCAGATATGGCATTTCCTCGCATGAATAATATAAGTTTT
A596Cscalaris   GGATTTGGTAATTGGTTTGTTCCTTTAATGATAGGTGCTCCAGATATGGCATTTCCTCGCATGAATAATATAAGTTTT
A292Cscalaris   GGATTTGGTAATTGGTTTGTTCCTTTAATGATAGGTGCTCCAGATATGGCATTTCCTCGCATGAATAATATAAGTTTT
B351CspWilson   GGATTTGGTAATTGGTTTGTTCCTTTAATGATAGGTGCTCCGGATATGGCGTTTCCTCGCATGAATAATATAAGTTTT
B319Csiliquosa  GGATTTGGTAATTGGTTTGTTCCTTTAATGATAGGTGCTCCAGATATGGCGTTTCCTCGCATGAATAATATAAGTTTT
B130Csiliquosa  GGATTTGGTAATTGGTTTGTTCCTTTAATGATAGGTGCTCCAGATATGGCGTTTCCTCGCATGAATAATATAAGTTTT
B143Csiliquosa  GGATTTGGTAATTGGTTTGTTCCTTTAATGATAGGTGCTCCAGATATGGCGTTTCCTCGCATGAATAATATAAGTTTT
B118Csiliquosa  GGATTTGGTAATTGGTTTGTTCCTTTAATGATAGGTGCTCCAGATATGGCGTTTCCTCGCATGAATAATATAAGTTTT
B325Cretorta    GGATTTGGTAATTGGTTTGTTCCTTTAATGATAGGTGCTCCAGATATGGCGTTTCCTCGCATGAATAATATAAGTTTT
B324Cretorta    GGATTTGGTAATTGGTTTGTTCCTTTAATGATAGGTGCTCCAGATATGGCGTTTCCTCGCATGAATAATATAAGTTTT

B601Ccephalorn TGGCTATTACCACCGTCTTTACTACTTCTTTTAGCCTCCTCATTAGTTGAATCCGGGGCTGGTACTGGTTGGACTGTT
BO91Ccephalorn TGGCTATTACCACCGTCTTTACTACTTCTTTTAGCCTCCTCATTAGTTGAATCCGGGGCTGGTACTGGTTGGACTGTT
B327Carpoconfl TGGTTATTGCCGCCGTCCCTAATACTTCTTTTAGCTTCTTCATTGGTTGAAGCGGGTGCTGGTACTGGTTGGACTGTT
B162Carpoconfl TGGTTATTGCCGCCGTCCCTTATACTTCTTTTAGCTTCTTCATTGGTTGAAGCGGGTGCTGGTACTGGTTGGACTGTT
B174Lquercifol TGGCTGTTACCGCCATCCTTAATACTTCTTTTAGCGTCTTCATTAGTGGAAGCCGGGGCAGGTACGGGTTGGACTGTT
B318Cmonilifer TGGCTGTTACCTCCCTCTTTAATACTTCTTTTAGCGTCCTCATTGGTAGAAGCTGGGGCAGGTACCGGTTGGACTGTT
B092Cmonilifer TGGCTGTTACCTCCCTCTTTAATACTTCTTTTAGCGTCCTCATTGGTAGAAGCTGGGGCAGGTACCGGTTGGACTGTT
B095Cmonilifer TGGCTGTTACCTCCCTCTTTAATACTTCTTTTAGCGTCCTCATTGGTAGAAGCTGGGGCAGGTACCGGTTGGACTGTT
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B120Cmonilifer TGGCTGTTACCTCCCTCTTTAATACTTCTTTTAGCGTCCTCATTGGTAGAAGCTGGGGCAGGTACCGGTTGGACTGTT
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B163Cmonilifor TGGCTATTGCCACCGTCTTTAATACTACTTTTGTCGTCTTCATTAGTGGAAGCCGGGGCGGGTACTGGTTGGACCGTT
B094Cretroflex TGGCTGTTACCTCCCTCTTTAATACTTCTTTTAGCGTCCTCATTAGTAGAAGCTGGGGCAGGTACCGGTTGGACTGTT
B100Cretroflex TGGCTGTTACCTCCCTCTTTAATACTTCTTTTAGCGTCCTCATTAGTAGAAGCTGGGGCAGGTACCGGTTGGACTGTT
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B099Cretroflex TGGCTGTTACCTCCCTCTTTAATACTTCTTTTAGCGTCCTCATTAGTAGAAGCCGGGGCAGGTACCGGTTGGACTGTT
B123Cretroflex TGGCTGTTACCTCCCTCTTTAATACTTCTTTTAGCGTCCTCATTAGTAGAAGCCGGGGCAGGTACCGGTTGGACTGTT
B124Cretroflex TGGCTGTTACCTCCCTCTTTAATACTTCTTTTAGCGTCCTCATTAGTAGAAGCCGGGGCAGGTACCGGTTGGACTGTT
B166Cretroflex TGGCTGTTACCTCCCTCTTTAATACTTCTTTTAGCGTCCTCATTAGTAGAAGCTGGGGCAGGTACCGGTTGGACTGTT
B108Cretroflex TGGCTGTTACCTCCATCTTTAATACTTCTTTTAGCCTCTTCATTAGTAGAAGCCGGGGCAGGTACTGGTTGGACTGTT
B617Cretroflex TGGCTGTTACCTCCCTCTTTAATACTTCTTTTAGCGTCCTCATTAGTAGAAGCCGGGGCAGGTACCGGTTGGACTGTT
B430Cretroflex TGGCTGTTACCTCCCTCTTTAATACTTCTTTTAGCGTCCTCATTAGTAGAAGCCGGGGCAGGTACCGGTTGGACTGTT
A615Cretroflex TGGCTGTTACCTCCCTCTTTAATACTTCTTTTAGCGTCCTCATTAGTAGAAGCCGGGGCAGGTACCGGTTGGACTGTT
B137Cretroflex TGGCTGTTACCTCCATCTTTAATACTTCTTTTAGCCTCTTCATTAGTAGAAGCCGGGGCAGGTACTGGTTGGACTGTT
B169Cretroflex TGGCTGTTACCTCCCTCTTTAATACTTCTTTTAGCGTCCTCATTAGTAGAAGCCGGGGCAGGTACCGGTTGGACTGTT
B425CretroflexaTGGCTGTTACCTCCCTCTTTAATACTTCTTTTAGCGTCCTCATTAGTAGAAGCCGGGGCAGGTACCGGTTGGACTGTT
B426Cretroflex TGGCTGTTACCTCCCTCTTTAATACTTCTTTTAGCGTCCTCATTAGTAGAAGCCGGGGCAGGTACCGGTTGGACTGTT
B335Cretroflex TGGCTGTTACCTCCCTCTTTAATACTTCTTTTAGCGTCCTCATTAGTAGAAGCCGGGGCAGGTACCGGTTGGACTGTT
B436Cretroflex TGGCTGTTACCTCCCTCTTTAATACTTCTTTTAGCGTCCTCATTAGTAGAAGCCGGGGCAGGTACCGGTTGGACTGTT
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A196Cretroflex TGGCTGTTACCTCCCTCTTTAATACTTCTTTTAGCGTCCTCATTAGTAGAAGCCGGGGCAGGTACCGGTTGGACTGTT
B412Cretroflex TGGCTGTTACCTCCCTCTTTAATACTTCTTTTAGCGTCCTCATTAGTAGAAGCTGGGGCAGGTACCGGTTGGACTGTT
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Appendix 4. (Continued)

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B121CretroflexaTGGCTGTTACCTCCCTCTTTAATACTTCTTTTAGCGTCCTCATTAGTAGAAGCCGGGGCAGGTACCGGTTGGACTGTT
B109Cretorta   TGGCTGTTACCTCCATCTTTAATACTTCTTTTAGCCTCTTCATTAGTAGAAGCCGGGGCAGGTACTGGTTGGACTGTT
B170Cretorta   TGGCTGTTACCTCCATCTTTAATACTTCTTTTAGCCTCTTCATTAGTAGAAGCCGGGGCAGGTACTGGTTGGACTGTT
B415Csiliquosa TGGCTGTTACCTCCGTCTTTAATACTTCTTTTAGCCTCTTCATTAGTAGAAGCCGGGGCGGGTACTGGTTGGACTGTT
B111Cpolycysti TGGCTTTTACCTCCCTCTTTAATACTTCTTTTAGCGTCCTCATTAGTAGAAGCTGGGGCAGGTACCGGTTGGACTGTT
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B116Ccongesta  TGGCTGTTACCTCCCTCTTTAATACTTCTTTTAGCGTCCTCATTAGTAGAAGCCGGGGCAGGTACCGGTTGGACTGTT
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B152Ccongesta  TGGCTGTTACCTCCCTCTTTAATACTTCTTTTAGCGTCCTCATTAGTAGAAGCCGGGGCAGGTACCGGTTGGACTGTT
B455Ccongesta  TGGCTGTTACCTCCCTCTTTAATACTTCTTTTAGCGTCCTCATTAGTAGAAGCCGGGGCAGGTACCGGTTGGACTGTT
B129Cretorta   TGGCTGTTACCTCCATCTTTAATACTTCTTTTAGCCTCTTCATTAGTAGAAGCCGGGGCAGGTACTGGTTGGACTGTT
B131Cretorta   TGGCTGTTACCTCCATCTTTAATACTTCTTTTAGCCTCTTCATTAGTAGAAGCCGGGGCAGGTACTGGTTGGACTGTT
B161CsubfarcinaTGGCTGTTACCTCCCTCTTTAATACTTCTTTTAGCGTCCTCATTAGTAGAAGCTGGGGCAGGTACCGGTTGGACTGTT
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B130Csiliquosa TGGCTGTTACCTCCGTCTTTAATACTTCTTTTAGCCTCTTCATTAGTAGAAGCCGGGGCGGGTACTGGTTGGACTGTT
B143Csiliquosa TGGCTGTTACCTCCGTCTTTAATACTTCTTTTAGCCTCTTCATTAGTAGAAGCCGGGGCGGGTACTGGTTGGACTGTT
B118Csiliquosa TGGCTGTTACCTCCGTCTTTAATACTTCTTTTAGCCTCTTCATTAGTAGAAGCCGGGGCGGGTACTGGTTGGACTGTT
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Appendix 4. (Continued)

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B601Ccephalorni TACCCCCCCTTAAGTGGTATTCAAGCACACTCGGGACCATCGGTTGATTTAGCTATTTTTAGCCTCCATTTATCGGGT
BO91Ccephalorni TACCCCCCCTTAAGTGGTATTCAAGCACACTCGGGACCATCGGTTGATTTAGCTATTTTTAGCCTCCATTTATCGGGT
B327Carpoconflu TACCCGCCGTTAaGTGGTATCCAAGCACACTCGGGTCCTTCTGTCGATTTAGCTATTTTTAGTCTTCATTTATCAGGT
B162Carpoconflu TACCCGCCGTTAAGTGGTATCCAAGCACACTCGGGTCCTTCTGTCGATTTAGCTATTTTTAGTCTTCATTTATCAGGT
B174Lquercifoli TACCCTCCTCTAAGTGGTATTCAAGCACATTCGGGACCATCGGTCGATTTAGCTATTTTTAGTCTTCACTTATCTGGT
B318Cmonilifera TATCCTCCCTTAAGCGGTATTCAAGCCCATTCAGGTCCATCGGTTGATTTAGCTATTTTTAGTCTTCATTTATCTGGT
B092Cmonilifera TATCCTCCCTTAAGCGGTATTCAAGCCCATTCAGGTCCATCGGTTGATTTAGCTATTTTTAGTCTTCATTTATCTGGT
B095Cmonilifera TATCCTCCCTTAAGCGGTATTCAAGCCCATTCAGGTCCATCGGTTGATTTAGCTATTTTTAGTCTTCATTTATCTGGT
B102Cmonilifera TATCCTCCCTTAAGCGGTATTCAAGCCCATTCAGGTCCATCGGTTGATTTAGCTATTTTTAGTCTTCATTTATCTGGT
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B101Cretroflexa TATCCTCCCTTAAGTGGTATTCAAGCCCATTCAGGGCCGTCGGTCGATTTAGCTATTTTTAGTCTTCATTTATCTGGT
B099Cretroflexa TATCCTCCCTTAAGTGGTATTCAAGCCCATTCAGGGCCGTCGGTCGATTTAGCTATTTTTAGTCTTCATTTATCTGGT
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B166Csubfarcina TATCCCCCCTTAAGTGGTATTCAAGCCCATTCAGGGCCGTCGGTTGATTTAGCTATTTTTAGTCTTCATTTATCTGGT
B108Cretorta    TATCCTCCCTTAAGTGGTATTCAAGCCCATTCGGGACCATCGGTTGATTTAGCTATTTTTAGTCTTCATTTATCTGGT
B617Cretroflexa TATCCTCCCTTAaGTGGTATTCAAGCCCATTCAGGGCCGTCGGTCGATTTAGCTATTTTTAGTCTTCATTTATCTGGT
B430Cretroflexa TATCCTCCCTTAAGTGGTATTCAAGCCCATTCAGGGCCGTCGGTTGATTTAGCTATTTTTAGTCTTCATTTATCTGGT
A615Cretroflexa TATCCTCCCTTAAGTGGTATTCAAGCCCATTCAGGGCCGTCGGTCGATTTAGCTATTTTTAGTCTTCATTTATCTGGT
B137Cretorta    TATCCTCCcTTAAGTGGTATTCAAGCCCATTCGGGACCATCGGTTGATTTAGCTATTTTTAGTCTTCATTTATCTGGT
B169Cretroflexa TATCCTCCCTTAAGTGGTATTCAAGCCCATTCAGGGCCGTCGGTTGATTTAGCTATTTTTAGTCTTCATTTATCTGGT
B425Cretroflexa TATCCTCCCTTAAGTGGTATTCAAGCCCATTCAGGGCCGTCGGTCGATTTAGCTATTTTTAGTCTTCATTTATCTGGT
B426Cretroflexa TATCCTCCCTTAAGTGGTATTCAAGCCCATTCAGGGCCGTCGGTCGATTTAGCTATTTTTAGTCTTCATTTATCTGGT
B335Ccongesta   TATCCTCCCTTAAGTGGTATTCAAGCCCATTCAGGGCCGTCGGTCGATTTAGCTATTTTTAGTCTTCATTTATCTGGT
B436Cretroflexa TATCCTCCCTTAaGTGGTATTCAAGCCCATTCAGGGCCGTCGGTCGATTTAGCTATTTTTAGTCTTCATTTATCTGGT
A154Cscalaris   TATCCTCCCTTAAGTAGTATTCAAGCCCATTCAGGGCCATCGGTTGATTTAGCTATTTTTAGTCTTCATTTATCTGGT
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B170Cretorta    TATCCTCCCTTAAGTGGTATTCAAGCCCATTCGGGACCATCGGTTGATTTAGCTATTTTTAGTCTTCATTTATCTGGT
B415Csiliquosa  TATCCTCCCTTAAGTGGTATTCAAGCCCATTCGGGACCATCGGTTGATTTAGCTATTTTTAGTCTTCATTTATCTGGT
B111Cpolycystid TATCCTCCCTTAAGTGGAATTCAAGCCCATTCAGGGCCATCGGTTGATTTAGCTATTTTTAGTCTTCATTTATCTGGT
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B152Ccongesta   TATCCTCCCTTAAGTGGTATTCAAGCCCATTCAGGGCCGTCGGTCGATTTAGCTATTTTTAGTCTTCATTTATCTGGT
B455Ccongesta   TATCCTCCCTTAAGTGGTATTCAAGCCCATTCAGGGCCGTCGGTTGATTTAGCTATTTTTAGTCTTCATTTATCTGGT
B129Cretorta    TATCCTCCCTTAAGTGGTATTCAAGCCCATTCGGGACCATCGGTTGATTTAGCTATTTTTAGTCTTCATTTATCTGGT
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B147Ctorulosa   TATCCTCCCTTAAGTGGTATTCAAGCCCATTCAGGGCCATCGGTTGATTTAGCTATTTTTAGTCTTCATTTATCCGGT
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Appendix 4. (Continued)

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A093Ctorulosa   TATCCTCCCTTAAGTGGTATTCAAGCCCATTCAGGGCCATCGGTTGATTTAGCTATTTTTAGTCTTCATTTATCCGGT
B320Ctorulosa   TATCCTCCCTTAAGTGGTATTCAAGCCCATTCAGGGCCATCGGTTGATTTAGCTATTTTTAGTCTTCATTTATCCGGT
A198Ctorulosa   TATCCTCCCTTAaGTGGTATTCAAGCCCATTCAGGGCCATCGGTTGATTTAGCTATTTTTAGTCTTCATTTATCCGGT
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B176Cscalaris   TATCCTCCCTTAAGTAGTATTCAAGCCCATTCAGGGCCATCGGTTGATTTAGCTATTTTTAGTCTTCATTTATCTGGT
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B325Cretorta   TATCCTCCCTTAaGTGGTATTCAAGCCCATTCGGGACCATCGGTTGATTTAGCTATTTTTAGTCTTCATTTATCTGGT
B324Cretorta   TATCCTCCCTTAAGTGGTATTCAAGCCCATTCGGGACCATCGGTTGATTTAGCTATTTTTAGTCTTCATTTATCTGGT

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BO91Ccephalorni GCTGCTTCTATTTTAGGTGCCATTAATTTTATTACAACTATTTTTAACATGCGTGCTCCTGGAATGAGCATGCATCGT
B327Carpoconflu GCAGCCTCTATTTTAGGTGCCATTAATTTTATTACAACCATTTTTAACATGCGTGCTCCTGGTATGAGCATGCACCGT
B162Carpoconflu GCAGCCTCTATTTTAGGTGCCATTAATTTTATTACAACCATTTTTAACATGCGTGCTCCTGGTATGAGCATGCACCGT
B174Lquercifoli GCGGCTTCTATTTTAGGTGCTATTAATTTCATAACGACTATTTTTAACATGCGTGCCCCGGGTATGAGCATGCACCGC
B318Cmonilifera GCAGCTTCTATTTTAGGTGCTATTAATTTCATAACAACTATTTTTAACATGCGTGCCCCAGGTATGAGCATGCACCGT
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Appendix 4. (Continued)

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B170Cretorta    GCAGCTTCTATTTTAGGCGCTATTAATTTCATAACAACTATTTTTAACATGCGTGCCCCGGGTATGAGTATGCACCGT
B415Csiliquosa  GCAGCTTCTATTTTAGGTGCCATTAATTTCATAACAACTATTTTTAACATGCGTGCCCCAGGTATGAGCATGCACCGT
B111Cpolycystid GCAGCCTCTATTTTAGGCGCTATTAATTTCATAACAACTATTTTTAACATGCGTGCCCCAGGTATGAGCATGCACCGT
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B416Ccongesta   GCAGCTTCTATTTTAGGCGCTATTAATTTCATAACAACTATTTTTAACATGCGTGCCCCAGGTATGAGCATGCACCGT
B176Cscalaris   GCAGCTTCTATTTTAGGCGCTATTAATTTCATAACAACTATTTTTAACATGCGTGCCCCAGGTATGAGCATGCACCGT
B178Cscalaris   GCAGCTTCTATTTTAGGCGCTATTAATTTCATAACAACTATTTTTAACATGCGTGCCCCAGGTATGAGCATGCACCGT
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B332Cscalaris   GCAGCTTCTATTTTAGGCGCTATTAATTTCATAACAACTATTTTTAACATGCGTGCCCCAGGTATGAGCATGCACCGT
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B351CspWilson   GCAGCTTCTATTTTAGGCGCTATTAATTTCATAACAACTATTTTTAACATGCGTGCCCCAGGTATGAGCATGCACCGT
B319Csiliquosa  GCAGCTTCTATTTTAGGTGCCATTAATTTCATAACAACTATTTTTAACATGCGTGCCCCAGGTATGAGCATGCACCGT
B130Csiliquosa  GCAGCTTCTATTTTAGGTGCCATTAATTTCATAACAACTATTTTTAACATGCGTGCCCCAGGTATGAGCATGCACCGT
B143Csiliquosa  GCAGCTTCTATTTTAGGTGCCATTAATTTCATAACAACTATTTTTAACATGCGTGCCCCAGGTATGAGCATGCACCGT
B118Csiliquosa  GCAGCTTCTATTTTAGGTGCCATTAATTTCATAACAACTATTTTTAACATGCGTGCCCCAGGTATGAGCATGCACCGT
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Appendix 4. (Continued)

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BO91Ccephalorni TTACCCTTATTCGTTTGGTCTGTATTAATAACAGCATTTTTACTTTTATTGTCGCTTCCTGTTTTAGCTGGAGGTATT
B327Carpoconflu TTACCTCTTTTTGTATGGTCTGTATTAATAACGGCATTTTTACTTTTGCTGTCTCTTCCTGTTTTAGCCGGTGGCATT
B162Carpoconflu TTACCTCTTTTTGTATGGTCTGTATTAATAACGGCATTTTTACTTTTGCTGTCTCTTCCTGTTTTAGCCGGTGGCATT
B174Lquercifoli TTACCGCTATTTGTATGGTCTGTACTGATAACAGCATTTTTACTTTTGCTATCTCTTCCTGTTTTAGCAGGGGGTATT
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B415Csiliquosa  TTACCTCTATTTGTATGGTCTGTGCTAATAACAGCATTTTTACTTTTACTGTCTCTTCCTGTTTTAGCTGGAGGTATT
B111Cpolycystid TTACCTCTATTTGTATGGTCTGTGCTAATAACAGCATTTTTACTTTTGCTGTCTCTTCCTGTTTTAGCTGGAGGTATT
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B147Ctorulosa   TTACCTCTATTTGTATGGTCTGTACTAATAACAGCATTTTTACTTTTATTGTCTCTTCCTGTTTTAGCTGGAGGTATT
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Appendix 4. (Continued)

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Appendix 4. (Continued)

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B170Cretorta        ACCATGCTTTTAACAGATCGTAATTTTAATACAACGTTTTTTGATCCAGCTGGTGGTGGTGATCCT
B415Csiliquosa       ACCATGCTTTTAACAGATCGTAATTTTAATACAACGTTTTTTGATCCAGCTGGTGGTGGTGATCCT
B111Cpolycystidea   ACCATGCTTTTAACGGATCGTAATTTTAATACAACGTTTTTTGATCCAGCCGGTGGTGGTGATCCT
B112Cpolycystidea   ACCATGCTTTTAACGGATCGTAATTTTAATACAACGTTTTTTGATCCAGCCGGTGGTGGTGATCCT
B148Cpolycystidea   ACCATGCTTTTAACGGATCGTAATTTTAATACAACGTTTTTTGATCCAGCCGGTGGTGGTGATCCT
B171Cpolycystidea   ACCATGCTTTTAACGGATCGTAATTTTAATACAACGTTTTTTGATCCAGCCGGTGGTGGTGATCCT
A168Cplatylobium    ACCATGCTTTTAACAGATCGTAATTTTAATACAACGTTTTTTGATCCAGCTGGTGGTGGTGATCCT
B140Cplatylobium    ACCATGCTTTTAACAGATCGTAATTTTAATACAACGTTTTTTGATCCAGCTGGTGGTGGTGATCCT
B104Cplatylobium    ACCATGCTTTTAACAGATCGTAATTTTAATACAACGTTTTTTGATCCAGCTGGTGGTGGTGATCCT
B116CconGesta       ACCATGCTTTTAACGGATCGTAATTTTAATACAACGTTTTTTGATCCAGCCGGGGGTGGTGATCCT
B115CconGesta       ACCATGCTTTTAACGGATCGTAATTTTAATACAACGTTTTTTGATCCAGCCGGTGGTGGTGATCCT
B152CconGesta       ACCATGCTTTTAACGGATCGTAATTTTAATACAACGTTTTTTGATCCAGCCGGTGGTGGTGATCCT
B455CconGesta       ACCATGCTTTTAACGGATCGTAATTTTAATACAACGTTTTTTGATCCAGCCGGTGGTGGTGATCCT
B129Cretorta        ACCATGCTTTTAACAGATCGTAATTTTAATACAACGTTTTTTGATCCAGCTGGTGGTGGTGATCCT
B131Cretorta        ACCATGCTTTTAACAGATCGTAATTTTAATACAACGTTTTTTGATCCAGCTGGTGGTGGTGATCCT
B161Csubfarcina     ACCATGCTTTTAACGGATCGTAATTTTAATACAACGTTTTTTGATCCAGCCGGTGGTGGTGATCCT
B626Cretroflexa     ACCATGCTTTTAACGGATCGTAATTTTAATACAACGTTTTTTGATCCAGCCGGTGGTGGTGATCCT
B146Csubfarcina     ACCATGCTTTTAACGGATCGTAATTTTAATACAACGTTTTTTGATCCAGCCGGTGGTGGTGATCCT
B146Csubfarcina     ACCATGCTTTTAACGGATCGTAATTTTAATACAACGTTTTTTGATCCAGCCGGTGGTGGTGATCCT
B128Csubfarccusp    ACCATGCTTTTAACGGATCGTAATTTTAATACAACGTTTTTTGATCCAGCCGGTGGTGGtGATCCT
B127Csubfarccusp    ACCATGCTTTTAACGGATCGTAATTTTAATACAACGTTTTTTGATCCAGCTGGTGGTGGTGATCCT
B159Cretroflexata   ACCATGCTTTTAACGGATCGTAATTTTAATACAACGTTTTTTGATCCAGCCGGTGGTGGTGATCCT
B134Csubfarcinata   ACCATGCTTTTAACGGATCGTAATTTTAATACAACGTTTTTTGATCCAGCCGGTGGTGGTGATCCT
B168Csubfarcinata   ACCATGCTTTTAACGGATCGTAATTTTAATACAACGTTTTTTGATCCAGCCGGTGGTGGTGATCCT
B136Csubfarcinata   ACCATGCTTTTAACGGATCGTAATTTTAATACAACGTTTTTTGATCCAGCCGGTGGTGGTGATCCT
B626Cretroflexata   ACCATGCTTTTAACGGATCGTAATTTTAATACAACGTTTTTTGATCCAGCCGGTGGTGGTGATCCT
B153Csubfarcinata   ACCATGCTTTTAACGGATCGTAATTTTAATACAACGTTTTTTGATCCAGCCGGTGGTGGTGATCCT
B110Csubfarcinata   ACCATGCTTTTAACGGATCGTAATTTTAATACAACGTTTTTTGATCCAGCCGGTGGTGGTGATCCT
B417Cretroflexata   ACCATGCTTTTAACGGATCGTAATTTTAATACAACGTTTTTTGATCCAGCCGGTGGTGGTGATCCT
B624Csubfarcinata   ACCATGCTTTTAACGGATCGTAATTTTAATACAACGTTTTTTGATCCAGCCGGTGGTGGTGATCCT
B428Ctorulosa       ACCATGCTTTTAACGGATCGTAATTTTAATACAACGTTTTTTGATCCAGCTGGTGGTGGTGATCCT
B103Ctorulosa       ACCATGCTTTTAACGGATCGTAATTTTAATACAACGTTTTTTGATCCAGCTGGTGGTGGTGATCCT
B165Ctorulosa       ACCATGCTTTTAACGGATCGTAATTTTAATACAACGTTTTTTGATCCAGCTGGTGGTGGTGATCCT
B125Ctorulosa       ACCATGCTTTTAACGGATCGTAATTTTAATACAACGTTTTTTGATCCAGCCGGTGGTGGTGATCCT
B147Ctorulosa       ACCATGCTTTTAACGGATCGTAATTTTAATACAACGTTTTTTGATCCAGCTGGTGGTGGTGATCCT
B179Ctorulosa       ACCATGCTTTTAACGGATCGTAATTTTAATACAACGTTTTTTGATCCAGCTGGTGGTGGTGATCCT
A271Ctorulosa       ACCATGCTTTTAACGGATCGTAATTTTAATACAACGTTTTTTGATCCAGCTGGTGGTGGTGATCCT
B175Ctorulosa       ACCATGCTTTTAACGGATCGTAATTTTAATACAACGTTTTTTGATCCAGCTGGTGGTGGTGATCCT
A093Ctorulosa       ACCATGCTTTTAACGGATCGTAATTTTAATACAACGTTTTTTGATCCAGCTGGTGGTGGTGATCCT
B320Ctorulosa       ACCATGCTTTTAACGGATCGTAATTTTAATACAACGTTTTTTGATCCAGCTGGTGGTGGTGATCCT
A198Ctorulosa       ACCATGCTTTTAACGGATCGTAATTTTAATACAACGTTTTTTGATCCAGCTGGTGGTGGTGATCCT
B316Ctorulosa       ACCATGCTTTTAACGGATCGTAATTTTAATACAACGTTTTTTGATCCAGCTGGTGGTGGTGATCCT
B167Ctorulosa       ACCATGCTTTTAACGGATCGTAATTTTAATACAACGTTTTTTGATCCAGCTGGTGGTGGTGATCCT
B145Ccongesta       ACCATGCTTTTAACGGATCGTAATTTTAATACAACGTTTTTTGATCCAGCCGGTGGTGGTGATCCT
B149Ccongesta       ACCATGCTTTTAACGGATCGTAATTTTAATACAACGTTTTTTGATCcAGCCGGTGGTGGTGATCCT
B416Ccongesta       ACCATGCTTTTAACGGATCGTAATTTTAATACAACGTTTTTTGATCCAGCCGGTGGTGGTGATCCT
B176Cscalaris       ACCATGCTTTTAACGGATCGTAATTTTAATACAACGTTTTTTGATCCAGCTGGTGGTGGTGATCCT
B178Cscalaris       ACCATGCTTTTAACGGATCGTAATTTTAATACAACGTTTTTTGATCCAGCTGGTGGTGGTGATCCT
A318Cscalaris       ACCATGCTTTTAACGGATCGTAATTTTAATACAACGTTTTTTGATCCAGCTGGTGGTGGTGATCCT
A469Cscalaris       ACCATGCTTTTAACGGATCGTAATTTTAATACAACGTTTTTTGATCCAGCTGGTGGTGGTGATCCT
B330Cscalaris       ACCATGCTTTTAACGGATCGTAATTTTAATACAACGTTTTTTGATCCAGCCGGTGGTGGTGATCCT
B333Cscalaris       ACCATGCTTTTAACGGATCGTAATTTTAATACAACGTTTTTTGATCCAGCTGGTGGTGGTGATCCT
B334Cscalaris       ACCATGCTTTTAACGGATCGTAATTTTAATACAACGTTTTTTGATCCAGCTGGTGGTGGTGATCCT
B336Cscalaris       ACCATGCTTTTAACGGATCGTAATTTTAATACAACGTTTTTTGATCCAGCTGGTGGTGGTGATCCT
B337Cscalaris       ACCATGCTTTTAACGGATCGTAATTTTAATACAACGTTTTTTGATCCAGCTGGTGGTGGTGATCCT
B338Cscalaris       ACCATGCTTTTAACGGATCGTAATTTTAATACAACGTTTTTTGATCCAGCTGGTGGTGGTGATCCT
B611Cscalaris       ACCATGCTTTTAACGGATCGTAATTTTAATACAACGTTTTTTGATCCAGCTGGTGGTGGTGATCCT
B612Cscalaris       ACCATGCTTTTAACGGATCGTAATTTTAATACAACGTTTTTTGATCCAGCTGGTGGTGGTGATCCT
B608Cscalaris       ACCATGCTTTTAACGGATCGTAATTTTAATACAACGTTTTTTGATCCAGCTGGTGGTGGTGATCCT
B607Cscalaris       ACCATGCTTTTAACGGATCGTAATTTTAATACAACGTTTTTTGATCCAGCTGGTGGTGGTGATCCT
B606Cscalaris       ACCATGCTTTTAACGGATCGTAATTTTAATACAACGTTTTTTGATCCAGCTGGTGGTGGTGATCCT
B177Cscalaris       ACCATGCTTTTAACGGATCGTAATTTTAATACAACGTTTTTTGATCCAGCTGGTGGTGGTGATCCT
B605Cscalaris       ACCATGCTTTTAACGGATCGTAATTTTAATACAACGTTTTTTGATCCAGCTGGTGGTGGTGATCCT
B332Cscalaris       ACCATGCTTTTAACGGATCGTAATTTTAATACAACGTTTTTTGATCCAGCTGGTGGTGGTGATCCT
A099Cscalaris       ACCATGCTTTTAACGGATCGTAATTTTAATACAACGTTTTTTGATCCAGCTGGTGGTGGTGATCCT
A614Cscalaris       ACCATGCTTTTAACGGATCGTAATTTTAATACAACGTTTTTTGATCCAGCTGGTGGTGGTGATCCT
A596Cscalaris       ACCATGCTTTTAACGGATCGTAATTTTAATACAACGTTTTTTGATCCAGCTGGTGGTGGTGATCCT
A292Cscalaris       ACCATGCTTTTAACGGATCGTAATTTTAATACAACGTTTTTTGATCCAGCTGGTGGTGGTGATCCT
B351CspWilson       ACCATGCTTTTAACGGATCGTAATTTTAATACAACGTTTTTTGATCCAGCTGGTGGTGGTGATCCT
B319Csiliquosa       ACCATGCTTTTAACAGATCGTAATTTTAATACAACGTTTTTTGATCCAGCTGGTGGTGGTGATCCT
B130Csiliquosa       ACCATGCTTTTAACAGATCGTAATTTTAATACAACGTTTTTTGATCCAGCTGGTGGTGGTGATCCT
B143Csiliquosa       ACCATGCTTTTAACAGATCGTAATTTTAATACAACGTTTTTTGATCCAGCTGGTGGTGGTGATCCT
B118Csiliquosa       ACCATGCTTTTAACAGATCGTAATTTTAATACAACGTTTTTTGATCCAGCTGGTGGTGGTGATCCT
B325Cretorta        ACCATGCTTTTAACAGATCGTAATTTTAATACAACGTTTTTTGATCCAGCTGGTGGTGGTGATCCT
B324Cretorta        ACCATGCTTTTAACAGATCGTAATTTTAATACAACGTTTTTTGATCCAGCTGGTGGTGGTGATCCT
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7.5 Appendix 5. Alignment of ITS sequences from *Cystophora.* Specimen names are truncated to FieldnumberSpecies.

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B174Lquercifoli ACCCACTCACCCGTCGGGGACGAGCGAGCGAGCGAGCGAG----------------------------------TC
B090Cmoniliform ACCCCCTCACCCGTCGGGGATGAGCAAGCGAGCGAGCGAGCGAGCGAGTGAGCGAGCGA---------ACGAGCCACC
B114Cexpansa    ACCCACTCACCCGTCGGGGACGAGCGAGCGAGCGAGCGAGCGA------------------------ATGGACCATC
B109Cretorta    ACCCACTCACCCGTCGGGGACGAGCGAGCGAGCGAGCGA----------------TCGA---------AAGGACCATC
B131Cretorta    ACCCACTCACCCGTCGGGGACGAGCGAGCGAGCGAGCGA----------------TCGA---------AAGGACCATC
B415Cretroflexa ACCCACTCACCCGTCGGGGACGAGCGAGCGAGCGAGCGAGCGA------------TCGA---------AAGGACCATC
B118Csiliquosa  ACCCACTCACCCGTCGGGGACGAGCGAGCGAGCGAGCGAGCGA------------TCGA---------AAGGACCATC
B143Csiliquosa  ACCCACTCACCCGTCGGGGACGAGCGAGCGAGCGAGCGAGCGA------------TCGA---------AAGGACCATC
B100Csubfarcina ACCCACTCACCCGTCGGGGACGAGCGAGCGA------------------------TCGA---------AAGGAC----
B146Csubfarcina ACCCACTCACCCGTCGGGGACGAGCGAGCGA------------------------TCGA---------AAGGAC----
B153Csubfarcina ACCCACTCACCCGTCGGGGACGAGCGAGCGA------------------------TCGA---------AAGGAC----
B110Csubfarcina ACCCACTCACCCGTCGGGGACGAGCGAGCGA------------------------TCGA---------AAGGAC----
B127Csubfarcina ACCCACTCACCCGTCGGGGACGAGCGAGCGA------------------------TCGA---------AAGGACCATC
B168Csubfarcina ACCCACTCACCCGTCGGGGACGAGCGAGCGA------------------------TCGA---------AAGGAC----
B136Csubfarcina ACCCACTCACCCGTCGGGGACGAGCGAGCGA------------------------TCGA---------AAGGAC----
B161Cretroflexa ACCCACTCACCCGTCGGGGACGAGCGAGCGA------------------------TCGA---------AAGGAC----
B103Ctorulosa   ACCCACTCACCCGTCGGGGACGAGCGAGCGA------------------------TCGA---------AAGGACCATC
B125Ctorulosa   ACCCACTCACCCGTCGGGGACGAGCGAGCGA------------------------TCGA---------AAGGAC----
B179Ctorulosa   ACCCACTCACCCGTCGGGGACGAGCGAGCGA------------------------TCGA---------AAGGACCATC
B165Ctorulosa   ACCCACTCACCCGTCGGGGACGAGCGAGCGA------------------------TCGA---------AAGGACCATC
B171Cpolycystid ACCCACTCACCCGTCGGGGACGAGCGAGCGAGCGAGCGA----------------TCGA---------AAGGACCATC
B112Cpolycystid ACCCACTCACCCGTCGGGGACGAGCGAGCGAGCGAGCGA----------------TCGA---------AAGGACCACC
B148Cpolycystid ACCCACTCACCCGTCGGGGACGAGCGAGCGAGCGAGCGA----------------TCGA---------AAGGACCACC
B111Cpolycystid ACCCACTCACCCGTCGGGGACGAGCGAGCGAGCGAGCGA----------------TCGA---------AAGGACCATC
B102Cmonilifera ACCCACTCACCCGTCGGGGACGAGCGAGCGAGCGAGCGAGCGA------------TCGA---------AAGGACCATC
B119Cmonilifera ACCCACTCACCCGTCGGGGACGAGCGAGCGAGCGAGCGA----------------TCGA---------AAGGACCATC
B318Cmonilifera ACCCACTCACCCGTCGGGGACGAGCGAGCGAGCGAGCGA----------------TCGA---------AAGGACCATC
B095CMONILIFERA ACCCACTCACCCGTCGGGGACGAGCGAGCGAGCGAGCGA----------------TCGA---------TAGGACCATC
B092Cmonilifera ------------------------------------------------------------------------------
B159Csubfarcina ACCCACTCACCCGTCGGGGACGAGCGAGCGAGCGA-------------------------------------ATC
A154Cscalaris   ---------------GGGGACGAGCGAGCGA------------------------TCGA----------------ATC
A292Cscalaris   ACCCACTCACCCGTCGGGGACGAGCGAGCGA------------------------TCGA----------------ATC
A469Cscalaris   ACCCACTCACCCGTCGGGGACGAGCGAGCGA------------------------TCGA----------------ATC
B178Cscalaris   ACCCACTCACCCGTCGGGGACGAGCGAGCGA------------------------TCGA----------------ATC
B321Csp         ACCCACTCACCCGTCGGGGACGAGCGAGCGA------------------------TCGA----------------ATC
B336Cdistenta   ACCCACTCACCCGTCGGGGACGAGCGAGCGA------------------------TCGA----------------ATC
B337Cdistenta   ACCCACTCACCCGTCGGGGACGAGCGAGCGA------------------------TCGA----------------ATC
B336Cscalaris   ACCCACTCACCCGTCGGGGACGAGCGAGCGA------------------------TCGA----------------ATC
B104Cplatylobiu ACCCACTCACCCGTCGGGGACGAGCGAGCGAGCGAGCGAGCGAGCTAACTAACTATCGATAGAAAGGAAAGGACCATC
A168Cplatylobiu ACCCACTCACCCGTCGGGGACGAGCGAGCGAGCGAGCGAGCGAGCTAACTAACTATCGATAGAAAGGAAAGGACCATC
B149Cycongesta  ACCCACTCACCCGTCGGGGACGAGCGAGCGAGCGAGCGA-----------------------------------ATC
B152Ccongesta   ACCCACTCACCCGTCGGGGATGAGCGAGCGAGCGAGCGA-----------------------------------ATC
B101Cretroflexa ACCCACTCACCCGTCGGGGACGAGCGAGCGAGCGA-------------------------------------ATC
A080Cretroflexa ACCCACTCACCCGTCGGGGACGAGCGAGCGAGCGA-------------------------------------ATC
A196Cretroflexa ACCCACTCACCCGTCGGGGACGAGCGAGCGAGCGA-------------------------------------ATC
A085Cretroflexa ACCCACTCACCCGTCGGGGACGAGCGAGCGAGCGA-------------------------------------ATC
B335Ccongesta   ACCCACTCACCCGTCGGGGACGAGCGAGCGAGCGA-------------------------------------ATC
B166Csubfarcina ACCCACTCACCCGTCGGGGACGAGCGAGCGA------------------------TCGA---------AAGGAC----
B116Ccongesta   ACCCACTCACCCGTCGGGGACGAGCGAGCGAGCGA-------------------------------------ATC
B115Ccongesta   ACCCACTCACCCGTCGGGGACGAGCGAGCGAGCGA-------------------------------------ATC
B121Cretroflexa ----------------------GCGAGCGAGCGA--------------------TCGA----------------ATC

B174Lquercifoli ACGC---------CCGCCCGCCCG--------------------TACCCGGGTGCGATGAACGAG---AAGCGGGGGG
B090Cmoniliform ACGCTCCGCTCGCCCGGCCGCCCGCCCGCCCGCCCGCCCGTTCGTTCCCGGGTGCGATGAACGAGG---AGCGGGGGG
B114Cexpansa    ACGCTCCGCTCGCCCGCCCGCCCG--------------------TTCCCGGGTGCGATGAACGAGGAGAAGCGGGGGG
B109Cretorta    AGGCTCCGCTCGCCCGCCCGCCCG--------------------TTCCCGGGTGCGATGAACGAGGAGAAGCGGGGGG
B131Cretorta    AGGCTCCGCTCGCCCGCCCGCCCG--------------------TTCCCGGGTGCGATGAACGAGGAGAAGCGGGGGG
B415Cretroflexa AGGCTCCGCTCGCCCGCCCGCCCG--------------------TTCCCGGGTGCGATGAACGAGGAGAAGCGGGGGG
B118Csiliquosa  AGGCTCCGCTCGCCCGCCCGCCCG--------------------TTCCCGGGTGCGATGAACGAGGAGAAGCGGGGGG
B143Csiliquosa  AGGCTCCGCTCGCCCGCCCGCCCG--------------------TTCCCGGGTGCGATGAACGAGGAGAAGCGGGGGG
B100Csubfarcina -------GCTCGCCCG----------------------------TTCCCGGGTGCGATGAACGAGGAGAAGCGGGGGG
B146Csubfarcina -------GCTCGCCCG----------------------------TTCCCGGGTGCGATGAACGAGGAGAAGCGGGGGG
B153Csubfarcina -------GCTCGCCCG----------------------------TTCCCGGGTGCGATGAACGAGGAGAAGCGGGGGG
B110Csubfarcina -------GCTCGCCCG----------------------------TTCCCGGGTGCGATGAACGAGGAGAAGCGGGGGG
B127Csubfarcina ACGCTCCGCTCGCCCGCCCGCCCG--------------------TTCCCGGGTGCGATGAACGAGGAGAAGCGGGGGG
B168Csubfarcina -------GCTCGCCCG----------------------------TTCCCGGGTGCGATGAACGAGGAGAAGCGGGGGG
B136Csubfarcina -------GCTCGCCCG----------------------------TTCCCGGGTGCGATGAACGAGGAGAAGCGGGGGG
B161Cretroflexa -------GCTCGCCCG----------------------------TTCCCGGGTGCGATGAACGAGGAGAAGCGGGGGG
B103Ctorulosa   ACGCTCCGCTCGCCCGCCCGCCCG--------------------TTCCCGGGTGCGATGAACGAGGAGAAGCGGGGGG
B125Ctorulosa   -------GCTCGCCCG----------------------------TTCCCGGGTGCGATGAACGAGGAGAAGCGGGGGG
B179Ctorulosa   ACGCTCCGCTCGCCCGCCCGCCCG--------------------TTCCCGGGTGCGATGAACGAGGAGAAGCGGGGGG
B165Ctorulosa   ACGCTCCGCTCGCCCGCCCGCCCG--------------------TTCCCGGGTGCGATGAACGAGGAGAAGCGGGGGG
B171Cpolycystid ACGCTCCGCTCGCCCG----------------------------TTCCCGGGTGCGATGAACGAGGAGAAGCGGGGGG
B112Cpolycystid ACGCTCCGCTCGCCCG----------------------------TTCCCGGGTGCGATGAACGAGGAGAAGCGGGGGG
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Appendix 5. (Continued)

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B148Cpolycystid ACGCTCCGCTCGCCCG---------------------------TTCCCGGGTGCGATGAACGAGGAGAAGCGGGGGG
B111Cpolycystid ACGCTCCGCTCGCCCG---------------------------TTCCCGGGTGCGATGAACGAGGAGAAGCGGGGGG
B102Cmonilifera ACGCTCCGCTCGCCCG---------------------------TTCCCGGGTGCGATGAACGAGGAGAAGCGGGGGG
B119Cmonilifera ACGCTCCGCTCGCCCG---------------------------TTCCCGGGTGCGATGAACGAGGAGAAGCGGGGGG
B318Cmonilifera ACGCTCCGCTCGCCCG---------------------------TTCCCGGGTGCGATGAACGAGGAGAAGCGGGGGG
B095CMONILIFERA ACGCTCCGTTCGCTCG---------------------------TTCGC---------------------------
B092Cmonilifera ---------TCGCCCGT---------------------------TTCCCGGGTGCGATGAACGAGGAGAAGCGGGGGG
B159Csubfarcina ACGCTCCGCTCGCCCG---------------------------TTCCCGGGTGCGATGAACGAGGAGAAGCGGGGGG
A154Cscalaris   ACGCTCCGCTCGCCCG---------------------------TTCCCGGGTGCGATGAACGAGGAGAAGCGGGGGG
A292Cscalaris   ACGCTCCGCTCGCCCG---------------------------TTCCCGGGTGCGATGAACGAGGAGAAGCGGGGGG
A469Cscalaris   ACGCTCCGCTCGCCCG---------------------------TTCCCGGGTGCGATGAACGAGGAGAAGCGGGGGG
B178Cscalaris   ACGCTCCGCTCGCCCG---------------------------TTCCCGGGTGCGATGAACGAGGAGAAGCGGGGGG
B321Csp         ACGCTCCGCTCGCCCG---------------------------TTCCCGGGTGCGATGAACGAGGAGAAGCGGGGGG
B336Cdistenta   ACGCTCCGCTCGCCCG---------------------------TTCCCGGGTGCGATGAACGAGGAGAAGCGGGGGG
B337Cdistenta   ACGCTCCGCTCGCCCG---------------------------TTCCCGGGTGCGATGAACGAGGAGAAGCGGGGGG
B336Cscalaris   ACGCTCCGCTCGCCCG---------------------------TTCCCGGGTGCGATGAACGAGGAGAAGCGGGGGG
B104Cplatylobiu ACGCTCCGCTCGCCCGCCCG-----------------------TTCCCGGGTGCGATGAACGAGGAGAAGCGGGGGG
A168Cplatylobiu ACGCTCCGCTCGCCCGCCCG-----------------------TTCCCGGGTGCGATGAACGAGGAGAAGCGGGGGG
B149Cycongesta  ACGCTCCGCTCGCCCG---------------------------GTTCCCGGGTGCGATGAACGAGGAGAAGCGGGGGG
B152Ccongesta   ACGCTCCGCTCGCCCG---------------------------TTCCCGGGTGCGATGAACGAGGAGAAGCGGGGGG
B101Cretroflexa ACGCTCCGCTCGCCCG---------------------------TTCCCGGGTGCGATGAACGAGGAGAAGCGGGGGG
A080Cretroflexa ACGCTCCGCTCGCCCG---------------------------TTCCCGGGTGCGATGAACGAGGAGAAGCGGGGGG
A196Cretroflexa ACGCTCCGCTCGCCCG---------------------------TTCCCGGGTGCGATGAACGAGGAGAAGCGGGGGG
A085Cretroflexa ACGCTCCGCTCGCCCG---------------------------TTCCCGGGTGCGATGAACGAGGAGAAGCGGGGGG
B335Ccongesta   ACGCTCCGCTCGCCCG---------------------------TTCCCGGGTGCGATGAACGAGGAGAAGCGGGGGG
B166Csubfarcina -------GCTCGCCCG---------------------------TTCCCGGGTGCGATGAACGAGGAGAAGCGGGGGG
B116Ccongesta   ACGCTCCGCTCGCCCG---------------------------TTCCCGGGTGCGATGAACGAGGAGAAGCGGGGGG
B115Ccongesta   ACGCTCCGCTCGCCCG---------------------------TTCCCGGGTGCGATGAACGAGGAGAAGCGGGGGG
B121Cretroflexa ACGCTCCGCTCGCCCG---------------------------TTCCCGGGTGCGATGAACGAGGAGAAGCGGGGGG

B174Lquercifoli ACGGAGCTGACG-GCTTCGTCCGCTCGTGAAAGGACCCCCTCCCTTCGGTGTGGCAGACTTGGGTGCTGCCGCGGTAC
B090Cmoniliform AGGAAGCTTTCTTGCTCCGTCCGCTCGTGAAAGGACCCCCTCCCTTCAGTGTGGCAGACTTGGGTGCTGCCGCGGTAC
B114Cexpansa    ACGGAGCTTTCTTGCTTCGTCCGCTCGTGGAAGGACCCCCTCCCTTCAGTGTGGGAGGCTTGGGTGCTGCCGCGGTAC
B109Cretorta    GCGGAGCTATCTTGCTTCGTCCACTCGTGAAAGGACCCCCTCCCTTCAGTGTGGCAGGCTTGGGTGCTGCCGCGGTAC
B131Cretorta    GCGGAGCTATCTTGCTTCGTCCACTCGTGAAAGGACCCCCTCCCTTCAGTGTGGCAGGCTTGGGTGCTGCCGCGGTAC
B415Cretroflexa GCGGAGCTATCTTGCTTCGTCCTCTCGTGAAAGGACCCCCTCCCTTCAGTGTGGCAGGCTTGGGTGCTGCCGCGGTAC
B118Csiliquosa  GCGGAGCTATCTTGCTTCGTCCTCTCGTGAAAGGACCCCCTCCCTTCAGTGTGGCAGGCTTGGGTGCTGCCGCGGTAC
B143Csiliquosa  GCGGAGCTATCTTGCTTCGTCCTCTCGTGAAAGGACCCCCTCCCTTCAGTGTGGCAGGCTTGGGTGCTGCCGCGGTAC
B100Csubfarcina GCGGAGCTATCTTGCTTCGTCCGCTCGTGAAAGGACCCCTTCCCTTCAGTGTGGCAGGCTTGGGTGCTGCCGCGGTAC
B146Csubfarcina GCGGAGCTATCTTGCTTCGTCCGCTCGTGAAAGGACCCCTTCCCTTCAGTGTGGCAGGCTTGGGTGCTGCCGCGGTAC
B153Csubfarcina GCGGAGCTATCTTGCTTCGTCCGCTCGTGAAAGGACCCCTTCCCTTCAGTGTGGCAGGCTTGGGTGCTGCCGCGGTAC
B110Csubfarcina GCGGAGCTATCTTGCTTCGTCCGCTCGTGAAAGGACCCCTTCCCTTCAGTGTGGCAGGCTTGGGTGCTGCCGCGGTAC
B127Csubfarcina GCGGAGCTATCTTGCTTCGTCCGCTCGTGAAAGGACCCCCTCCCTTCAGTGTGGCAGGCTTGGGTGCTGCCGCGGTAC
B168Csubfarcina GCGGAGCTATCTTGCTTCGTCCGCTCGTGAAAGGACCCCTTCCCTTCAGTGTGGCAGGCTTGGGTGCTGCCGCGGTAC
B136Csubfarcina GCGGAGCTATCTTGCTTCGTCCGCTCGTGAAAGGACCCCTTCCCTTCAGTGTGGCAGGCTTGGGTGCTGCCGCGGTAC
B161Cretroflexa GCGGAGCTATCTTGCTTCGTCCGCTCGTGAAAGGACCCCTTCCCTTCAGTGTGGCAGGCTTGGGTGCTGCCGCGGTAC
B103Ctorulosa   GCGGAGCTATCTTGCTTCGTCCGCTCGTGAAAGGACCCCCTCCCTTCAGTGTGGCAGGCTTGGGTGCTGCCGCGGTAC
B125Ctorulosa   GCGGAGCTATCTTGCTTCGTCCGCTCGTGAAAGGACCCCTTCCCTTCAGTGTGGCAGGCTTGGGTGCTGCCGCGGTAC
B179Ctorulosa   GCGGAGCTATCTTGCTTCGTCCGCTCGTGAAAGGACCCCCTCCCTTCAGTGTGGCAGGCTTGGGTGCTGCCGCGGTAC
B165Ctorulosa   GCGGAGCTATCTTGCTTCGTCCGCTCGTGAAAGGACCCCCTCCCTTCAGTGTGGCAGGCTTGGGTGCTGCCGCGGTAC
B171Cpolycystid GCGGAGCTATCTTGCTTCGTCCGCTCGTGAAAGGACCCCCTCCCTTCAGTGTGGCAGGCTTGGGTGCTGCCGCGGTAC
B112Cpolycystid GCGGAGCTATCTTGCTTCGTCCGCTCGTGAAAGGACCCCCTCCCTTCAGTGTGGCAGGCTTGGGTGCTGCCGCGGTAC
B148Cpolycystid GCGGAGCTATCTTGCTTCGTCCGCTCGTGAAAGGACCCCCTCCCTTCAGTGTGGCAGGCTTGGGTGCTGCCGCGGTAC
B111Cpolycystid GCGGAGCTATCTTGCTTCGTCCGCTCGTGAAAGGACCCCCTCCCTTCAGTGTGGCAGGCTTGGGTGCTGCCGCGGTAC
B102Cmonilifera GCGGAGCAATCTTGCTTCGTCCGCTCGTGAAAGGACCCCCTCCCTTCAGTGTGGCAGGCTTGGGTGCTGCCGCGGTAC
B119Cmonilifera GCGGAGCAATCTTGCTTCGTCCGCTCGTGAAAGGACCCCCTCCCTTCAGTGTGGCAGGCTTGGGTGCTGCCGCGGTAC
B318Cmonilifera GCGGAGCAATCTTGCTTCGTCCGCTCGTGAAAGGACCCCCTCCCTTCAGTGTGGCAGGCTTGGGTGCTGCCGCGGTAC
B095CMONILIFERA ---------------------------------------------------------------------------
B092Cmonilifera GCGGAGCAATCTTGCTTCGTCCGCTCGTGAAAGGACCCCCTCCCTTCAGTGTGGCAGGCTTGGGTGCTGCCGCGGTAC
B159Csubfarcina GCGGAGCTATCTTGCTTCGTCCGCTCGTGAAAGGACCCCCTCCCTTCAGTGTGGCAGGCTTGGGTGCTGCCGCGGTAC
A154Cscalaris   GCGGAGCTATCTTGCTTCGTCCGCTCGTGAAAGGACCCCCTCCCTTCAGTGTGGCAGGCTTGGGTGCTGCCGCGGTAC
A292Cscalaris   GCGGAGCTATCTTGCTTCGTCCGCTCGTGAAAGGACCCCCTCCCTTCAGTGTGGCAGGCTTGGGTGCTGCCGCGGTAC
A469Cscalaris   GCGGAGCTATCTTGCTTCGTCCGCTCGTGAAAGGACCCCCTCCCTTCAGTGTGGCAGGCTTGGGTGCTGCCGCGGTAC
B178Cscalaris   GCGGAGCTATCTTGCTTCGTCCGCTCGTGAAAGGACCCCCTCCCTTCAGTGTGGCAGGCTTGGGTGCTGCCGCGGTAC
B321Csp         GCGGAGCTATCTTGCTTCGTCCGCTCGTGAAAGGACCCCCTCCCTTCAGTGTGGCAGGCTTGGGTGCTGCCGCGGTAC
B336Cdistenta   GCGGAGCTATCTTGCTTCGTCCGCTCGTGAAAGGACCCCCTCCCTTCAGTGTGGCAGGCTTGGGTGCTGCCGCGGTAC
B337Cdistenta   GCGGAGCTATCTTGCTTCGTCCGCTCGTGAAAGGACCCCCTCCCTTCAGTGTGGCAGGCTTGGGTGCTGCCGCGGTAC
B336Cscalaris   GCGGAGCTATCTTGCTTCGTCCGCTCGTGAAAGGACCCCCTCCCTTCAGTGTGGCAGGCTTGGGTGCTGCCGCGGTAC
B104Cplatylobiu GCGGAGCTATCTTGCTTCGTCCGCTCGTGAAAGGACCCCCTCCCTTCAGTGTGGCAGGCTTGGGTGCTGCCGCGGTAC
A168Cplatylobiu GCGGAGCTATCTTGCTTCGTCCGCTCGTGAAAGGACCCCCTCCCTTCAGTGTGGCAGGCTTGGGTGCTGCCGCGGTAC
B149Cycongesta  GCGGAGCTATCTTGCTTCGTCCGCTCGTGAAAGGACCCCCTCCCTTCAGTGTGGCAGGCTTGGGTGCTGCCGCGGTAC
B152Ccongesta   GCGGAGCTATCTTGCTTCGTCCGCTCGTGAAAGGACCCCCTCCCTTCAGTGTGGMAGGCTTGGGTGCTGCCGCGGTAC
B101Cretroflexa GCGGAGCTATCTTGCTTCGTCCGCTCGTGAAAGGACCCCCTCCCTTCAGTGTGGCAGGCTTGGGTGCTGCCGCGGTAC
A080Cretroflexa GCGGAGCTATCTTGCTTCGTCCGCTCGTGAAAGGACCCCCTCCCTTCAGTGTGGCAGGCTTGGGTGCTGCCGCGGTAC
A196Cretroflexa GCGGAGCTATCTTGCTTCGTCCGCTCGTGAAAGGACCCCCTCCCTTCAGTGTGGCAGGCTTGGGTGCTGCCGCGGTAC
A085Cretroflexa GCGGAGCTATCTTGCTTCGTCCGCTCGTGAAAGGACCCCCTCCCTTCAGTGTGGCAGGCTTGGGTGCTGCCGCGGTAC
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Appendix 5. (Continued)

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B335Ccongesta   GCGGAGCTATCTTGCTTCGTCCGCTCGTGAAAGGACCCCCTCCCTTCAGTGTGGCAGGCTTGGGTGCTGCCGCGGTAC
B166Csubfarcina GCGGAGCTATCTTGCTTCGTCCGCTCGTGAAAGGACCCCTTCCCTTCAGTGTGGCAGGCTTGGGTGCTGCCGCGGTAC
B116Ccongesta   GCGGAGCTATCTTGCTTCGTCCGCTCGTGAAAGGACCCCCTCCCTTCAGTGTGGCAGGCTTGGGTGCTGCCGCGGTAC
B115Ccongesta   GCGGAGCTATCTTGCTTCGTCCGCTCGTGAAAGGACCCCCTCCCTTCAGTGTGGCAGGCTTGGGTGCTGCCGCGGTAC
B121Cretroflexa GCGGAGCTATCTTGCTTCGTCCGCTCGTGAAAGGACCCCCTCCCTTCAGTGTGGCAGGCTTGGGTGCTGCCGCGGTAC

B174Lquercifoli TGGAGTGGGGAGGCTCGGGAGCGCCCGCAACCC-TCTCCGAGTGGGGCCGCCTTGTCGGGGCGGGGAGGGCCCGAGGT
B090Cmoniliform TGGAGTGGGGAGGCTCGGGAGCGCCCGCAACCCCTCTCCGAGTGGGGCCGCCTTGGCGTGGCGGAGAGGGCTCGAGGT
B114Cexpansa    TGGACGGGGGAGGCTCGGGAGCGCCCGCAACCCCTCTCCGAGTGGGGCCGCCTTGGCGGGGCGGAgAGGGCCCGAGGT
B109Cretorta    TGGACGGGGGAGGCTCGGGAGCGCCCACAACCCCTCTCCGAGTGGGGCCGCCTTGGCGGGGCGGAGAGGGCCCGAGGT
B131Cretorta    TGGACGGGGGAGGCTCGGGAGCGCCCACAACCCCTCTCCGAGTGGGGCCGCCTTGGCGGGGCGGAGAGGGCCCGAGGT
B415Cretroflexa TGGGCGGGGGAGGCTCGGGAGCGCCCACAACCCCTCTCCGAGTGGGGCCGCCTTGGCGGGGCGGAGAGGGCCCGAGGT
B118Csiliquosa  TGGACGGGGGAGGCTCGGGAGCGCCCACAACCCCTCTCCGAGTGGGGCCGCCTTGGCGGGGCGGAGAGGGCCCGAGGT
B143Csiliquosa  TGGACGGGGGAGGCTCGGGAGCGCCCACAACCCCTCTCCGAGTGGGGCCGCCTTGGCGGGGCGGAGAGGGCCCGAGGT
B100Csubfarcina TGGACGGGGGCGGCTCGGGAGCGCCCGCAACCCCTCTCCGAGTGGGGCCGCCTTGGCGGGGCGGAGAGGGCCCGAGGT
B146Csubfarcina TGGACGGGGGCGGCTCGGGAGCGCCCGCAACCCCTCTCCGAGTGGGGCCGCCTTGGCGGGGCGGAGAGGGCCCGAGGT
B153Csubfarcina TGGACGGGGGCGGCTCGGGAGCGCCCGCAACCCCTCTCCGAGTGGGGCCGCCTTGGCGGGGCGGAGAGGGCCCGAGGT
B110Csubfarcina TGGACGGGGGCGGCTCGGGAGCGCCCGCAACCCCTCTCCGAGTGGGGCCGCCTTGGCGGGGCGGAGAGGGCCCGAGGT
B127Csubfarcina TGGACGGGGGCGGCTCGGGAGCGCCCGCAACCCCTCTCCGAGTGGGGCCGCCTTGGCGGGGCGGAGAGGGCCCGAGGT
B168Csubfarcina TGGACGGGGGCGGCTCGGGAGCGCCCGCAACCCCTCTCCGAGTGGGGCCGCCTTGGCGGGGCGGAGAGGGCCCGAGGT
B136Csubfarcina TGGACGGGGGCGGCTCGGGAGCGCCCGCAACCCCTCTCCGAGTGGGGCCGCCTTGGCGGGGCGGAGAGGGCCCGAGGT
B161Cretroflexa TGGACGGGGGCGGCTCGGGAGCGCCCGCAACCCCTCTCCGAGTGGGGCCGCCTTGGCGGGGCGGAGAGGGCCCGAGGT
B103Ctorulosa   TGGACGGGGGCGGCTCGGGAGCGCCCGCAACCCCTCTCCGAGTGGGGCCGCCTTGGCGGGGCGGAGAGGGCCCGAGGT
B125Ctorulosa   TGGACGGGGGCGGCTCGGGAGCGCCCGCAACCCCTCTCCGAGTGGGGCCGCCTTGGCGGGGCGGAGAGGGCCCGAGGT
B179Ctorulosa   TGGACGGGGGCGGCTCGGGAGCGCCCGCAACCCCTCTCCGAGTGGGGCCGCCTTGGCGGGGCGGAGAGGGCCCGAGGT
B165Ctorulosa   TGGACGGGGGCGGCTCGGGAGCGCCCGCAACCCCTCTCCGAGTGGGGCCGCCTTGGCGGGGCGGAGAGGGCCCGAGGT
B171Cpolycystid TGGACGGGGGCGGCTCGGGAGCGCCCGCAACCCCTCTCCGAGTGGGGCCGCCTTGGCGGGGCGGAGAGGGCCCGAGGT
B112Cpolycystid TGGACGGGGGCGGCTCGGGAGCGCCCGCAACCCCTCTCCGAGTGGGGCCGCCTTGGCGGGGCGGAGAGGGCCCGAGGT
B148Cpolycystid TGGACGGGGGCGGCTCGGGAGCGCCCGCAACCCCTCTCCGAGTGGGGCCGCCTTGGCGGGGCGGAGAGGGCCCGAGGT
B111Cpolycystid TGGACGGGGGCGGCTCGGGAGCGCCCGCAACCCCTCTCCGAGTGGGGCCGCCTTGGCGGGGCGGAGAGGGCCCGAGGT
B102Cmonilifera TGGACGGGGGCGGCTCGGGAGCGCCCGCAACCCCTCTCCGAGTGGGGCCGCCTTGGCGGGGCGGAGAGGGCCCGAGGT
B119Cmonilifera TGGACGGGGGCGGCTCGGGAGCGCCCGCAACCCCTCTCCGAGTGGGGCCGCCTTGGCGGGGCGGAGAGGGCCCGAGGT
B318Cmonilifera TGGACGGGGGCGGCTCGGGAGCGCCCGCAACCCCTCTCCGAGTGGGGCCGCCTTGGCGGGGCGGAGAGGGCCCGAGGT
B095CMONILIFERA ----------------------------ACCCCTCTCCGAGTGGGGCCGCCTTGGCGGGGCGGAGAGGGCCCGAGGT
B092Cmonilifera TGGACGGGGGCGGCTCGGGAGCGCCCGC-ACCCCTCTCCGAGTGGGGCCGCCTTGGCGGGGCGGAGAGGGCCCGAGGT
B159Csubfarcina TGGACGGGGGCGGCTCGGGAGCGCCCGCAACCCCTCTCCGAGTGGGGCCGCCTTGGCGGGGCGGAGAGGGCCCGAGGT
A154Cscalaris   TGGACGGGGGCGGCTCGGGAGCGCCCGCAACCCCTCTCCGAGTGGGGCCGCCTTGGCGGGGCGGAGAGGGCCCGAGGT
A292Cscalaris   TGGACGGGGGCGGCTCGGGAGCGCCCGCAACCCCTCTCCGAGTGGGGCCGCCTTGGCGGGGCGGAGAGGGCCCGAGGT
A469Cscalaris   TGGACGGGGGCGGCTCGGGAGCGCCCGCAACCCCTCTCCGAGTGGGGCCGCCTTGGCGGGGCGGAGAGGGCCCGAGGT
B178Cscalaris   TGGACGGGGGCGGCTCGGGAGCGCCCGCAACCCCTCTCCGAGTGGGGCCGCCTTGGCGGGGCGGAGAGGGCCCGAGGT
B321Csp         TGGACGGGGGCGGCTCGGGAGCGCCCGCAACCCCTCTCCGAGTGGGGCCGCCTTGGCGGGGCGGAGAGGGCCCGAGGT
B336Cdistenta   TGGACGGGGGCGGCTCGGGAGCGCCCGCAACCCCTCTCCGAGTGGGGCCGCCTTGGCGGGGCGGAGAGGGCCCGAGGT
B337Cdistenta   TGGACGGGGGCGGCTCGGGAGCGCCCGCAACCCCTCTCCGAGTGGGGCCGCCTTGGCGGGGCGGAGAGGGCCCGAGGT
B336Cscalaris   TGGACGGGGGCGGCTCGGGAGCGCCCGCAACCCCTCTCCGAGTGGGGCCGCCTTGGCGGGGCGGAGAGGGCCCGAGGT
B104Cplatylobiu TGGACGGGGGCGGCTCGGGAGCGCCCACAACCCCTCTCCGAGTGGGGCCGCCTTGGCGGGGCGGAGAGGGCCCGAGGT
A168Cplatylobiu TGGACGGGGGCGGCTCGGGAGCGCCCACAACCCCTCTCCGAGTGGGGCCGCCTTGGCGGGGCGGAGAGGGCCCGAGGT
B149Cycongesta  TGGACGGGGGCGGCTCGGGAGCGCCCGCAACCCCTCTCCGAGTGGGGCCGCCTTGGCGGGGCGGAGAGGGCCCGAGGT
B152Ccongesta   TGGACGGGGGCGGCTCGGGAGCGCCCGCAACCCCTCTCCGAGTGGGGCCGCCTTGGCGGGGCGGAGAGGGCCCGAGGT
B101Cretroflexa TGGACGGGGGCGGCTCGGGAGCGCCCGCAACCCCTCTCCGAGTGGGGCCGCCTTGGCGGGGCGGAGAGGGCCCGAGGT
A080Cretroflexa TGGACGGGGGCGGCTCGGGAGCGCCCGCAACCCCTCTCCGAGTGGGGCCGCCTTGGCGGGGCGGAGAGGGCCCGAGGT
A196Cretroflexa TGGACGGGGGCGGCTCGGGAGCGCCCGCAACCCCTCTCCGAGTGGGGCCGCCTTGGCGGGGCGGAGAGGGCCCGAGGT
A085Cretroflexa TGGACGGGGGCGGCTCGGGAGCGCCCGCAACCCCTCTCCGAGTGGGGCCGCCTTGGCGGGGCGGAGAGGGCCCGAGGT
B335Ccongesta   TGGACGGGGGCGGCTCGGGAGCGCCCGCAACCCCTCTCCGAGTGGGGCCGCCTTGGCGGGGCGGAGAGGGCCCGAGGT
B166Csubfarcina TGGACGGGGGCGGCTCGGGAGCGCCCGCAACCCCTCTCCGAGTGGGGCCGCCTTGGCGGGGCGGAGAGGGCCCGAGGT
B116Ccongesta   TGGACGGGGGCGGCTCGGGAGCGCCCGCAACCCCTCTCCGAGTGGGGCCGCCTTGGCGGGGCGGAGAGGGCCCGAGGT
B115Ccongesta   TGGACGGGGGCGGCTCGGGAGCGCCCGCAACCCCTCTCCGAGTGGGGCCGCCTTGGCGGGGCGGAGAGGGCCCGAGGT
B121Cretroflexa TGGACGGGGGCGGCTCGGGAGCGCCCGCAACCCCTCTCCGAGTGGGGCCGCCTTGGCGGGGCGGAGAGGGCCCGAGGT

B174Lquercifoli --CGCTGCATCG--CACAATGCGCT-GCACTCCTCGCGAAGATCCCAGTGGCTCGGTCGG------------------
B090Cmoniliform --CACTGC----------ACGCGGT-GCACTCCTCGCGAAGATCCCAGTGGCTCCGTCGGTCGATCGGTCGGTCGGTC
B114Cexpansa    --CACTGCATCGCGCACGACGCGGtGGCACTCCT??CGAAGATCTCAGTGGCTCGGTCGG----TCGGTCGGTCGGTC
B109Cretorta    --CACTGCATCGCGCACGACGCGGT-GCACTCCTCGCGAAGATCCCAGTGGCTCGGTCGA------------------
B131Cretorta    --CACTGCATCGCGCACGACGCGGT-GCACTCCTCGCGAAGATCCCAGTGGCTCGGTCGA------------------
B415Cretroflexa --CACTGCATCGCGCACGACGCGGT-GCACTCCTCGCGAAGATCCCAGTGGCTCGGTCGA------------------
B118Csiliquosa  --CACTGCATCGCGCACGACGCGGT-GCACTCCTCGCGAAGATCCCAGTGGCTCGGTCGA------------------
B143Csiliquosa  --CACTGCATCGCGCACGACGCGGT-GCACTCCTCGCGAAGATCCCAGTGGCTCGGTCGA------------------
B100Csubfarcina CTCACTGCATCGCGCACGACGCGGT-GCACTCCTCGCGAAGATCCCAGTGGCTCGGTCGG------------------
B146Csubfarcina CTCACTGCATCGCGCACGACGCGGT-GCACTCCTCGCGAAGATCCCAGTGGCTCGGTCGG------------------
B153Csubfarcina CTCACTGCATCGCGCACGACGCGGT-GCACTCCTCGCGAAGATCCCAGTGGCTCGGTCGG------------------
B110Csubfarcina CTCACTGCATCGCGCACGACGCGGT-GCACTCCTCGCGAAGATCCCAGTGGCTCGGTCGG------------------
B127Csubfarcina --CACTGCATCGCGCACGACGCGGT-GCACTCCTCGCGAAGATCCCAGTGGCTCGGTCGG------------------
B168Csubfarcina CTCACTGCATCGCGCACGACGCGGT-GCACTCCTCGCGAAGATCCCAGTGGCTCGGTCGG------------------
B136Csubfarcina CTCACTGCATCGCGCACGACGCGGT-GCACTCCTCGCGAAGATCCCAGTGGCTCGGTCGG------------------
B161Cretroflexa CTCACTGCATCGCGCACGACGCGGT-GCACTCCTCGCGAAGATCCCAGTGGCTCGGTCGG------------------
B103Ctorulosa   --CACTGCATCGCGCACGACGCGGT-GCACTCCTCGCGAAGATCCCAGTGGCTCGGTCGG------------------
B125Ctorulosa   CTCACTGCATCGCGCACGACGCGGT-GCACTCCTCGCGAAGATCCCAGTGGCTCGGTCGG------------------
```

Appendix 5. (Continued)

```
B179Ctorulosa   --CACTGCATCGCGCACGACGCGGT-GCACTCCTCGCGAAGATCCCAGTGGCTCGGTCGG----------------CC
B165Ctorulosa   --CACTGCATCGCGCACGACGCGGT-GCACTCCTCGCGAAGATCCCAGTGGCTCGGTCGG------------------
B171Cpolycystid --CACTGCATCGCGCACGACGCGGT-GCACTCCTCGCGAAGATCCCAGTGGCTCGGTCGG------------------
B112Cpolycystid --CACTGCATCGCGCACGACGCGGT-GCACTCCTCGCGAAGATCCCAGTGGCTCGGTCGG------------------
B148Cpolycystid --CACTGCATCGCGCACGACGCGGT-GCACTCCTCGCGAAGATCCCAGTGGCTCGGTCGG------------------
B111Cpolycystid --CACTGCATCGCGCACGACGCGGT-GCACTCCTCGCGAAGATCCCAGTGGCTCGGTCGG------------------
B102Cmonilifera --CACTGCATCGCGCACGACGCGGT-GCACTCCTCGCGAAGATCCCAGTGGCTCGGTCGG------------CCGGCC
B119Cmonilifera --CACTGCATCGCGCACGACGCGGT-GCACTCCTCGCGAAGATCCCAGTGGCTCGGTCGG----------------CC
B318Cmonilifera --CACTGCATCGCGCACGACGCGGT-GCACTCCTCGCGAAGATCCCAGTGGCTCGGTCGG----------------CC
B095CMONILIFERA --CACTGCATCGCGCACGACGCGGT-GCACTCCTCGCGAAGATCCCAGTGGCTCGGTCGG------------CCGGCC
B092Cmonilifera --CACTGCATCGCGCACGACGCGGT-GCACTCCTCGCGAAGATCCCAGTGGCTCGGTCGG------------CCGGCC
B159Csubfarcina --CACTGCATCGCGCACGACGCGGT-GCACTCCTCGCGAAGATCCCAGTGGCTCGGTCGG------------------
A154Cscalaris   --CACTGCATCGCGCACGACGCGGT-GCACTCCTCGCGAAGATCCCAGTGGCTCGGTCGG------------------
A292Cscalaris   --CACTGCATCGCGCACGACGCGGT-GCACTCCTCGCGAAGATCCCAGTGGCTCGGTCGG------------------
A469Cscalaris   --CACTGCATCGCGCACGACGCGGT-GCACTCCTCGCGAAGATCCCAGTGGCTCGGTCGG------------------
B178Cscalaris   --CACTGCATCGCGCACGACGCGGT-GCACTCCTCGCGAAGATCCCAGTGGCTCGGTCGG------------------
B321Csp         --CACTGCATCGCGCACGACGCGGT-GCACTCCTCGCGAAGATCCCAGTGGCTCGGTCGG------------------
B336Cdistenta   --CACTGCATCGCGCACGACGCGGT-GCACTCCTCGCGAAGATCCCAGTGGCTCGGTCGG------------------
B337Cdistenta   --CACTGCATCGCGCACGACGCGGT-GCACTCCTCGCGAAGATCCCAGTGGCTCGGTCGG------------------
B336Cscalaris   --CACTGCATCGCGCACGACGCGGT-GCACTCCTCGCGAASATCCCAGTGGCTCGGTCGG------------------
B104Cplatylobiu --CACTGCATCGCGCACGACGCGGT-GCACTCCTCGCGAAGATCCCAGTGGCTCGGTCGA------------------
A168Cplatylobiu --CACTGCATCGCGCACGACGCGGT-GCACTCCTCGCGAAGATCCCAGTGGCTCGGTCGA------------------
B149Cycongesta  --CACTGCATCGCGCACGACGCGGT-GCACTCCTCGCGAAGATCCCAGTGGCTCGGTCGG------------------
B152CconGesta   --CACTGCATCGCGCACGACGCGGT-GCACTCCTCGCGAAGATCCCAGTGGCTCGGTCGG------------------
B101Cretroflexa --CACTGCATCGCGCACGACGCGGT-GCACTCCTCGCGAAGATCCCAGTGGCTCGGTCGG------------------
A080Cretroflexa --CACTGCATCGCGCACGACGCGGT-GCACTCCTCGCGAAGATCCCAGTGGCTCGGTCGG------------------
A196Cretroflexa --CACTGCATCGCGCACGACGCGGT-GCACTCCTCGCGAAGATCCCAGTGGCTCGGTCGG------------------
A085Cretroflexa --CACTGCATCGCGCACGACGCGGT-GCACTCCTCGCGAAGATCCCAGTGGCTCGGTCGG------------------
B335Cconcesta   --CACTGCATCGCGCACGACGCGGT-GCACTCCTCGCGAAGATCCCAGTGGCTCGGTCGG------------------
B166Csubfarcina CTCACTGCATCGCGCACGACGCGGT-GCACTCCTCGCGAAGATCCCAGTGGCTCGGTCGG------------------
B116Cconcesta   --CACTGCATCGCGCACGACGCGGT-GCACTCCTCGCGAAGATCCCAGTGGCTCGGTCGG------------------
B115Cconcesta   --CACTGCATCGCGCACGACGCGGT-GCACTCCTCGCGAAGATCCCAGTGGCTCGGTCGG------------------
B121Cretroflexa --CACTGCATCGCGCACGACGCGGT-GCACTCCTCGCGAAGATCCCAGTGGCTCGGTCGG------------------

B174Lquercifoli --CCGGTCGGTCGGCCTGGAGCCAGGTTGTCCTCTTCCTCCCGCACCATCGTTATCTTTTGTTGCTTGCCGCCGC---
B090Cmoniliform GGTCGGTCGGTCGGTCTGGAGCCACAGTGTCCTCTTTCCCTTGCAACACCATTACTTTTCGTTGCCTGCCGCCGCCTT
B114Cexpansa    GGTCGGTCGGTCGGCCTGGAGCCGAAGTGTCCTCTTCCCCTTGCAACATTATTACTTTTCGTTGCATGCCGCCGC---
B109Cretorta    --TCGGTCGGTCGGCCTGGAGCCGGAGTGTCCTCTTCCCCTTGCAACATCATTA-TTTTCGTTGCCTGCCGCCGC---
B131Cretorta    --TCGGTCGGTCGGCCTGGAGCCGGAGTGTCCTCTTCCCCTTGCAACATCATTA-TTTTCGTTGCCTGCCGCCGC---
B415Cretroflexa --TCGGTCGGTCGGCCTGGAGCCGGAGTGTCCTCTTCCCCTTGCAACATCATTA-TTTTCGTTGCCTGCCGCCGC---
B118Csiliquosa  --TCGGTCGGTCGGCCTGGAGCCGGAGTGTCCTCTTCCCCTTGCAACATCATTA-TTTTCGTTGCCTGCCGCCGC---
B143Csiliquosa  --TCGGTCGGTCGGCCTGGAGCCGGAGTGTCCTCTTCCCCTTGCAACATCATTA-TTTTCGTTGCCTGCCGCCGC---
B100Csubfarcina --CCGGTCGGTCGGCCTGGAGCCGGAGTGTCCTCTTCCCCTCGCAACATCATTACTTTTCGTTGCCTGCCGCCGC---
B146Csubfarcina --CCGGTCGGTCGGCCTGGAGCCGGAGTGTCCTCTTCCCCTCGCAACATCATTACTTTTCGTTGCCTGCCGCCGC---
B153Csubfarcina --CCGGTCGGTCGGCCTGGAGCCGGAGTGTCCTCTTCCCCTCGCAACATCATTACTTTTCGTTGCCTGCCGCCGC---
B110Csubfarcina --CCGGTCGGTCGGCCTGGAGCCGGAGTGTCCTCTTCCCCTCGCAACATCATTACTTTTCGTTGCCTGCCGCCGC---
B127Csubfarcina --CCGGTCGGTCGGCCTGGAGCCGGAGTGTCCTCTTCCCCTCGCAACATCATTACTTTTCGTTGCCTGCCGCCGC---
B168Csubfarcina --CCGGTCGGTCGGCCTGGAGCCGGAGTGTCCTCTTCCCCTCGCAACATCATTACTTTTCGTTGCCTGCCGCCGC---
B136Csubfarcina --CCGGTCGGTCGGCCTGGAGCCGGAGTGTCCTCTTCCCCTCGCAACATCATTACTTTTCGTTGCCTGCCGCCGC---
B161Cretroflexa --CCGGTCGGTCGGCCTGGAGCCGGAGTGTCCTCTTCCCCTCGCAACATCATTACTTTTCGTTGCCTGCCGCCGC---
B103Ctorulosa   --CCGGTCGGTCGGCCTGGAGCCGGAGTGTCCTCTTCCCCTCGCAACATCATTACTTTTCGTTGCCTGCCGCCGC---
B125Ctorulosa   --CCGGTCGGTCGGCCTGGAGCCGGAGTGTCCTCTTCCCCTCGCAACATCATTACTTTTCGTTGCCTGCCGCCGC---
B179Ctorulosa   GGTCGGTCGGTCGGCCTGGAGCCGGAGTGTCCTCTTCCCCTCGCAACATCATTACTTTTCGTTGCCTGCCGCCGC---
B165Ctorulosa   --CCGGTCGGTCGGCCTGGAGCCGGAGTGTCCTCTTCCCCTCGCAACATCATTACTTTTCGTTGCCTGCCGCCGC---
B171Cpolycystid --CCGGTCGGTCGGCCTGGAGCCGGAGTGTCCTCTTCCCCTCGCAACATCATTACTTTTCGTTGCCTGCCGCCGC---
B112Cpolycystid --CCGGTCGGTCGGCCTGGAGCCGGAGTGTCCTCTTCCCCTCGCAACATCATTACTTTTCGTTGCCTGCCGCCGC---
B148Cpolycystid --CCGGTCGGTCGGCCTGGAGCCGGAGTGTCCTCTTCCCCTCGCAACATCATTACTTTTCGTTGCCTGCCGCCGC---
B111Cpolycystid --CCGGTCGGTCGGCCTGGAGCCGGAGTGTCCTCTTCCCCTCGCAACATCATTACTTTTCGTTGCCTGCCGCCGC---
B102Cmonilifera GGTCGGTCGGTCGGCCTGGAGCCGGAGTGTCCTCTTCCCCTCGCAACATCATTACTTTTCGTTGCCTGCCGCCGC---
B119Cmonilifera GGTCGGTCGGTCGGCCTGGAGCCGGAGTGTCCTCTTCCCCTCGCAACATCATTACTTTTCGTTGCCTGCCGCCGC---
B318Cmonilifera GGTCGGTCGGTCGGCCTGGAGCCGGAGTGTCCTCTTCCCCTCGCAACATCATTACTTTTCGTTGCCTGCCGCCGC---
B095CMONILIFERA GGTCGGTCGGTCGGCCTGGAGCCGGAGTGTCCTCTTCCCCTCGCAACATCATTACTTTTCGTTGCCTGCCGCCGC---
B092Cmonilifera GGTCGGTCGGTCGGCCTGGAGCCGGAGTGTCCTCTTCCCCTCGCAACATCATTACTTTTCGTTGCCTGCCGCCGC---
B159Csubfarcina --CCGGTCGGTCGGCCTGGAGCCGGAGTGTCCTCTTCCCCTCGCAACATCATTACTTTTCGTTGCCTGCCGCCGC---
A154Cscalaris   --CCGGTCGGTCGGCCTCGAGCTGGAGTGTCCTCTTCCCCTCGCAACATTATTACTTTTCGTTGCCTGCCGCCGC---
A292Cscalaris   --CCGGTCGGTCGGCCTCGAGCTGGAGTGTCCTCTTCCCCTCGCAACATTATTACTTTTCGTTGCCTGCCGCCGC---
A469Cscalaris   --CCGGTCGGTCGGCCTCGAGCTGGAGTGTCCTCTTCCCCTCGCAACATTATTACTTTTCGTTGCCTGCCGCCGC---
B178Cscalaris   --CCGGTCGGTCGGCCTCGAGCTGGAGTGTCCTCTTCCCCTCGCAACATTATTACTTTTCGTTGCCTGCCGCCGC---
B321Csp         --CCGGTCGGTCGGCCTCGAGCTGGAGTGTCCTCTTCCCCTCGCAACATTATTACTTTTCGTTGCCTGCCGCCGC---
B336Cdistenta   --CCGGTCGGTCGGCCTCGAGCTGGAGTGTCCTCTTCCCCTCGCAACATTATTACTTTTCGTTGCCTGCCGCCGC---
B337Cdistenta   --CCGGTCGGTCGGCCTCGAGCTGGAGTGTCCTCTTCCCCTCGCAACATTATTACTTTTCGTTGCCTGCCGCCGC---
B336Cscalaris   --CCGGTCGGTCGGCCTCGAGCTGGAGTGTCCTCTTCCCCTCGCAACATTATTACTTTTCGTTGCCTGCCGCCGC---
B104Cplatylobiu --TCGGTCGGTCGGCCTGCAGCCGGAGTGTCCTCTTCCCCTTGCAACATGATTA-TTTTCGTTGCCTGCCGCCGC---
A168Cplatylobiu --TCGGTCGGTCGGCCTGCAGCCGGAGTGTCCTCTTCCCCTTGCAACATGATTA-TTTTCGTTGCCTGCCGCCGC---
B149Cycongesta  --CCGGTCGGTCGGCCTGGAGCCGGAGTGTCCTCTTCCCCTCGCAACATCATTACTTTTCGTTGCCTGCCGCCGC---
B152Cconcesta   --CCGGTCGGTCGGCCTGGAGCCGGAGTGTCCTCTTCCCCTCGCAACATCATTACTTTTCGTTGCCTGCCGCCGC---
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Appendix 5. (Continued)

```
B101Cretroflexa --CCGGTCGGTCGGCCTGGAGCCGGAGTGTCCTCTTCCCCTCGCAACATCATTACTTTTCGTTGCCTGCCGCCGC---
A080Cretroflexa --CCGGTCGGTCGGCCTGGAGCCGGAGTGTCCTCTTCCCCTCGCAACATCATTACTTTTCGTTGCCTGCCGCCGC---
A196Cretroflexa --CCGGTCGGTCGGCCTGGAGCCGGAGTGTCCTCTTCCCCTCGCAACATCATTACTTTTCGTTGCCTGCCGCCGC---
A085Cretroflexa --CCGGTCGGTCGGCCTGGAGCCGGAGTGTCCTCTTCCCCTCGCAACATCATTACTTTTCGTTGCCTGCCGCCGC---
B335Ccongesta   --CCGGTCGGTCGGCCTGGAGCCGGAGTGTCCTCTTCCCCTCGCAACATCATTACTTTTCGTTGCCTGCCGCCGC---
B166Csubfarcina --CCGGTCGGTCGGCCTGGAGCCGGAGTGTCCTCTTCCCCTCGCAACATCATTACTTTTCGTTGCCTGCCGCCGC---
B116Ccongesta   --CCGGTCGGTCGGCCTGGAGCCGGAGTGTCCTCTTCCCCTCGCAACATCATTACTTTTCGTTGCCTGCCGCCGC---
B115Ccongesta   --CCGGTCGGTCGGCCTGGAGCCGGAGTGTCCTCTTCCCCTCGCAACATCATTACTTTTCGTTGCCTGCCGCCGC---
B121Cretroflexa --CCGGTCGGTCGGCCTGGAGCCGGAGTGTCCTCTTCCCCTCGCAACATCATTACTTTTCGTTGCCTGCCGCCGC---

B174Lquercifoli GCCGTCGTCCCG-AAAAGGTGATGGTGGCGGTCGCGAGCAGTGGCAGC---GATT----GTTGCGGGAGGGAGAGCGA
B090Cmoniliform GCCATCGTCCCC-AAAAGATGATGGTGGTGGTCGTGA----------------TTGGCTGTTGTGGGAGGGAGAGTGA
B114Cexpansa    GCCATCATCCCCCAAAAGGTGATGGTGGCGGTCGCGAGTGATGGAGA------TTAGCTGTTGTGGGAGGGAGAGGGA
B109Cretorta    GCCATCGTCCCC-AAAATATGATGGTGGCGGTCGCGAGTGATGGAGA------TTAGCTGTTGTGGGAGGGAGAGTGA
B131Cretorta    GCCATCGTCCCC-AAAATATGATGGTGGCGGTCGCGAGTGATGGAGA------TTAGCTGTTGTGGGAGGGAGAGTGA
B415Cretroflexa GCCATCGTCCCC-AAAATATGATGGTGGCGGTCGCGAGTGATGGAGA------TTAGCTGTTGTGGGAGGGAGAGTGA
B118Csiliquosa  GCCATCGTCCCC-AAAATATGATGGTGGCGGTCGCGAGTGATGGAGA------TTAGCTGTTGTGGGAGGGAGAGTGA
B143Csiliquosa  GCCATCGTCCCC-AAAATATGATGGTGGCGGTCGCGAGTGATGGAGA------TTAGCTGTTGTGGGAGGGAGAGTGA
B100Csubfarcina GCCATCG--------------ATGGTGTCGGTCGCGAGTGATGGAGA---TTATTAGCTGTTGTGGGAGGGAGAGTGA
B146Csubfarcina GCCATCG--------------ATGGTGTCGGTCGCGAGTGATGGAGA---TTATTAGCTGTTGTGGGAGGGAGAGTGA
B153Csubfarcina GCCATCG--------------ATGGTGTCGGTCGCGAGTGATGGAGA---TTATTAGCTGTTGTGGGAGGGAGAGTGA
B110Csubfarcina GCCATCG--------------ATGGTGTCGGTCGCGAGTGATGGAGA---TTATTAGCTGTTGTGGGAGGGAGAGTGA
B127Csubfarcina GCCATCG--------------ATGGTGTCGGTCGCGAGTGATGGAGA---TTATTAGCTGTTGTGGGAGGGAGAGTGA
B168Csubfarcina GCCATCG--------------ATGGTGTCGGTCGCGAGTGATGGAGA---TTATTAGCTGTTGTGGGAGGGAGAGTGA
B136Csubfarcina GCCATCG--------------ATGGTGTCGGTCGCGAGTGATGGAGA---TTATTAGCTGTTGTGGGAGGGAGAGTGA
B161Cretroflexa GCCATCG--------------ATGGTGTCGGTCGCGAGTGATGGAGA---TTATTAGCTGTTGTGGGAGGGAGAGTGA
B103Ctorulosa   GCCATCG--------------ATGGTGTCGGTCGCGAGTGATGGAGA---TTATTAGCTGTTGTGGGAGGGAGAGTGA
B125Ctorulosa   GCCATCG--------------ATGGTGTCGGTCGCGAGTGATGGAGA---TTATTAGCTGTTGTGGGAGGGAGAGTGA
B179Ctorulosa   GCCATCG--------------ATGGTGTCGGTCGCGAGTGATGGAGA---TTATTAGCTGTTGTGGGAGGGAGAGTGA
B165Ctorulosa   GCCATCG--------------ATGGTGTCGGTCGCGAGTGATGGAGA---TTATTAGCTGTTGTGGGAGGGAGAGTGA
B171Cpolycystid GCCATCGCCCCC-AAAATATGATGGTGGCGGTCGCGAGTGATGGAGA---TTATTAGCTGTTGTGGGAGGGAGAGTGA
B112Cpolycystid GCCATCGCCCCC-AAAATATGATGGTGGCGGTCGCGAGTGATGGAGA---TTATTAGCTGTTGTGGGAGGGAGAGTGA
B148Cpolycystid GCCATCGCCCCC-AAAATATGATGGTGGCGGTCGCGAGTGATGGAGA---TTATTAGCTGTTGTGGGAGGGAGAGTGA
B111Cpolycystid GCCATCGCCCCC-AAAATATGATGGTGGCGGTCGCGAGTGATGGAGA---TTATTAGCTGTTGTGGGAGGGAGAGTGA
B102Cmonilifera GCCATCGCCCCC-AAAATATGATGGTGGCGGTCGCGAGCGATGGAGA---TTATTAGCTGTTGTGGGAGGGAGAGTGA
B119Cmonilifera GCCATCGCCCCC-AAAATATGATGGTGGCGGTCGCGAGCGATGGAGA---TTATTAGCTGTTGTGGGAGGGAGAGTGA
B318Cmonilifera GCCATCGCCCCC-AAAATATGATGGTGGCGGTCGCGAGCGATGGAGA---TTATTAGCTGTTGTGGGAGGGAGAGTGA
B095CMONILIFERA GCCATCGCCCCC-AAAATATGATGGTGGCGGTCGCGAGCGATGGAGA---TTATTAGCTGTTGTGGGAGGGAGAGTGA
B092Cmonilifera GCCATCGCCCCC-AAAATATGATGGTGGCGGTCGCGAGCGATGGAGA---TTATTAGCTGTTGTGGGAGGGAGAGTGA
B159Csubfarcina GCCATCG--------------ATGGTGTCGGTCGCGAGTGATGGAGACTATTATTAGCTGTTGTGGGAGGGAGAGTGA
A154Cscalaris   GCCATCG--------------ATGGTGTCGGTCGCGAGTGATGGAGA---TTATTAGCTGTTGTGGGAGGGAGAGTGA
A292Cscalaris   GCCATCG--------------ATGGTGTCGGTCGCGAGTGATGGAGA---TTATTAGCTGTTGTGGGAGGGAGAGTGA
A469Cscalaris   GCCATCG--------------ATGGTGTCGGTCGCGAGTGATGGAGA---TTATTAGCTGTTGTGGGAGGGAGAGTGA
B178Cscalaris   GCCATCG--------------ATGGTGTCGGTCGCGAGTGATGGAGA---TTATTAGCTGTTGTGGGAGGGAGAGTGA
B321Csp         GCCATCG--------------ATGGTGTCGGTCGCGAGTGATGGAGA---TTATTAGCTGTTGTGGGAGGGAGAGTGA
B336Cdistenta   GCCATCG--------------ATGGTGTCGGTCGCGAGTGATGGAGA---TTATTAGCTGTTGTGGGAGGGAGAGTGA
B337Cdistenta   GCCATCG--------------ATGGTGTCGGTCGCGAGTGATGGAGA---TTATTAGCTGTTGTGGGAGGGAGAGTGA
B336Cscalaris   GCCATCG--------------ATGGTGTCGGTCGCGAGTGATGGAGA---TTATTAGCTGTTGTGGGAGGGAGAGTGA
B104Cplatylobiu ACCATCGTCCCC-GAAATATGACGGTGGCGGTCGCGAGTGGTGGAGA------TTAGCTGTTGCGGGAGGGAGAGTGA
A168Cplatylobiu ACCATCGTCCCC-AAAATATGACGGTGGCGGTCGCGAGTGGTGGAGA------TTAGCTGTTGCGGGAGGGAGAGTGA
B149Cycongesta  GCCATCG--------------ATGGTGTCGGTCGCGAGTGATGGAGACTATTATTAGCTGTTGTGGGAGGGAGAGTGA
B152Ccongesta   GCCATCG--------------ATGGTGTCGGTCGCGAGTGATGGAGACTATTATTAGCTGTTGTGGGAGGGAGAGTGA
B101Cretroflexa GCCATCG--------------ATGGTGTCGGTCGCGAGTGATGGAGACTATTATTAGCTGTTGTGGGAGGGAGAGTGA
A080Cretroflexa GCCATCG--------------ATGGTGTCGGTCGCGAGTGATGGAGACTATTATTAGCTGTTGTGGGAGGGAGAGTGA
A196Cretroflexa GCCATCG--------------ATGGTGTCGGTCGCGAGTGATGGAGACTATTATTAGCTGTTGTGGGAGGGAGAGTGA
A085Cretroflexa GCCATCG--------------ATGGTGTCGGTCGCGAGTGATGGAGACTATTATTAGCTGTTGTGGGAGGGAGAGTGA
B335Ccongesta   GCCATCG--------------ATGGTGTCGGTCGCGAGTGATGGAGACTATTATTAGCTGTTGTGGGAGGGAGAGTGA
B166Csubfarcina GCCATCG--------------ATGGTGTCGGTCGCGAGTGATGGAGA---TTATTAGCTGTTGTGGGAGGGAGAGTGA
B116Ccongesta   GCCATCG--------------ATGGTGTCGGTCGCGAGTGATGGAGACTATTATTAGCTGTTGTGGGAGGGAGAGTGA
B115Ccongesta   GCCATCG--------------ATGGTGTCGGTCGCGAGTGATGGAGACTATTATTAGCTGTTGTGGGAGGGAGAGTGA
B121Cretroflexa GCCATCG--------------ATGGTGTCGGTCGCGAGTGATGGAGACTATTATTAGCTGTTGTGGGAGGGAGAGTGA

B174Lquercifoli ATTCGAATGAATGGGCAAGTAACACGTCGCGGAGGT--CACGGCGTGGGGCCGGGCGCGGTCGGATC-CATTA-----
B090Cmoniliform ATTAGAATGAATGGGCAAGTAGCACGTCGTGGAGAT--CAGGGCGTGGGGCCGGGCGCGATCGGATC-----------
B114Cexpansa    GTTAGAATGAATGGGCAAGTAGCACGTCGTGGAGGT--CATGGCGTGGGGTCGGGCGCGATCG--------------
B109Cretorta    ATTAGAATGAATGGGCAAGTAGCTCGTCGCGGAGGT--CATGGCGTGGGGTCGGGCGCGATCGGATC-TACT------
B131Cretorta    ATTAGAATGAATGGGCAAGTAGCTCGTCGCGGAGGT--CATGGCGTGGGGTCGGGCGCGATCGGATC-TACT------
B415Cretroflexa ATTAGAATGAATGGGCAAGTAGCTCGTCGCGGAGGT--CATGGCGTGGGGTCGGGCGCGATCGGATC-TACT------
B118Csiliquosa  ATTAGAATGAATGGGCAAGTAGCTCGTCGCGGAGGT--CATGGCGTGGGGTCGGGCGCGATCGGATC-TACT------
B143Csiliquosa  ATTAGAATGAATGGGCAAGTAGCTCGTCGCGGAGGT--CATGGCGTGGGGTCGGGCGCGATCGGATC-TACT------
B100Csubfarcina ATTAGAATGAATGGGCAAGTAGCTCGTCGCGGAGGTGTCATGGCGTGGGGTCGGTCGCGATCGGATC-TACT------
B146Csubfarcina ATTAGAATGAATGGGCAAGTAGCTCGTCGCGGAGGTGTCATGGCGTGGGGTCGGTCGCGATCGGATC-TACT------
B153Csubfarcina ATTAGAATGAATGGGCAAGTAGCTCGTCGCGGAGGTGTCATGGCGTGGGGTCGGTCGCGATCGGATC-TACT------
B110Csubfarcina ATTAGAATGAATGGGCAAGTAGCTCGTCGCGGAGGTGTCATGGCGTGGGGTCGGTCGCGATCGGATC-TACT------
B127Csubfarcina ATTAGAATGAATGGGCAAGTAGCTCGTCGCGGAGGT--CATGGCGTGGGGTCGGTCGCGATCGGATC-TACT------
B168Csubfarcina ATTAGAATGAATGGGCAAGTAGCTCGTCGCGGAGGTGTCATGGCGTGGGGTCGGTCGCGATCGGATC-TACT------
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Appendix 5. (Continued)

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B136Csubfarcina ATTAGAATGAATGGGCAAGTAGCTCGTCGCGGAGGTGTCATGGCGTGGGGTCGGTCGCGATCGGATC-TACT------
B161Cretroflexa ATTAGAATGAATGGGCAAGTAGCTCGTCGCGGAGGTGTCATGGCGTGGGGTCGGTCGCGATCGGATC-TACT------
B103Ctorulosa   ATTAGAATGAATGGGCAAGTAGCTCGTCGCGGAGGT--CATGGCGTGGGGTCGGTCGCGATCGGATC-TACT------
B125Ctorulosa   ATTAGAATGAATGGGCAAGTAGCTCGTCGCGGAGGTGTCATGGCGTGGGGTCGGTCGCGATCGGATC-TACT------
B179Ctorulosa   ATTAGAATGAATGGGCAAGTAGCTCGTCGCGGAGGT--CATGGCGTGGGGTCGGTCGCGACCGGATC-TACT------
B165Ctorulosa   ATTAGAATGAATGGGCAAGTAGCTCGTCGCGGAGGT--CATGGCGTGGGGTCGGTCGCGATCGGATC-TACT------
B171Cpolycystid ATTAGAATGAATGGGCAAGTAGCTCGTCGCGGAGGT--CATGGCGTGGGGTCGGGCGCGATCGGATCCTACG------
B112Cpolycystid ATTAGAATGAATGGGCAAGTAGCTCGTCGCGGAGGT--CATGGCGTGGGGTCGGGCGCGATCGGATCCTACG------
B148Cpolycystid ATTAGAATGAATGGGCAAGTAGCTCGTCGCGGAGGT--CATGGCGTGGGGTCGGGCGCGATCGGATCCTACG------
B111Cpolycystid ATTAGAATGAATGGGCAAGTAGCTCGTCGCGGAGGT--CATGGCGTGGGGTCGGGCGCGATCGGATCCTACG------
B102Cmonilifera ATTAGAATGAATGGGCAAGTAGCTCGTCGCGGAGGT--CATGGCGTGGGGTCGGGCGCGATCGGATC-TACT------
B119Cmonilifera ATTAGAATGAATGGGCAAGTAGCTCGTCGCGGAGGT--CATGGCGTGGGGTCGGGCGCGATCGGATC-TACT------
B318Cmonilifera ATTAGAATGAATGGGCAAGTAGCTCGTCGCGGAGGT--CATGGCGTGGGGTCGGGCGCGATCGGATC-TACT------
B095CMONILIFERA ATTAGAATGAATGGGCAAGTAGCTCGTCGCGGAGGT--CATGGCGTGGGGTCGGGCGCGATCGGATC-TACT------
B092Cmonilifera ATTAGAATGAATGGGCAAGTAGCTCGTCGCGGAGGT--CATGGCGTGGGGTCGGGCGCGATCGGATC-TACT------
B159Csubfarcina ATTAGAATGAATGGGCAAGTAGCTCGTCGCGGAGGT--CATGGCGTGGGGTCGGTCGCGATCGGATC-TACT------
A154Cscalaris   ATTAGAATGAATGGGCAAGTAGCTCGTCGCGGAGGTGTCATGGCGTGGGGTCGGGCGCGATCGGATC-TACTACTGTT
A292Cscalaris   ATTAGAATGAATGGGCAAGTAGCTCGTCGCGGAGGTGTCATGGCGTGGGGTCGGGCGCGATCGGATC-TACTACTGTT
A469Cscalaris   ATTAGAATGAATGGGCAAGTAGCTCGTCGCGGAGGTGTCATGGCGTGGGGTCGGGCGCGATCGGATC-TACTACTGTT
B178Cscalaris   ATTAGAATGAATGGGCAAGTAGCTCGTCGCGGAGGTGTCATGGCGTGGGGTCGGGCGCGATCGGATC-TACTACTGTT
B321Csp         ATTAGAATGAATGGGCAAGTAGCTCGTCGCGGAGGTGTCATGGCGTGGGGTCGGGCGCGATCGGATC-TACTACTGTT
B336Cdistenta   ATTAGAATGAATGGGCAAGTAGCTCGTCGCGGAGGTGTCATGGCGTGGGGTCGGGCGCGATCGGATC-TACTACTGTT
B337Cdistenta   ATTAGAATGAATGGGCAAGTAGCTCGTCGCGGAGGTGTCATGGCGTGGGGTCGGGCGCGATCGGATC-TACTACTGTT
B336Cscalaris   ATTAGAATGAATGGGCAAGTAGCTCGTCGCGGAGGTGTCATGGCGTGGGGTCGGGCGCGATCGGATC-TACTACTGTT
B104Cplatylobiu ATTAGAATGAATGGGCAAGTAGCTCGTCGCGGAGGT--CATGGCGTGGGGTCGGGCGCGATCGGATC-----------
A168Cplatylobiu ATTAGAATGAATGGGCAAGTAGCTCGTCGCGGAGGT--CATGGCGTGGGGTCGGGCGCGATCGGATC-----------
B149Cycongesta  ATTAGAATGAATGGGCAAGTAGCTCGTCGCGGAGGT--CATGGCGTGGGGTCGGTCGCGATCGGATC-TACT------
B152Ccongesta   ATTAGAATGAATGGGCAAGTAGCTCGTCGCGGAGGT--CATGGCGTGGGGTCGGTCGCGATCGGATC-TACT------
B101Cretroflexa ATTAGAATGAATGGGCAAGTAGCTCGTCGCGGAGGT--CATGGCGTGGGGTCGGTCGCGATCGGATC-TACT------
A080Cretroflexa ATTAGAATGAATGGGCAAGTAGCTCGTCGCGGAGGT--CATGGCGTGGGGTCGGTCGCGATCGGATC-TACT------
A196Cretroflexa ATTAGAATGAATGGGCAAGTAGCTCGTCGCGGAGGT--CATGGCGTGGGGTCGGTCGCGATCGGATC-TACT------
A085Cretroflexa ATTAGAATGAATGGGCAAGTAGCTCGTCGCGGAGGT--CATGGCGTGGGGTCGGTCGCGATCGGATC-TACT------
B335Ccongesta   ATTAGAATGAATGGGCAAGTAGCTCGTCGCGGAGGT--CATGGCGTGGGGTCGGTCGCGATCGGATC-TACT------
B166Csubfarcina ATTAGAATGAATGGGCAAGTAGCTCGTCGCGGAGGTGTCATGGCGTGGGGTCGGTCGCGATCGGATC-TACT------
B116Ccongesta   ATTAGAATGAATGGGCAAGTAGCTCGTCGCGGAGGT--CATGGCGTGGGGTCGGTCGCGATCGGATC-TACT------
B115Ccongesta   ATTAGAATGAATGGGCAAGTAGCTCGTCGCGGAGGT--CATGGCGTGGGGTCGGTCGCGATCGGATC-TACT------
B121Cretroflexa ATTAGAATGAATGGGCAAGTAGCTCGTCGCGGAGGT--CATGGCGTGGGGTCGGTCGCGATCGGATC-TACT------

B174Lquercifoli --TGTGTCCGGTCGTGTGCGGCCTCAGTCGACCTTCTTGGCGGCGGCT---TGCTGGCGCGGTGGT---CGG------
B090Cmoniliform ---GTGTCTGGTCGTGGGCGGCCTCAGTCGACCTTGGCGGCGGCGGTT---CGCTGGCGCGGTGGTGGGCGGTGCCGG
B114Cexpansa    ----TGTCTGGTCGTGGGCGGCCYCAGTCGACCTTCGCGGCGGCG------CGCTGGCGCGGTGGTGGGCGGGGCGAG
B109Cretorta    GTTGTGTCTGGTCGTGGGCGGCCTTCGTCGACCTTCGCGGCGGCGGCG---CGCTGGCGCGTTGGTGGGTGGGGCGAG
B131Cretorta    GTTGTGTCTGGTCGTGGGCGGCCTTCGTCGACCTTCGCGGCGGCGGCG---CGCTGGCGCGTTGGTGGGTGGGGCGAG
B415Cretroflexa GTTGTGTCTGGTCGTGGGCGGCCTTCGTCGACCTTCGCGGCGGCGGCG---CGCTGGCGCGTTGGTGGGTGGGGCGAG
B118Csiliquosa  GTTGTGTCTGGTCGTGGGCGGCCTTCGTCGACCTTCGCGGCGGCGGCG---CGCTGGCGCGTTGGTGGGTGGGGCGAG
B143Csiliquosa  GTTGTGTCTGGTCGTGGGCGGCCTTCGTCGACCTTCGCGGCGGCGGCG---CGCTGGCGCGTTGGTGGGTGGGGCGAG
B100Csubfarcina GT--------GTCGTGGGCGGCCTCAGTCGTTCTTCGCGGCGGCGGCGGCGCGCTGGCGCGTTGGTGGGTGGGTTCAG
B146Csubfarcina GT--------GTCGTGGGCGGCCTCAGTCGTTCTTCGCGGCGGCGGCGGCGCGCTGGCGCGTTGGTGGGTGGGGCGAG
B153Csubfarcina GT--------GTCGTGGGCGGCCTCAGTCGTTCTTCGCGGCGGCGGCGGCGCGCTGGCGCGTTGGTGGGTGGGGCGAG
B110Csubfarcina GT--------GTCGTGGGCGGCCTCAGTCGTTCTTCGCGGCGGCGGCGGCGCGCTGGCGCGTTGGTGGGTGGGGCGAG
B127Csubfarcina GTTGTGTCTGGTCGTGGGCGGCCTCAGTCGTTCTTCGCGGCGGCGGCGGCGCGCTGGCGCGTTGGTGGGTGGGGCGAG
B168Csubfarcina GT--------GTCGTGGGCGGCCTCAGTCGTTCTTCGCGGCGGCGGCGGCGCGCTGGCGCGTTGGTGGGTGGGGCGAG
B136Csubfarcina GT--------GTCGTGGGCGGCCTCAGTCGTTCTTCGCGGCGGCGGCGGCGCGCTGGCGCGTTGGTGGGTGGGGCGAG
B161Cretroflexa GT--------GTCGTGGGCGGCCTCAGTCGTTCTTCGCGGCGGCGGCGGCGCGCTGGCGCGTTGGTGGGTGGGGCGAG
B103Ctorulosa   GTTGTGTCTGGTCGTGGGCGGCCTCAGTCGTTCTTCGCGGCGGCGGCGGCGCGCTGGCGCGTTGGTGGGTGGGGCGAG
B125Ctorulosa   GT--------GTCGTGGGCGGCCTCAGTCGTTCTTCGCGGCGGCGGCGGCGCGCTGGCGCGTTGGTGGGTGGGGCGAG
B179Ctorulosa   GTTGTGTCTGGTCGTGGGCGGCCTCAGTCGTTCTTCGCGGCGGCGGCGGCGCGCTGGCGCGTTGGTGGGTGGGGCGAG
B165Ctorulosa   GTTGTGTCTGGTCGTGGGCGGCCTCAGTCGTTCTTCGCGGCGGCGGCGGCGCGCTGGCGCGTTGGTGGGTGGGGCGAG
B171Cpolycystid GTTGTGTCTGGTCGTCGGCGGCCTCAGTCGGCCTTCGCGGCGGCGGCGGCGCGCTGGCGCGTTGGTGGGTGGGGCGAG
B112Cpolycystid GTTGTGTCTGGTCGTCGGCGGCCTCAGTCGGCCTTCGCGGCGGCGGCGGCGCGCTGGCGCGTTGGTGGGTGGGGCGAG
B148Cpolycystid GTTGTGTCTGGTCGTCGGCGGCCTCAGTCGGCCTTCGCGGCGGCGGCGGCGCGCTGGCGCGTTGGTGGGTGGGGCGAG
B111Cpolycystid GTTGTGTCTGGTCGTCGGCGGCCTCAGTCGGCCTTCGCGGCGGCGGCGGCGCGCTGGCGCGTTGGTGGGTGGGGCGAG
B102Cmonilifera GTTGTGTCTGGTCGTGGGCGGCCTCAGTCGACCTTCGCGGCGGCGGCGGCGCGCTGGCGCGTTGGTGGGTGGGGCGAG
B119Cmonilifera GTTGTGTCTGGTCGTGGGCGGCCTCAGTCGACCTTCGCGGCGGCGGCGGCGCGCTGGCGCGTTGGTGGGTGGGGCGAG
B318Cmonilifera GTTGTGTCTGGTCGTGGGCGGCCTCAGTCGACCTTCGCGGCGGCGGCGGCGCGCTGGCGCGTTGGTGGGTGGGGCGAG
B095CMONILIFERA GTTGTGTCTGGTCGTGGGCGGCCTCAGTCGACCTTCGCGGCGGCGGCGGCGCGCTGGCGCGTTGGTGGGTGGGGCGAG
B092Cmonilifera GTTGTGTCTGGTCGTGGGCGGCCTCAGTCGACCTTCGCGGCGGCGGCGGCGCGCTGGCGCGTTGGTGGGTGGGGCGAG
B159Csubfarcina GTTGTGTCTGGTCGTGGGCGGCCTCAGTCGTTCTTCGCGGCGGCGGCGGCGCGCTGGCGCGTTGGTGGGTGGGGCGAG
A154Cscalaris   GTTGTGTCTGGTCGTGGGCGGCCTCAGTCGGCCTTCGCGGCGGCGGCGGCGCGCTGGCGCGTTGGTGGGTGGGGCGAG
A292Cscalaris   GTTGTGTCTGGTCGTGGGCGGCCTCAGTCGGCCTTCGCGGCGGCGGCGGCGCGCTGGCGCGTTGGTGGGTGGGGCGAG
A469Cscalaris   GTTGTGTCTGGTCGTGGGCGGCCTCAGTCGGCCTTCGCGGCGGCGGCGGCGCGCTGGCGCGTTGGTGGGTGGGGCGAG
B178Cscalaris   GTTGTGTCTGGTCGTGGGCGGCCTCAGTCGGCCTTCGCGGCGGCGGCGGCGCGYTGGCGCGTTGGTGGGTGGGGCGAG
B321Csp         GTTGTGTCTGGTCGTGGGCGGCCTCAGTCGGCCTTCGCGGCGGCGGCGGCGCGCTGGCGCGTTGGTGGGTGGGGCGAG
B336Cdistenta   GTTGTGTCTGGTCGTGGGCGGCCTCAGTCGGCCTTCGCGGCGGCGGCGGCGCGCTGGCGCGTTGGTGGGTGGGGCGAG
B337Cdistenta   GTTGTGTCTGGTCGTGGGCGGCCTCAGTCGGCCTTCGCGGCGGCGGCGGCGCGCTGGCGCGTTGGTGGGTGGGGCGAG
B336Cscalaris   GTTGTGTCTGGTCGTGGGCGGCCTCAGTCGGCCTTCGCGGCGGCGGCGGCGCGCTGGCGCGTTGGTGGGTGGGGCGAG
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Appendix 5. (Continued)

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B104Cplatylobim ------TCTGGTCGTGGGCGGCCTTCGTCGACCTTCGCGGCGGCGGCG---CGCTGGCGCGTTGGTGGGTGGGGCGAG
A168Cplatylobiu ------TCTGGTCGTGGGCGGCCTTCGTCGACCTTCGCGGCGGCGGCG---CGCTGGCGCGTTGGTGGGTGGGGCGAG
B149Cycongesta  GTTGTGTCTGGTCGTGGGCGGCCTCAGTCGTTCTTCGCGGCGGCGGCGGCGCGCTGGCGCGTTGGTGGGTGGGGCGAG
B152Ccongesta   GTTGTGTCTGGTCGTGGGCGGCCTCAGTCGTTCTTCGCGGCGGCGGCGGCGCGCTGGCGCGTTGGTGGGTGGGGCGAG
B101Cretroflexa GTTGTGTCTGGTCGTGGGCGGCCTCAGTCGTTCTTCGCGGCGGCGGCGGCGCGCTGGCGCGTTGGTGGGTGGGGCGAG
A080Cretroflexa GTTGTGTCTGGTCGTGGGCGGCCTCAGTCGTTCTTCGCGGCGGCGGCGGCGCGCTGGCGCGTTGGTGGGTGGGGCGAG
A196Cretroflexa GTTGTGTCTGGTCGTGGGCGGCCTCAGTCGTTCTTCGCGGCGGCGGCGGCGCGCTGGCGCGTTGGTGGGTGGGGCGAG
A085Cretroflexa GTTGTGTCTGGTCGTGGGCGGCCTCAGTCGTTCTTCGCGGCGGCGGCGGCGCGCTGGCGCGTTGGTGGGTGGGGCGAG
B335Ccongesta   GTTGTGTCTGGTCGTGGGCGGCCTCAGTCGTTCTTCGCGGCGGCGGCGGCGCGCTGGCGCGTTGGTGGGTGGGGCGAG
B166Csubfarcina GT--------GTCGTGGGCGGCCTCAGTCGTTCTTCGCGGCGGCGGCGGCGCGCTGGCGCGTTGGTGGGTGGGGCGAG
B116Ccongesta   GTTGTGTCTGGTCGTGGGCGGCCTCAGTCGTTCTTCGCGGCGGCGGCGGCGCGCTGGCGCGTTGGTGGGTGGGGCGAG
B115Ccongesta   GTTGTGTCTGGTCGTGGGCGGCCTCAGTCGTTCTTCGCGGCGGCGGCGGCGCGCTGGCGCGTTGGTGGGTGGGGCGAG
B121Cretroflexa GTTGTGTCTGGTCGTGGGCGGCCTCAGTCGTTCTTCGCGGCGGCGGCGGCGCGCTGGCGCGTTGGTGGGTGGGGCGAG

B174Lquercifoli --------------ACGTGCGT----CCTGCTTCCT-----------GCCGCGTGCAGCAAGTAATCATTACCAGTTT
B090Cmoniliform GTACGCGTGTAGGCACGTGCGC----CCAACTTCCTCC----CCACCGCCGCGTGCAGGGAGCAAATATTAGCAGTTT
B114Cexpansa    GTACGCGCGTACGTACGTACGTGCTCCCAACTCCCTCC----TTCCCATCGCGTGCAGCGAGCAAATATTACCAGTGT
B109Cretorta    GTAGGCGTGTACGTACGTGCGC----CCAACTCCCTCC----TTTCCATCGCGTGCAGCGGGCRAAAATTACCAGTTT
B131Cretorta    GTAGGCGTGTACGTACGTGCGC----CCAACTCCCTCC----TTTCCATCGCGTGCAGCGGGCAAAAATTACCAGTTT
B415Cretroflexa GTAGGCGTGTACGTACGTGCGC----CCAACTCCCTCC----TTTCCATCGCGTGCAGCGAGCAAAAATTACCAGTTT
B118Csiliquosa  GTAGGCGTGTACGTACGTGCGC----CCAACTCCCTCC----TTTCCATCGCGTGCAGCGAGCAAAAATTACCAGTTT
B143Csiliquosa  GTAGGCGTGTACGTACGTGCGC----CCAACTCCCTCC----TTTCCATCGCGTGCAGCGAGCAAAAATTACCAGTTT
B100Csubfarcina GTAGGCGTGTACGTACGTGCGC----CCAACTCCCTCCTTC-TTTCCATCGCGTGCAGCGAGCAAAAATTACCAGTTT
B146Csubfarcina GTAGGCGTGTACGTACGTGCGC----CCAACTCCCTCCTTC-TTTCCATCGCGTGCAGCGAGCAAAAATTACCAGTTT
B153Csubfarcina GTAGGCGTGTACGTACGTGCGC----CCAACTCCCTCCTTC-TTTCCATCGCGTGCAGCGAGCAAAAATTACCAGTTT
B110Csubfarcina GTAGGCGTGTACGTACGTGCGC----CCAACTCCCTCCTTC-TTTCCATCGCGTGCAGCGAGCAAAAATTACCAGTTT
B127Csubfarcina GTAGGCGTGTACGTACGTGCGC----CCAACTCCCTCCTTC-TTTCCATCGCGTGCAGCGAGCAAAAATTACCAGTTT
B168Csubfarcina GTAGGCGTGTACGTACGTGCGC----CCAACTCCCTCCTTC-TTTCCATCGCGTGCAGCGAGCAAAAATTACCAGTTT
B136Csubfarcina GTAGGCGTGTACGTACGTGCGC----CCAACTCCCTCCTTC-TTACCATCGCGTGCAGCGAGCAAAAATTACCAGTTT
B161Cretroflexa GTAGGCGTGTACGTACGTGCGC----CCAACTCCCTCCTTC-TTTCCATCGCGTGCAGCGAGCAAAAATTACCAGTTT
B103Ctorulosa   GTAGGCGTGTACGTACGTGCGC----CCAACTCCCTCCTTC-TTTCCATCGCGTGCAGCGAGCAAAAATTACCAGTTT
B125Ctorulosa   GTAGGCGTGTACGTACGTGCGC----CCAACTCCCTCCTTC-TTTCCATCGCGTGCAGCGAGCAAAAATTACCAGTTT
B179Ctorulosa   GTAGGCGTGTACGTACGTGCGC----CCAACTCCCTCCTTC-TTTCCATCGCGTGCAGCGAGCAAAAATTACCAGTTT
B165Ctorulosa   GTAGGCGTGTACGTACGTGCGC----CCAACTCCCTCCTTC-TTTCCATCGCGTGCAGCGAGCAAAAATTACCAGTTT
B171Cpolycystid GTAGGCGTGTACGTACGTGCGC----CCAACTCCCTCC----TTTCCATCGCGTGCAGCGAGCAAAAATTACCAGTTT
B112Cpolycystid GTAGGCGTGTACGTACGTGCGC----CCAACTCCCTCC----TTTCCATCGCGTGCAGCGAGCAAAAATTACCAGTTT
B148Cpolycystid GTAGGCGTGTACGTACGTGCGC----CCAACTCCCTCC----TTTCCATCGCGTGCAGCGAGCAAAAATTACCAGTTT
B111Cpolycystid GTAGGCGTGTACGTACGTGCGC----CCAACTCCCTCC----TTTCCATCGCGTGCAGCGAGCAAAAATTACCAGTTT
B102Cmonilifera GTAGGCGTGTACGTACGTGCGC----CCAACTCCCTCC----TTTCCATCGCGTGCAGCGAGCAAAAATTACCAGTTT
B119Cmonilifera GTAGGCGTGTACGTACGTGCGC----CCAACTCCCTCC----TTTCCATCGCGTGCAGCGAGCAAAAATTACCAGTTT
B318Cmonilifera GTAGGCGTGTACGTACGTGCGC----CCAACTCCCTCC----TTTCCATCGCGTGCAGCGAGCAAAAATTACCAGTTT
B095CMONILIFERA GTAGGCGTGTACGTACGTGCGC----CCAACTCCCTCC----TTTCCATCGCGTGCAGCGAGCAAAAATTACCAGTTT
B092Cmonilifera GTAGGCGTGTACGTACGTGCGC----CCAACTCCCTCC----TTTCCATCGCGTGCAGCGAGCAAAAATTACCAGTTT
B159Csubfarcina GTAGGCGTGTACGTACGTGCGC----CCAACTCCCTCCTTC-TTTCCATCGCGTGCAGCGAGCAAATATTACCAGTTT
A154Cscalaris   GTAGGCGTGTACGTACGTGCGCGCGCCCAACTAACTCCCTCCTTTCCATCGCGTGCAGCGAGCAAAAATTACCAGTTT
A292Cscalaris   GTAGGCGTGTACGTACGTGCGCGCGCCCAACTAACTCCCTCCTTTCCATCGCGTGCAGCGAGCAAAAATTACCAGTTT
A469Cscalaris   GTAGGCGTGTACGTACGTGCGCGCGCCCAACTAACTCCCTCCTTTCCATCGCGTGCAGCGAGCAAAAATTACCAGTTT
B178Cscalaris   GTAGGCGTGTACGTACGTGCGCGCGCCCAACTAACTCCCTCCTTTCCATCGCGTGCAGCGAGCAAAAATTACCAGTTT
B321Csp         GTAGGCGTGTACGTACGTGCGCGCGCCCAACTAACTCCCTCCTTTCCATCGCGTGCAGCGAGCAAAAATTACCAGTTT
B336Cdistenta   GTAGGCGTGTACGTACGTGCGCGCGCCCAACTAACTCCCTCCTTTCCATCGCGTGCAGCGAGCAAAAATTACCAGTTT
B337Cdistenta   GTAGGCGTGTACGTACGTGCGCGCGCCCAACTAACTCCCTCCTTTCCATCGCGTGCAGCGAGCAAAAATTACCAGTTT
B336Cscalaris   GTAGGCGTGTACGTACGTGCGCGCGCCCAACTAACTCCCTCCTTTCCATCGCGTGCAGCGAGCAAAAATTACCAGTTT
B104Cplatylobiu GTAGGCCTGTACGTACGTGCGC----CCAACTCCCTCC----TCTCCATCGCGTGCAGCGAGCAAAAATTACCAGTTT
A168Cplatylobiu GTAGGCCTGTACGTACGTGCGC----CCAACTCCCTCC----TCTCCATCGCGTGCAGCGAGCAAAAATTACCAGTTT
B149Cycongesta  GTAGGCGTGTACGTACGTGCGC----CCAACTCCCTCCTTC-TTTCCATCGCGTGCAGCGAGCAAATATTACCAGTTT
B152Ccongesta   GTAGGCGTGTACGTACGTGCGC----CCAACTCCCTCCTTC-TTTCCATCGCGTGCAGCGAGCAAATATTACCAGTTT
B101Cretroflexa GTAGGCGTGTACGTACGTGCGC----CCAACTCCCTCCTTC-TTTCCATCGCGTGCAGCGAGCAAATATTACCAGTTT
A080Cretroflexa GTAGGCGTGTACGTACGTGCGC----CCAACTCCCTCCTTC-TTTCCATCGCGTGCAGCGAGCAAATATTACCAGTTT
A196Cretroflexa GTAGGCGTGTACGTACGTGCGC----CCAACTCCCTCCTTC-TTTCCATCGCGTGCAGCGAGCAAATATTACCAGTTT
A085Cretroflexa GTAGGCGTGTACGTACGTGCGC----CCAACTCCCTCCTTC-TTTCCATCGCGTGCAGCGAGCAAATATTACCAGTTT
B335Ccongesta   GTAGGCGTGTACGTACGTGCGC----CCAACTCCCTCCTTC-TTTCCATCGCGTGCAGCGAGCAAATATTACCAGTTT
B166Csubfarcina GTAGGCGTGTACGTACGTGCGC----CCAACTCCCTCCTTC-TTTCCATCGCGTGCAGCGAGCAAAAATTACCAGTTT
B116Ccongesta   GTAGGCGTGTACGTACGTGCGC----CCAACTCCCTCCTTC-TTTCCATCGCGTGCAGCGAGCAAATATTACCAGTTT
B115Ccongesta   GTAGGCGTGTACGTACGTGCGC----CCAACTCCCTCCTTC-TTTCCATCGCGTGCAGCGAGCAAATATTACCAGTTT
B121Cretroflexa GTAGGCGTGTACGTACGTGCGC----CCAACTCCCTCCTTC-TTTCCATCGCGTGCAGCGAGCAAATATTACCAGTTT

B174Lquercifoli ----CTCTGATCTGACGCGAGGGAGGGGGCGTGGCTGCCCGACGCGGTGGCTGCAGCCCCC-TCC---CGGA----GC
B090Cmoniliform GTTTCTCTGAACTGACCCGAGGGAGGGA-----------------GGGGGCTACAGGCCCC-TCCCCCCGGA----TC
B114Cexpansa    TTTTCTCTGAACTGTCCCGAGGGA--------------------GGGGGCTAC-GGCCCCCTCCTCCCGGA----GC
B109Cretorta    TTTTCTCTGGACTGACCCGAGGGA--------------------CGGGGGCTAC-GGCCCC-CCCTCCCGGA----GC
B131Cretorta    TTTTCTCTGGACTGACCCGAGGGA--------------------CGGGGGCTAC-GGCCCC-CCCTCCCGGA----GC
B415Cretroflexa TTTTCTCTGGACTGACCCGAGGGA--------------------GGGGGCTAC-GGCCCC-CCCTCCCGGA----GC
B118Csiliquosa  TTTTCTCTGGACTGACCCGAGGGA--------------------GGGGGCTAC-GGCCCC-CCCTCCCGGA----GC
B143Csiliquosa  TTTTCTCTGGACTGACCCGAGGGA--------------------GGGGGCTAC-GGCCCC-CCCTCCCGGA----GC
B100Csubfarcina TTTTCTCTGGACTGACCCGAAGGA--------------------GGGGGCTACAGGCCCC----TCCCGGAGCGGTC
B146Csubfarcina TTTTCTCTGGACTGACCCGAAGGA--------------------GGGGGCTACAGGCCCC----TCCCGGAGCGGTC
```

Appendix 5. (Continued)

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B153Csubfarcina TTTTCTCTGGACTGACCCGAAGGA--------------------GGGGGCTACAGGCCCC----TCCCGGAGCGGTC
B110Csubfarcina TTTTCTCTGGACTGACCCGAAGGA--------------------GGGGGCTACAGGCCCC----TCCCGGAGCGGTC
B127Csubfarcina TTTTCTCTGGACTGACCCGAAGGA--------------------GGGGGCTACAGGCCCC----TCCCGGAGCGGTC
B168Csubfarcina TTTTCTCTGGACTGACCCGAAGGA--------------------GGGGGCTACAGGCCCC----TCCCGGAGCGGTC
B136Csubfarcina TTTTCTCTGGACTGACCCGAAGGA--------------------GGGGGCTACAGGCCCC----TCCCGGAGCGGTC
B161Cretroflexa TTTTCTCTGGACTGACCCGAAGGA--------------------GGGGGCTACAGGCCCC----TCCCGGAGCGGTC
B103Ctorulosa   TTTTCTCTGGACTGACCCGAAGGA--------------------GGGGGCTACAGGCCCC----TCCCGGAGCGGTC
B125Ctorulosa   TTTTCTCTGGACTGACCCGAAGGA--------------------GGGGGCTACAGGCCCC----TCCCGGAGCGGTC
B179Ctorulosa   TTTTCTCTGGACTGACCCGAAGGA--------------------GGGGGCTACAGGCCCC----TCCCGGAGCGGTC
B165Ctorulosa   TTTTCTCTGGACTGACCCGAAGGA--------------------GGGGGCTACAGGCCCC----TCCCGGAGCGGTC
B171Cpolycystid TTTTCTCTGGACTGACCCGAGGGA--------------------GGGGGCTGCAGGCCCC----TCCCGGA----GC
B112Cpolycystid TTTTCTCTGGACTGACCCGAGGGA--------------------GGGGGCTGCAGGCCCC----TCCCGGA----GC
B148Cpolycystid TTTTCTCTGGACTGACCCGAGGGA--------------------GGGGGCTGCAGGCCCC----TCCCGGA----GC
B111Cpolycystid TTTTCTCTGGACTGACCCGAGGGA--------------------GGGGGCTGCAGGCCCC----TCCCGGA----GC
B102Cmonilifera TTTTCTCTGGACTGACCCGAGGGA--------------------GGGGGCTACAGGCCCC----TCCCGGA----GC
B119Cmonilifera TTTTCTCTGGACTGACCCGAGGGA--------------------GGGGGCTACAGGCCCC----TCCCGGA----GC
B318Cmonilifera TTTTCTCTGGACTGACCCGAGGGA--------------------GGGGGCTACAGGCCCC----TCCCGGA----GC
B095CMONILIFERA TTTTCTCTGGACTGACCCGAGGGA--------------------GGGGGCTACAGGCCCC----TCCCGGA----GC
B092Cmonilifera TTTTCTCTGGACTGACCCGAGGGA--------------------GGGGGCTACAGGCCCC----TCCCGGA----GC
B159Csubfarcina TTTTCTCTGGACTGACCCGAAGGA--------------------GGGGGCTACAGGCCCC----TCCCGGAGCGGTC
A154Cscalaris   TTTTCTCTGGACTGACCCGAGGGA--------------------GGGGGCTACAGGCCCC----TCCCGGA----GC
A292Cscalaris   TTTTCTCTGGACTGACCCGAGGGA--------------------GGGGGCTACAGGCCCC----TCCCGGA----GC
A469Cscalaris   TTTTCTCTGGACTGACCCGAGGGA--------------------GGGGGCTACAGGCCCC----TCCCGGA----GC
B178Cscalaris   TTTTCTCTGGACTGACCCGAGGGA--------------------GGGGGCTACAGGCCCC----TCCCGGA----GC
B321Csp         TTTTCTCTGGACTGACCCGAGGGA--------------------GGGGGCTACAGGCCCC----TCCCGGA----GC
B336Cdistenta   TTTTCTCTGGRCTGACCCGAGGGA--------------------GGGGGCTACAGGCCCC----TCCCGGA----GC
B337Cdistenta   TTTTCTCTGGACTGACCCGAGGGA--------------------GGGGGCTACAGGCCCC----TCCCGGA----GC
B336Cscalaris   TTTTCTCTGGACTGACCCGAGGGA--------------------GGGGGCTACAGGCCCC----TCCCGGA----GC
B104Cplatylobiu TTTTCTCCGGACTGCCCCGAGGGA--------------------CGGGGCTACAGGCCCC---CTCCCGGA----GC
A168Cplatylobiu TTTTCTCCGGACTGCCCCGAGGGA--------------------CGGGGCTACAGGCCCC---CTCCCGGA----GC
B149Cycongesta  TTTTCTCTGGACTGACCCGAAGGA--------------------GGGGGCTACAGGCCCC----TCCCGGAGCGGTC
B152CconGesta   TTTTCTCTGGACTGACCCGAAGGA--------------------GGGGGCTACAGGCCCC----TCCCGGAGCGGTC
B101Cretroflexa TTTTCTCTGGACTGACCCGAAGGA--------------------GGGGGCTACAGGCCCC----TCCCGGAGCGGTC
A080Cretroflexa TTTTCTCTGGACTGACCCGAAGGA--------------------GGGGGCTACAGGCCCC----TCCCGGAGCGGTC
A196Cretroflexa TTTTCTCTGGACTGACCCGAAGGA--------------------GGGGGCTACAGGCCCC----TCCCGGAGCGGTC
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B335Ccongesta   TTTTCTCTGGACTGACCCGAAGGA--------------------GGGGGCTACAGGCCCC----TCCCGGAGCGGTC
B166Csubfarcina TTTTCTCTGGACTGACCCGAAGGA--------------------GGGGGCTACAGGCCCC----TCCCGGAGCGGTC
B116Ccongesta   TTTTCTCTGGACTGACCCGAAGGA--------------------GGGGGCTACAGGCCCC----TCCCGGAGCGGTC
B115Ccongesta   TTTTCTCTGGACTGACCCGAAGGA--------------------GGGGGCTACAGGCCCC----TCCCGGAGCGGTC
B121Cretroflexa TTTTCTCTGGACTGACCCGAAGGA--------------------GGGGGCTACAGGCCCC----TCCCGGAGCGGTC

B174Lquercifoli GGTCCAATCTATAATTAGCGTAAAACTTTCAGCGACGGATGTCTTGGCTCCCACAACGATGAAGAACGCAGCGAAATG
B090Cmoniliform GGTCCAATCTTTAATTAGCGTAAAACTTTCAGCGACGGATGTCTTGGCTCCCACAACGATGAAGAACGCAGCGAAATG
B114Cexpansa    GGTCCAATCTCTAATTAGCGTAAAACTTTCAGCGACGGATGTCTTGGCTCCCACAACGATGAAGAACGCAGCGAAATG
B109Cretorta    GGTCCAATCTCTAATTAGCGTAAAACTTTCAGCGACGGATGTCTTGGCTCCCACAACGATGAAGAACGCAGCGAAATG
B131Cretorta    GGTCCAATCTCTAATTAGCGTAAAACTTTCAGCGACGGATGTCTTGGCTCCCACAACGATGAAGAACGCAGCGAAATG
B415Cretroflexa GGTCCAATCTCTAATTAGCGTAAAACTTTCAGCGACGGATGTCTTGGCTCCCACAACGATGAAGAACGCAGCGAAATG
B118Csiliquosa  GGTCCAATCTCTAATTAGCGTAAAACTTTCAGCGACGGATGTCTTGGCTCCCACAACGATGAAGAACGCAGCGAAATG
B143Csiliquosa  GGTCCAATCTCTAATTAGCGTAAAACTTTCAGCGACGGATGTCTTGGCTCCCACAACGATGAAGAACGCAGCGAAATG
B100Csubfarcina GGTCCAATCTCTAATTAGCGTAAAACTTTCAGCGACGGATGTCTTGGCTCCCACAACGATGAAGAACGCAGCGAAATG
B146Csubfarcina GGTCCAATCTCTAATTAGCGTAAAACTTTCAGCGACGGATGTCTTGGCTCCCACAACGATGAAGAACGCAGCGAAATG
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B127Csubfarcina GGTCCAATCTCTAATTAGCGTAAAACTTTCAGCGACGGATGTCTTGGCTCCCACAACGATGAAGAACGCAGCGAAATG
B168Csubfarcina GGTCCAATCTCTAATTAGCGTAAAACTTTCAGCGACGGATGTCTTGGCTCCCACAACGATGAAGAACGCAGCGAAATG
B136Csubfarcina GGTCCAATCTCTAATTAGCGTAAAACTTTCAGCGACGGATGTCTTGGCTCCCACAACGATGAAGAACGCAGCGAAATG
B161Cretroflexa GGTCCAATCTCTAATTAGCGTAAAACTTTCAGCGACGGATGTCTTGGCTCCCACAACGATGAAGAACGCAGCGAAATG
B103Ctorulosa   GGTCCAATCTCTAATTAGCGTAAAACTTTCAGCGACGGATGTCTTGGCTCCCACAACGATGAAGAACGCAGCGAAATG
B125Ctorulosa   GGTCCAATCTCTAATTAGCGTAAAACTTTCAGCGACGGATGTCTTGGCTCCCACAACGATGAAGAACGCAGCGAAATG
B179Ctorulosa   GGTCCAATCTCTAATTAGCGTAAAACTTTCAGCGACGGATGTCTTGGCTCCCACAACGATGAAGAACGCAGCGAAATG
B165Ctorulosa   GGTCCAATCTCTAATTAGCGTAAAACTTTCAGCGACGGATGTCTTGGCTCCCACAACGATGAAGAACGCAGCGAAATG
B171Cpolycystid GGTCCAATCTCTAATTAGCGTAAAACTTTCAGCGACGGATGTCTTGGCTCCCACAACGATGAAGAACGCAGCGAAATG
B112Cpolycystid GGTCCAATCTCTAATTAGCGTAAAACTTTCAGCGACGGATGTCTTGGCTCCCACAACGATGAAGAACGCAGCGAAATG
B148Cpolycystid GGTCCAATCTCTAATTAGCGTAAAACTTTCAGCGACGGATGTCTTGGCTCCCACAACGATGAAGAACGCAGCGAAATG
B111Cpolycystid GGTCCAATCTCTAATTAGCGTAAAACTTTCAGCGACGGATGTCTTGGCTCCCACAACGATGAAGAACGCAGCGAAATG
B102Cmonilifera GGTCCAATCTCTAATTAGCGTAAAACTTTCAGCGACGGATGTCTTGGCTCCCACAACGATGAAGAACGCAGCGAAATG
B119Cmonilifera GGTCCAATCTCTAATTAGCGTAAAACTTTCAGCGACGGATGTCTTGGCTCCCACAACGATGAAGAACGCAGCGAAATG
B318Cmonilifera GGTCCAATCTCTAATTAGCGTAAAACTTTCAGCGACGGATGTCTTGGCTCCCACAACGATGAAGAACGCAGCGAAATG
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B092Cmonilifera GGTCCAATCTCTAATTAGCGTAAAACTTTCAGCGACGGATGTCTTGGCTCCCACAACGATGAAGAACGCAGCGAAATG
B159Csubfarcina GGTCCAATCTCTAATTAGCGTAAAACTTTCAGCGACGGATGTCTTGGCTCCCACAACGATGAAGAACGCAGCGAAATG
A154Cscalaris   GGTCCAATCTCTAATTAGCGTAAAACTTTCAGCGACGGATGTCTTGGCTCCCACAACGATGAAGAACGCAGCGAAATG
A292Cscalaris   GGTCCAATCTCTAATTAGCGTAAAACTTTCAGCGACGGATGTCTTGGCTCCCACAACGATGAAGAACGCAGCGAAATG
A469Cscalaris   GGTCCAATCTCTAATTAGCGTAAAACTTTCAGCGACGGATGTCTTGGCTCCCACAACGATGAAGAACGCAGCGAAATG
B178Cscalaris   GGTCCAATCTCTAATTAGCGTAAAACTTTCAGCGACGGATGTCTTGGCTCCCACAACGATGAAGAACGCAGCGAAATG
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Appendix 5. (Continued)

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B321Csp          GGTCCAATCTCTAATTAGCGTAAAACTTTCAGCGACGGATGTCTTGGCTCCCACAACGATGAAGAACGCAGCGAAATG
B336Cdistenta    GGTCCAATCTCTAATTAGCGTAAAACTTTCAGCGACGGATGTCTTGGCTCCCACAACGATGAAGAACGCAGCGAAATG
B337Cdistenta    GGTCCAATCTCTAATTAGCGTAAAACTTTCAGCGACGGATGTCTTGGCTCCCACAACGATGAAGAACGCAGCGAAATG
B336Cscalaris    GGTCCAATCTCTAATTAGCGTAAAACTTTCAGCGACGGATGTCTTGGCTCCCACAACGATGAAGAACGCAGCGAAATG
B104Cplatylobiu GGTCCAATCTCTAATTAGCGTAAAACTTTCAGCGACGGATGTCTTGGCTCCCACAACGATGAAGAACGCAGCGAAATG
A168Cplatylobiu GGTCCAATCTCTAATTAGCGTAAAACTTTCAGCGACGGATGTCTTGGCTCCCACAACGATGAAGAACGCAGCGAAATG
B149Cycongesta   GGTCCAATCTCTAATTAGCGTAAAACTTTCAGCGACGGATGTCTTGGCTCCCACAACGATGAAGAACGCAGCGAAATG
B152Ccongesta    GGTCCAATCTCTAATTAGCGTAAAACTTTCAGCGACGGATGTCTTGGCTCCCACAACGATGAAGAACGCAGCGAAATG
B101Cretroflexa GGTCCAATCTCTAATTAGCGTAAAACTTTCAGCGACGGATGTCTTGGCTCCCACAACGATGAAGAACGCAGCGAAATG
A080Cretroflexa GGTCCAATCTCTAATTAGCGTAAAACTTTCAGCGACGGATGTCTTGGCTCCCACAACGATGAAGAACGCAGCGAAATG
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A085Cretroflexa GGTCCAATCTCTAATTAGCGTAAAACTTTCAGCGACGGATGTCTTGGCTCCYACAACGATGAAGAACGCAGCGAAATG
B335Ccongesta    GGTCCAATCTCTAATTAGCGTAAAACTTTCAGCGACGGATGTCTTGGCTCCCACAACGATGAAGAACGCAGCGAAATG
B166Csubfarcina GGTCCAATCTCTAATTAGCGTAAAACTTTCAGCGACGGATGTCTTGGCTCCCACAACGATGAAGAACGCAGCGAAATG
B116Ccongesta    GGTCCAATCTCTAATTAGCGTAAAACTTTCAGCGACGGATGTCTTGGCTCCCACAACGATGAAGAACGCAGCGAAATG
B115Ccongesta    GGTCCAATCTCTAATTAGCGTAAAACTTTCAGCGACGGATGTCTTGGCTCCCACAACGATGAAGAACGCAGCGAAATG
B121Cretroflexa GGTCCAATCTCTAATTAGCGTAAAACTTTCAGCGACGGATGTCTTGGCTCCCACAACGATGAAGAACGCAGCGAAATG

B174Lquercifoli CGATACGTCTTGCGACTTGCAGAATCCCGTGAATCATCGAAACTTTGAACGCACCTTGCGCTCCCGGGATATGCCTGG
B090Cmoniliform CGATACGTCTTGCGACTTGCAGAATCCCGTGAATCATCGAAACTTTGAACGCACCTTGCGCTCCCGGGATATGCCTGG
B114Cexpansa    CGATACGTCtTGCGACTTGCAGAATCCCGTGAATCATCGAAACTTTGAACGCACCTTGCGCTCCCGGGATATGCCTGG
B109Cretorta    CGATACGTCTTGCGACTTGCAGAATCCCGTGAATCATCGAAACTTTGAACGCACCTTGCGCTCCCGGGATATGCCTGG
B131Cretorta    CGATACGTCTTGCGACTTGCAGAATCCCGTGAATCATCGAAACTTTGAACGCACCTTGCGCTCCCGGGATATGCCTGG
B415Cretroflexa CGATACGTCTTGCGACTTGCAGAATCCCGTGAATCATCGAAACTTTGAACGCACCTTGCGCTCCCGGGATATGCCTGG
B118Csiliquosa  CGATACGTCTTGCGACTTGCAGAATCCCGTGAATCATCGAAACTTTGAACGCACCTTGCGCTCCCGGGATATGCCTGG
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B100Csubfarcina CGATACGTCTTGCGACTTGCAGAATCCCGTGAATCATCGAAACTTTGAACGCACCTTGCGCTCCCGGGATATGCCTGG
B146Csubfarcina CGATACGTCTTGCGACTTGCAGAATCCCGTGAATCATCGAAACTTTGAACGCACCTTGCGCTCCCGGGATATGCCTGG
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B136Csubfarcina CGATACGTCtTGCGACTTGCAGAATCCCGTGAATCATCGAAACTTTGAACGCACCTTGCGCTCCCGGGATATGCCTGG
B161Cretroflexa CGATACGTCTTGCGACTTGCAGAATCCCGTGAATCATCGAAACTTTGAACGCACCTTGCGCTCCCGGGATATGCCTGG
B103Ctorulosa   CGATACGTCTTGCGACTTGCAGAATCCCGTGAATCATCGAAACTTTGAACGCACCTTGCGCTCCCGGGATATGCCTGG
B125Ctorulosa   CGATACGTCTTGCGACTTGCAGAATCCCGTGAATCATCGAAACTTTGAACGCACCTTGCGCTCCCGGGATATGCCTGG
B179Ctorulosa   CGATACGTCTTGCGACTTGCAGAATCCCGTGAATCATCGAAACTTTGAACGCACCTTGCGCTCCCGGGATATGCCTGG
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B321Csp         CGATACGTCTTGCGACTTGCAGAATCCCGTGAATCATCGAAACTTTGAACGCACCTTGCGCTCCCGGGATATGCCTGG
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B335Ccongesta   CGATACGTCTTGCGACTTGCAGAATCCCGTGAATCATCGAAACTTTGAACGCACCTTGCGCTCCCGGGATATGCCTGG
B166Csubfarcina CGATACGTCTTGCGACTTGCAGAATCCCGTGAATCATCGAAACTTTGAACGCACCTTGCGCTCCCGGGATATGCCTGG
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B121Cretroflexa CGATACGTCTTGCGACTTGCAGAATCCCGTGAATCATCGAAACTTTGAACGCACCTTGCGCTCCCGGGATATGCCTGG

B174Lquercifoli GAGCATGCTTGTCGGGGAGGAGGAGGCGAAAAATCGCCCACCGCTTTGGAGT-------------------CCCTCCG
B090Cmoniliform GAGCATGCTTGTCGGGGAGGAGGAGGCGAAAAATCGCCCACCGCTTCGGAA-------------------CCCTCCG
B114Cexpansa    GAGCATGCTTGTCGGGGAGGAGGAGGCGAAAAATCGCCCACGGCCTTGGAGGGAGTTAATAAT-------CCCCTCCG
B109Cretorta    GAGCATGCTTGTCGGGGAGGAGGAGGCGAAAAATCGCCCACGGCCTTGGAGTGGGTTAATAAAAT-----CCCCTCCG
B131Cretorta    GAGCATGCTTGTCGGGGAGGAGGAGGCGAAAAATCGCCCACGGCCTTGGAGTGGGTTAATAAAAT-----CCCCTCCG
B415Cretroflexa GAGCATGCTTGTCGGGGAGGAGGAGGCGAAAAATCGCCCACGGCCTTGGAGTGGGTTAATAAAAT-----CCCCTCCG
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Appendix 5. (Continued)

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A292Cscalaris   GAGCATGCTTGTCGGGGAGGAGGAGGCGAAAAATCGCCCACGGTCTTGGAGTGAGTTAATAATATATTATCCCCTCCG
A469Cscalaris   GAGCATGCTTGTCGGGGAGGAGGAGGCGAAAAATCGCCCACGGTCTTGGAGTGAGTTAATAATATATTATCCCCTCCG
B178Cscalaris   GAGCATGCTTGTCGGGGAGGAGGAGGCGAAAAATCGCCCACGGTCTTGGAGTGAGTTAATAATATATTATCCCCTCCG
B321Csp         GAGCATGCTTGTCGGGGAGGAGGAGGCGAAAAATCGCCCACGGTCTTGGAGTGAGTTAATAATATATTATCCCCTCCG
B336Cdistenta   GAGCATGCTTGTCGGGGAGGAGGAGGCGAAAAATCGCCCACGGTCTTGGAGTGAGTTAATAATATATTATCCCCTCCG
B337Cdistenta   GAGCATGCTTGTCGGGGAGGAGGAGGCGAAAAATCGCCCACGGTCTTGGAGTGAGTTAATAATATATTATCCCCTCCG
B336Cscalaris   GAGCATGCTTGTCGGGGAGGAGGAGGCGAAAAATCGCCCACGGTCTTGGAGTGAGTTAATAATATATTATCCCCTCCG
B104Cplatylobiu GAGCATGCTTGTCGGGGAGGAGGAGGCGAAAAATCGCCCACGGCCTTGGAGTGGGTTA-TAATA-A-TATCCCCTCCG
A168Cplatylobiu GAGCATGCTTGTCGGGGAGGAGGAGGCGAAAAATCGCCCACGGCCTTGGAGTGGGTTA-TAATA-A-TATCCCCTCCG
B149Cycongesta  GAGCATGCTTGTCGGGGAGGAGGAGGCGAAAAATCGCCCACGGTCTTGGAGTGAGTTAATAATAT--TATCCCCTCCG
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B101Cretroflexa GAGCATGCTTGTCGGGGAGGAGGAGGCGAAAAATCGCCCACGGTCTTGGAGTGAGTTAATAATAT--TATCCCCTCCG
A080Cretroflexa GAGCATGCTTGTCGGGGAGGAGGAGGCGAAAAATCGCCCACGGTCTTGGAGTGAGTTAATAATAT--TATCCCCTCCG
A196Cretroflexa GAGCATGCTTGTCGGGGAGGAGGAGGCGAAAAATCGCCCACGGTCTTGGAGTGAGTTAATAATAT--TATCCCCTCCG
A085Cretroflexa GAGCATGCTTGTCGGGGAGGAGGAGGCGAAAAATCGCCCACGGTCTTGGAGTGAGTTAATAATAT--TATCCCCTCCG
B335Ccongesta   GAGCATGCTTGTCGGGGAGGAGGAGGCGAAAAATCGCCCACGGTCTTGGAGTGAGTTAATAATAT--TATCCCCTCCG
B166Csubfarcina GAGCATGCTTGTCGGGGAGGAGGAGGCGAAAAATCGCCCACGGTCTTGGAGTGAGTTAATAATAT--TATCCCCTCCG
B116Ccongesta   GAGCATGCTTGTCGGGGAGGAGGAGGCGAAAAATCGCCCACGGTCTTGGAGTGAGTTAATAATAT--TATCCCCTCCG
B115Ccongesta   GAGCATGCTTGTCGGGGAGGAGGAGGCGAAAAATCGCCCACGGTCTTGGAGTGAGTTAATAATAT--TATCCCCTCCG
B121Cretroflexa GAGCATGCTTGTCGGGGAGGAGGAGGCGAAAAATCGCCCACGGTCTTGGAGTGGGTTAATAATAT--TATCCCCTCCG

B174Lquercifoli AGGCGGTGGCGCGGACTCTGCGTGTTCCGGGGGACGA--------CGGAAGTACGTCGT----CCCCCGAGCTCACCC
B090Cmoniliform AGGCGGTGGCGCGGACTCTGCGTGTTCCGGGGGACGA--------CGAGAGTACGTCGT----ACCCTGAGTTCACCC
B114Cexpansa    AGGCGGTGGCGCGGACTCTGCGTGTTCCGGGGGACGAGACGACGACGAGAGTACGTCGTCGTTCCCCTGAGTTCACCC
B109Cretorta    AGGCGGTGGCGCGGACTCTGCGTGTCCCGGGGGACGAGACGACGACGAAAGTACGTCGT---TCCCCTGAGTTCACCC
B131Cretorta    AGGCGGTGGCGCGGACTCTGCGTGTCCCGGGGGACGAGACGACGACGAAAGTACGTCGT---TCCCCTGAGTTCACCC
B415Cretroflexa AGGCGGTGGCGCGGACTCTGCGTGTCCCGGGGGACGAGACGACGACGAAAGTACGTCGT---TCCCCTGAGTTCACCC
B118Csiliquosa  AGGCGGTGGCGCGGACTCTGCGTGTCCCGGGGGACGAGACGACGACGAAAGTACGTCGT---TCCCCTGAGTTCACCC
B143Csiliquosa  AGGCGGTGGCGCGGACTCTGCGTGTCCCGGGGGACGAGACGACGACGAAAGTACGTCGT---TCCCCTGAGTTCACCC
B100Csubfarcina AGGCGGTGGCGCGGACTCTGCGTGTCCCGGGGGACGAGACGACGACGAAAGTACGTCGT---TCCCCTGAGTTCACCC
B146Csubfarcina AGGCGGTGGCGCGGACTCTGCGTGTCCCGGGGGACGAGACGACGACGAAAGTACGTCGT---TCCCCTGAGTTCACCC
B153Csubfarcina AGGCGGTGGCGCGGACTCTGCGTGTCCCGGGGGACGAGACGACGACGAAAGTACGTCGT---TCCCCTGAGTTCACCC
B110Csubfarcina AGGCGGTGGCGCGGACTCTGCGTGTCCCGGGGGACGAGACGACGACGAAAGTACGTCGT---TCCCCTGAGTTCACCC
B127Csubfarcina AGGCGGTGGCGCGGACTCTGCGTGTCCCGGGGGACGAGACGACGACGAAAGTACGTCGT---TCCCCTGAGTTCACCC
B168Csubfarcina AGGCGGTGGCGCGGACTCTGCGTGTCCCGGGGGACGAGACGACGACGAAAGTACGTCGT---TCCCCTGAGTTCACCC
B136Csubfarcina AGGCGGTGGCGCGGACTCTGCGTGTCCCGGGGGACGAGACGACGACGAAAGTACGTCGT---TCCCCTGAGTTCACCC
B161Cretroflexa AGGCGGTGGCGCGGACTCTGCGTGTCCCGGGGGACGAGACGACGACGAAAGTACGTCGT---TCCCCTGAGTTCACCC
B103Ctorulosa   AGGCGGTGGCGCGGACTCTGCGTGTCCCGGGGGACGAGACGACGACGAAAGTACGTCGT---TCCCCTGAGTTCACCC
B125Ctorulosa   AGGCGGTGGCGCGGACTCTGCGTGTCCCGGGGGACGAGACGACGACGAAAGTACGTCGT---TCCCCTGAGTTCACCC
B179Ctorulosa   AGGCGGTGGCGCGGACTCTGCGTGTCCCGGGGGACGAGACGACGACGAAAGTACGTCGT---TCCCCTGAGTTCACCC
B165Ctorulosa   AGGCGGTGGCGCGGACTCTGCGTGTCCCGGGGGACGAGACGACGACGAAAGTACGTCGT---TCCCCTGAGTTCACCC
B171Cpolycystid AGGCGGTGGCGCGGACTCTGCGTGTTCCGGGGGACGAGACGGCGACGAAAGTACGTCGT---TCCCCTGAGTTCACCC
B112Cpolycystid AGGCGGTGGCGCGGACTCTGCGTGTTCCGGGGGACGAGACGGCGACGAAAGTACGTCGT---TCCCCTGAGTTCACCC
B148Cpolycystid AGGCGGTGGCGCGGACTCTGCGTGTTCCGGGGGACGAGACGGCGACGAAAGTACGTCGT---TCCCCTGAGTTCACCC
B111Cpolycystid AGGCGGTGGCGCGGACTCTGCGTGTTCCGGGGGACGAGACGGCGACGAAAGTACGTCGT---TCCCCTGAGTTCACCC
B102Cmonilifera AGGCGGTGGCGCGGACTCTGCGTGTCCCGGGGGACGAGACGACGACGAAAGTACGTCGT---TCCCCTGAGTTCACCC
B119Cmonilifera AGGCGGTGGCGCGGACTCTGCGTGTCCCGGGGGACGAGACGACGACGAAAGTACGTCGT---TCCCCTGAGTTCACCC
B318Cmonilifera AGGCGGTGGCGCGGACTCTGCGTGTCCCGGGGGACGAGACGACGACGAAAGTACGTCGT---TCCCCTGAGTTCACCC
B095CMONILIFERA AGGCGGTGGCGCGGACTCTGCGTGTCCCGGGGGACGAGACGACGACGAAAGTACGTCGT---TCCCCTGAGTTCACCC
B092Cmonilifera AGGCGGTGGCGCGGACTCTGCGTGTCCCGGGGGACGAGACGACGACGAAAGTACGTCGT---TCCCCTGAGTTCACCC
B159Csubfarcina AGGCGGTGGCGCGGACTCTGCGTGTTCCGGGGGACGAGACGACGACGAAAGTACGTCGT---TCCCCTGAGTTCACCC
```

Appendix 5. (Continued)

```
A154Cscalaris   AGGCGGTGGCGCGGACTCTGCGTGTTCCGGGGGACGAGACGACGACGAAAGTTCGTCGT---TCCCCTGAGTTCACCC
A292Cscalaris   AGGCGGTGGCGCGGACTCTGCGTGTTCCGGGGGACGAGACGACGACGAAAGTTCGTCGT---TCCCCTGAGTTCACCC
A469Cscalaris   AGGCGGTGGCGCGGACTCTGCGTGTTCCGGGGGACGAGACGACGACGAAAGTTCGTCGT---TCCCCTGAGTTCACCC
B178Cscalaris   AGGCGGTGGCGCGGACTCTGCGTGTTCCGGGGGACGAGACGACGACGAAAGTTCGTCGT---TCCCCTGAGTTCACCC
B321Csp         AGGCGGTGGCGCGGACTCTGCGTGTTCCGGGGGACGAGACGACGACGAAAGTTCGTCGT---TCCCCTGAGTTCACCC
B336Cdistenta   AGGCGGTGGCGCGGACTCTGCGTGTTCCGGGGGACGAGACGACGACGAAAGTTCGTCGT---TCCCCTGAGTTCACCC
B337Cdistenta   AGGCGGTGGCGCGGACTCTGCGTGTTCCGGGGGACGAGACGACGACGAAAGTTCGTCGT---TCCCCTGAGTTCACCC
B336Cscalaris   AGGCGGTGGCGCGGACTCTGCGTGTTCCGGGGGACGAGACGACGACGAAAGTTCGTCGT---TCCCCTGAGTTCACCC
B104Cplatylobiu AGGCGGTGGCGCGGACTCTGCGTGTCCCGGGGGACGA------------GTACGTCGT---TCCCCTGAGTTCACCC
A168Cplatylobiu AGGCGGTGGCGCGGACTCTGCGTGTCCCGGGGGACGA------------GTACGTCGT---TCCCCTGAGTTCACCC
B149Cycongesta  AGGCGGTGGCGCGGACTCTGCGTGTTCCGGGGGACGAGACGACGACGAAAGTACGTCGT---TCCCCTGAGTTCACCC
B152Ccongesta   AGGCGGTGGCGCGGACTCTGCGTGTTCCGGGGGACGAGACGACGACGAAAGTACGTCGT---TCCCCTGAGTTCACCC
B101Cretroflexa AGGCGGTGGCGCGGACTCTGCGTGTTCCGGGGGACGAGACGACGACGAAAGTACGTCGT---TCCCCTGAGTTCACCC
A080Cretroflexa AGGCGGTGGCGCGGACTCTGCGTGTTCCGGGGGACGAGACGACGACGAAAGTACGTCGT---TCCCCTGAGTTCACCC
A196Cretroflexa AGGCGGTGGCGCGGACTCTGCGTGTTCCGGGGGACGAGACGACGACGAAAGTACGTCGT---TCCCCTGAGTTCACCC
A085Cretroflexa AGGCGGTGGCGCGGACTCTGCGTGTTCCGGGGGACGAGACGACGACGAAAGTACGTCGT---TCCCCTGAGTTCACCC
B335Ccongesta   AGGCGGTGGCGCGGACTCTGCGTGTTCCGGGGGACGAGACGACGACGAAAGTACGTCGT---TCCCCTGAGTTCACCC
B166Csubfarcina AGGCGGTGGCGCGGACTCTGCGTGTCCCGGGGGACGAGACGACGACGAAAGTACGTCGT---TCCCCTGAGTTCACCC
B116Ccongesta   AGGCGGTGGCGCGGACTCTGCGTGTTCCGGGGGACGAGACGACGACGAAAGTACGTCGT---TCCCCTGAGTTCACCC
B115Ccongesta   AGGCGGTGGCGCGGACTCTGCGTGTTCCGGGGGACGAGACGACGACGAAAGTACGTCGT---TCCCCTGAGTTCACCC
B121Cretroflexa AGGCGGTGGCGCGGACTCTGCGTGTTCCGGGGGACGAGACGACGACGAAAGTACGTCGT---TCCCCTGAGTTCACCC

B174Lquercifoli AAGGCTGGAGGGCAACCGACCGGCCGGCCA----CGAGTTGCGGCG---CCGGAGGCGTTCGCTTCAAGGCCCCCCCC
B090Cmoniliform AAGGCTGGAGGGCAACCGACCGGCCGGCCGGCCACGAGTTGCGGCG---CTGGAGGCGTTCACTTCGA--TGCCCTCC
B114Cexpansa    AAGGCTGGAGGGCAACCGACCGACCGGCCGACCACGAGTCG------------AGGCGTTCGCTTCTAA-CGCCCTTC
B109Cretorta    AAGGCTGGAGGGCAACCGACCGGCCGGCCA----CGAGTTGCGGCG---CCGGAGGCGTTCGCTTCAA--CGCCCTTC
B131Cretorta    AAGGCTGGAGGGCAACCGACCGGCCGGCCA----CGAGTTGCGGCG---CCGGAGGCGTTCGCTTCAA--CGCCCTTC
B415Cretroflexa AAGGCTGGAGGGCAACCGACCGGCCGGCCA----CGAGTTGCGGCGGCGCCGGAGGCGTTCGCTTCAA--CGCCCTTC
B118Csiliquosa  AAGGCTGGAGGGCAACCGACCGGCCGGCCA----CGAGTTGCGGCGGCGCCGGAGGCGTTCGCTTCAA--CGCCCTTC
B143Csiliquosa  AAGGCTGGAGGGCAACCGACCGGCCGGCCA----CGAGTTGCGGCGGCGCCGGAGGCGTTCGCTTCAA--CGCCCTTC
B100Csubfarcina AAGGCTGGAGGGCAACCGACCGGCCGGCCA----CGAGTTGCGGCG---CCGGAGGCGTTCGCTTCAA--CGCCCTTC
B146Csubfarcina AAGGCTGGAGGGCAACCGACCGGCCGGCCA----CGAGTTGCGGCG---CCGGAGGCGTTCGCTTCAA--CGCCCTTC
B153Csubfarcina AAGGCTGGAGGGCAACCGACCGGCCGGCCA----CGAGTTGCGGCG---CCGGAGGCGTTCGCTTCAA--CGCCCTTC
B110Csubfarcina AAGGCTGGAGGGCAACCGACCGGCCGGCCA----CGAGTTGCGGCG---CCGGAGGCGTTCGCTTCAA--CGCCCTTC
B127Csubfarcina AAGGCTGGAGAGCAACCGACCGGCCGGCCA----CGAGTTGCGGCG---CCGGAGGCGTTCGCTTCAA--CGCCCTTC
B168Csubfarcina AAGGCTGGAGGGCAACCGACCGGCCGGCCA----CGAGTTGCGGCG---CCGGAGGCGTTCGCTTCAA--CGCCCTTC
B136Csubfarcina AAGGCTGGAGGGCAACCGACCGGCCGGCCA----CGAGTTGCGGCG---CCGGAGGCGTTCGCTTCAA--CGCCCTTC
B161Cretroflexa AAGGCTGGAGGGCAACCGACCGGCCGGCCA----CGAGTTGCGGCG---CCGGAGGCGTTCGCTTCAA--CGCCCTTC
B103Ctorulosa   AAGGCTGGAGAGCAACCGACCGGCCGGCCA----CGAGTTGCGGCG---CCGGAGGCGTTCGCTTCAA--CGCCCTTC
B125Ctorulosa   AAGGCTGGAGGGCAACCGACCGGCCGGCCA----CGAGTTGCGGCG---CCGGAGGCGTTCGCTTCAA--CGCCCTTC
B179Ctorulosa   AAGGCTGGAGAGCAACCGACCGGCCGGCCA----CGAGTTGCGGCG---CCGGAGGCGTTCGCTTCAA--CGCCCTTC
B165Ctorulosa   AAGGCTGGAGAGCAACCGACCGGCCGGCCA----CGAGTTGCGGCG---CCGGAGGCGTTCGCTTCAA--CGCCCTTC
B171Cpolycystid AAGGCTGGAGGGCAACCGACCGGCCGGCCT----CGAGTTGCGGCG---CCGGAGGCGTTCGCTTCAA--CGCCCTTC
B112Cpolycystid AAGGCTGGAGGGCAACCGACCGGCCGGCCT----CGAGTTGCGGCG---CCGGAGGCGTTCGCTTCAA--CGCCCTTC
B148Cpolycystid AAGGCTGGAGGGCAACCGACCGGCCGGCCT----CGAGTTGCGGCG---CCGGAGGCGTTCGCTTCAA--CGCCCTTC
B111Cpolycystid AAGGCTGGAGGGCAACCGACCGGCCGGCCT----CGAGTTGCGGCG---CCGGAGGCGTTCGCTTCAA--CGCCCTTC
B102Cmonilifera AAGGTTGGAGGGCAACCGACCGGCCGGCCG----CGAGTTGCGGCG---CCGGAGGCGTTCGCTTCAA--CGCCCTTC
B119Cmonilifera AAGGTTGGAGGGCAACCGACCGGCCGGCCG----CGAGTTGCGGCG---CCGGAGGCGTTCGCTTCAA--CGCCCTTC
B318Cmonilifera AAGGTTGGAGGGCAACCGACCGGCCGGCCG----CGAGTTGCGGCG---CCGGAGGCGTTCGCTTCAA--CGCCCTTC
B095CMONILIFERA AAGGTTGGAGGGCAACCGACCGGCCGGCCG----CGAGTTGCGGCG---CCGGAGGCGTTCGCTTCAA--CGCCCTTC
B092Cmonilifera AAGGTTGGAGGGCAACCGACCGGCCGGCCG----CGAGTTGCGGCG---CCGGAGGCGTTCGCTTCAA--CGCCCTTC
B159Csubfarcina AAGGCTGGAGGGCAACCGACCGGCCGGCCG----CGAGTTGCGGCG---CCAGAGGCGTTCGCTTCAA--CGCCCTTC
A154Cscalaris   AAGGCTGGAGGGCAACCGACCGGCCGGCCG----CGAGTTGCGGCG---CCAGAGGCGTTCGCTTCAA--CGCCCTTC
A292Cscalaris   AAGGCTGGAGGGCAACCGACCGGCCGGCCG----CGAGTTGCGGCG---CCAGAGGCGTTCGCTTCAA--CGCCCTTC
A469Cscalaris   AAGGCTGGAGGGCAACCGACCGGCCGGCCG----CGAGTTGCGGCG---CCAGAGGCGTTCGCTTCAA--CGCCCTTC
B178Cscalaris   AAGGCTGGAGGGCAACCGACCGGCCGGCCG----CGAGTTGCGGCG---CCAGAGGCGTTCGCTTCAA--CGCCCTTC
B321Csp         AAGGCTGGAGGGCAACCGACCGGCCGGCCG----CGAGTTGCGGCG---CCAGAGGCGTTCGCTTCAA--CGCCCTTC
B336Cdistenta   AAGGCTGGAGGGCAACCGACCGGCCGGCCG----CGAGTTGCGGCG---CCAGAGGCGTTCGCTTCAA--CGCCCTTC
B337Cdistenta   AAGGCTGGAGGGCAACCGACCGGCCGGCCG----CGAGTTGCGGCG---CCAGAGGCGTTCGCTTCAA--CGCCCTTC
B336Cscalaris   AAGGCTGGAGGGCAACCGACCGGCCGGCCG----CGAGTTGCGGCG---CCAGAGGCGTTCGCTTCAA--CGCCCTTC
B104Cplatylobiu AAGGCTGGAGGGCAACCGACCGGCCGGCCA----CGAGTTGCGGCG---CCTTAGGCGTTCGCTTCAA--CGCCCTTC
A168Cplatylobiu AAGGCTGGAGGGCAACCGACCGGCCGGCCA----CGAGTTGCGGCG---CCTTAGGCGTTCGCTTCAA--CGCCCTTC
B149Cycongesta  AAGGCTGGAGGGCAACCGACCGGCCGGCCG----CGAGTTGCGGCG---CCAGAGGCGTTCGCTTCAA--CGCCCTTC
B152Ccongesta   AAGGCTGGAGGGCAACCGACCGGCCGGCCG----CGAGTTGCGGCG---CCAGAGGCGTTCGCTTCAA--CGCCCTTC
B101Cretroflexa AAGGCTGGAGGGCAACCGACCGGCCGGCCG----CGAGTTGCGGCG---CCAGAGGCGTTCGCTTCAA--CGCCCTTC
A080Cretroflexa AAGGCTGGAGGGCAACCGACCGGCCGGCCG----CGAGTTGCGGCG---CCAGAGGCGTTCGCTTCAA--CGCCCTTC
A196Cretroflexa AAGGCTGGAGGGCAACCGACCGGCCGGCCG----CGAGTTGCGGCG---CCAGAGGCGTTCGCTTCAA--CGCCCTTC
A085Cretroflexa AAGGCTGGAGGGCAACCGACCGGCCGGCCG----CGAGTTGCGGCG---CCAGAGGCGTTCGCTTCAA--CGCCCTTC
B335Ccongesta   AAGGCTGGAGGGCAACCGACCGGCCGGCCG----CGAGTTGCGGCG---CCAGAGGCGTTCGCTTCAA--CGCCCTTC
B166Csubfarcina AAGGCTGGAGGGCAACCGACCGGCCGGCCA----CGAGTTGCGGCG---CCGGAGGCGTTCGCTTCAA--CGCCCTTC
B116Ccongesta   AAGGCTGGAGGGCAACCGACCGGCCGGCCG----CGAGTTGCGGCG---CCAGAGGCGTTCGCTTCAA--CGCCCTTC
B115Ccongesta   AAGGCTGGAGGGCAACCGACCGGCCGGCCG----CGAGTTGCGGCG---CCAGAGGCGTTCGCTTCAA--CGCCCTTC
B121Cretroflexa AAGGCTGGAGGGCAACCGACCGGCCGGCCG----CGAGTTGCGGCG---CCAGAGGCGTTCGCTTCAA--CGCCCTTC

B174Lquercifoli GGACGAGCCGTTGTTGACGGCGCCCCCTTCGCGGGGCGGGGACACGACGGGTCGCTCGGGGTG-GA-GCTTGGACGAG
B090Cmoniliform GGACGAGCCGTTGTTGACGGCGCCCCCTTCGCGGGGCGGGGACACGACGGGTCGCTCGGGGTGA---GCTTGGACGAG
```

Appendix 5. (Continued)

```
B114Cexpansa    GGACGAGCCGTTGTTGACGGCGCCCCCTTCGCGGGGCGGGGACACGACGGGTCGCTCGGGGTGGGAAGCTTAGAC---
B109Cretorta    GGACGAGCTGTTGTTGACGGCGCCCCCTTCGCGGGGCGGGGACACGACGGGTCGCTCGGGGTGAG------------
B131Cretorta    GGACGAGCTGTTGTTGACGGCGCCCCCTTCGCGGGGCGGGGACACGACGGGTCGCTCGGGGTGAG------------
B415Cretroflexa GGACGAGCTGTTGTTGACGGCGCCCCCTTCGCGGGGCGGGGACACGACGGGTCGCTCGGGGTGAG------------
B118Csiliquosa  GGACGAGCTGTTGTTGACGGCGCCCCCTTCGCGGGGCGGGGACACGACGGGTCGCTCGGGGTGAG------------
B143Csiliquosa  GGACGAGCTGTTGTTGACGGCGCCCCCTTCGCGGGGCGGGGACACGACGGGTCGCTCGGGGTGAG------------
B100Csubfarcina GGACGAGCTGTTGTTGACGGCGCCCCCTTCGCGGGGCGGGGACACGACGGGTCGCTCGGGGTGAT------------
B146Csubfarcina GGACGAGCTGTTGTTGACGGCGCCCCCTTCGCGGGGCGGGGACACGACGGGTCGCTCGGGGTGAT------------
B153Csubfarcina GGACGAGCTGTTGTTGACGGCGCCCCCTTCGCGGGGCGGGGACACGACGGGTCGCTCGGGGTGAT------------
B110Csubfarcina GGACGAGCTGTTGTTGACGGCGCCCCCTTCGCGGGGCGGGGACACGACGGGTCGCTCGGGGTGAT------------
B127Csubfarcina GGACGAGCTGTTGTTGACGGCGCCCCCTTCGCGGGGCGGGGACACGACGGGTCGCTCGGGGTGAT------------
B168Csubfarcina GGACGAGCTGTTGTTGACGGCGCCCCCTTCGCGGGGCGGGGACACGACGGGTCGCTCGGGGTGAT------------
B136Csubfarcina GGACGAGCTGTTGTTGACGGCGCCCCCTTCGCGGGGCGGGGACACGACGGGTCGCTCGGGGTGAT------------
B161Cretroflexa GGACGAGCTGTTGTTGACGGCGCCCCCTTCGCGGGGCGGGGACACGACGGGTCGCTCGGGGTGAT------------
B103Ctorulosa   GGACGAGCTGTTGTTGACGGCGCCCCCTTCGCGGGGCGGGGACACGACGGGTCGCTCGGGGTGAT------------
B125Ctorulosa   GGACGAGCTGTTGTTGACGGCGCCCCCTTCGCGGGGCGGGGACACGACGGGTCGCTCGGGGTGAT------------
B179Ctorulosa   GGACGAGCTGTTGTTGACGGCGCCCCCTTCGCGGGGCGGGGACACGACGGGTCGCTCGGGGTGAT------------
B165Ctorulosa   GGACGAGCTGTTGTTGACGGCGCCCCCTTCGCGGGGCGGGGACACGACGGGTCGCTCGGGGTGAT------------
B171Cpolycystid GGACGAGCTGTTGTTGACGGCGCCCCCTTCGCGGGGCGGGGACACGACGGGTCGCTCGGGGTGAT------------
B112Cpolycystid GGACGAGCTGTTGTTGACGGCGCCCCCTTCGCGGGGCGGGGACACGACGGGTCGCTCGGGGTGAT------------
B148Cpolycystid GGACGAGCTGTTGTTGACGGCGCCCCCTTCGCGGGGCGGGGACACGACGGGTCGCTCGGGGTGAT------------
B111Cpolycystid GGACGAGCTGTTGTTGACGGCGCCCCCTTCGCGGGGCGGGGACACGACGGGTCGCTCGGGGTGAT------------
B102Cmonilifera GGACGAGCTGTTGTTGACGGCGCCCCCTTCGCGGGGCGGGGACACGACGGGTCGCTCGGGGTGAT------------
B119Cmonilifera GGACGAGCTGTTGTTGACGGCGCCCCCTTCGCGGGGCGGGGACACGACGGGTCGCTCGGGGTGAT------------
B318Cmonilifera GGACGAGCTGTTGTTGACGGCGCCCCCTTCGCGGGGCGGGGACACGACGGGTCGCTCGGGGTGAT------------
B095CMONILIFERA GGACGAGCTGTTGTTGACGGCGCCCCCTTCGCGGGGCGGGGACACGACGGGTCGCTCGGGGTGAT------------
B092Cmonilifera GGACGAGCTGTTGTTGACGGCGCCCCCTTCGCGGGGCGGGGACACGACGGGTCGCTCGGGGTGAT------------
B159Csubfarcina GGACGAGCTGTTGTTGACGGCGCCCCCTTCGCGGGGCGGGGACACGACGGGTCGCTCGGGGTGAT------------
A154Cscalaris   GGACGAGCTGTTGTTGACGGCGCCCCCTTCGCGGGGCGGGGACACGACGGGTCGCTCGGGGTGAT------------
A292Cscalaris   GGACGAGCTGTTGTTGACGGCGCCCCCTTCGCGGGGCGGGGACACGACGGGTCGCTCGGGGTGAT------------
A469Cscalaris   GGACGAGCTGTTGTTGACGGCGCCCCCTTCGCGGGGCGGGGACACGACGGGTCGCTCGGGGTGAT------------
B178Cscalaris   GGACGAGCTGTTGTTGACGGCGCCCCCTTCGCGGGGCGGGGACACGACGGGTCGCTCGGGGTGAT------------
B321Csp         GGACGAGCTGTTGTTGACGGCGCCCCCTTCGCGGGGCGGGGACACGACGGGTCGCTCGGGGTGAT------------
B336Cdistenta   GGACGAGCTGTTGTTGACGGCGCCCCCTTCGCGGGGCGGGGACACGACGGGTCGCTCGGGGTGAT------------
B337Cdistenta   GGACGAGCTGTTGTTGACGGCGCCCCCTTCGCGGGGCGGGGACACGACGGGTCGCTCGGGGTGAT------------
B336Cscalaris   GGACGAGCTGTTGTTGACGGCGCCCCCTTCGCGGGGCGGGGACACGACGGGTCGCTCGGGGTGAT------------
B104Cplatylobiu GGACGAGCTGTTGTTGACGGCGCCCCCTTCGCGGGGCGGGGACACGACGGGTCGCTCGGGGTGAG------------
A168Cplatylobiu GGACGAGCTGTTGTTGACGGCGCCCCCTTCGCGGGGCGGGGACACGACGGGTCGCTCGGGGTGAG------------
B149Cycongesta  GGACGAGCTGTTGTTGACGGCGCCCCCTTCGCGGGGCGGGGACACGACGGGTCGCTCGGGGTGAT------------
B152Cconcesta   GGACGAGCTGTTGTTGACGGCGCCCCCTTCGCGGGGCGGGGACACGACGGGTCGCTCGGGGTGAT------------
B101Cretroflexa GGACGAGCTGTTGTTGACGGCGCCCCCTTCGCGGGGCGGGGACACGACGGGTCGCTCGGGGTGAT------------
A080Cretroflexa GGACGAGCTGTTGTTGACGGCGCCCCCTTCGCGGGGCGGGGACACGACGGGTCGCTCGGGGTGAT------------
A196Cretroflexa GGACGAGCTGTTGTTGACGGCGCCCCCTTCGCGGGGCGGGGACACGACGGGTCGCTCGGGGTGAT------------
A085Cretroflexa GGACGAGCTGTTGTTGACGGCGCCCCCTTCGCGGGGCGGGGACACGACGGGTCGCTCGGGGTGAT------------
B335Cconcesta   GGACGAGCTGTTGTTGACGGCGCCCCCTTCGCGGGGCGGGGACACGACGGGTCGCTCGGGGTGAT------------
B166Csubfarcina GGACGAGCTGTTGTTGACGGCGCCCCCTTCGCGGGGCGGGGACACGACGGGTCGCTCGGGGTGAT------------
B116Cconcesta   GGACGAGCTGTTGTTGACGGCGCCCCCTTCGCGGGGCGGGGACACGACGGGTCGCTCGGGGTGAT------------
B115Cconcesta   GGACGAGCTGTTGTTGACGGCGCCCCCTTCGCGGGGCGGGGACACGACGGGTCGCTCGGGGTGAT------------
B121Cretroflexa GGACGAGCTGTTGTTGACGGCGCCCCCTTCGCGGGGCGGGGACACGACGGGTCGCTCGGGGTGAT------------

B174Lquercifoli GACAGCGTCGCTCGATCGCTC----------------GAGGGCCGGAAGGGAGGTAGGCTCGTGA----CGAC-GA--
B090Cmoniliform GACAGCGTCGCTCGATCGCTCGATCGCTCGATCGCTCGAGGGCCGGAAGGGAGGTAGGCTCGTG-----CGACT----
B114Cexpansa    ------------------------------------GAGG-CCGGAAGGGAGGTAGGCTCGTGA----GGACTG---
B109Cretorta    ------------------------------------GAGGGCCGGAAGGGAGGTAAGCTCGTGA----GGACTG---
B131Cretorta    ------------------------------------GAGGGCCGGAAGGGAGGTAAGCTCGTGA----GGACTG---
B415Cretroflexa ------------------------------------GAGGGCCGGAAGGGAGGTAAGCTCGTGA----GGACTG---
B118Csiliquosa  ------------------------------------GAGGGCCGGAAGGGAGGTAAGCTCGTGA----GGACTG---
B143Csiliquosa  ------------------------------------GAGGGCCGGAAGGGAGGTAAGCTCGTGA----GGACTG---
B100Csubfarcina ------------------------------------GAGGGCCGGAAGGGAGGTAGGCTCGTGA----GGACTGACT
B146Csubfarcina ------------------------------------GAGGGCCGGAAGGGAGGTAGGCTCGTGA----GGACTGACT
B153Csubfarcina ------------------------------------GAGGGCCGGAAGGGAGGTAGGCTCGTGA----GGACTGACT
B110Csubfarcina ------------------------------------GAGGGCCGGAAGGGAGGTAGGCTCGTGA----GGACTGACT
B127Csubfarcina ------------------------------------GAGGGCCGGAAGGGAGGTAGGCTCGTGA----GGACTGACT
B168Csubfarcina ------------------------------------GAGGGCCGGAAGGGAGGTAGGCTCGTGA----GGACTGACT
B136Csubfarcina ------------------------------------GAGGGCCGGAAGGGAGGTAGGCTCGTGA----GGACTGACT
B161Cretroflexa ------------------------------------GAGGGCCGGAAGGGAGGTAGGCTCGTGA----GGACTGACT
B103Ctorulosa   ------------------------------------GAGGGCCGGAAGGGAGGTAGGCTCGTGA----GGACTGACT
B125Ctorulosa   ------------------------------------GAGGGCCGGAAGGGAGGTAGGCTCGTGA----GGACTGACT
B179Ctorulosa   ------------------------------------GAGGGCCGGAAGGGAGGTAGGCTCGTGA----GGACTGACT
B165Ctorulosa   ------------------------------------GAGGGCCGGAAGGGAGGTAGGCTCGTGA----GGACTGACT
B171Cpolycystid ------------------------------------GAGGGCCGGAAGGGAGGTAGGCTCGTGA----GGACTGA--
B112Cpolycystid ------------------------------------GAGGGCCGGAAGGGAGGTAGGCTCGTGA----GGACTGA--
B148Cpolycystid ------------------------------------GAGGGCCGGAAGGGAGGTAGGCTCGTGA----GGACTGA--
B111Cpolycystid ------------------------------------GAGGGCCGGAAGGGAGGTAGGCTCGTGA----GGACTGA--
B102Cmonilifera ------------------------------------GAGGGCCGGAAGGGAGGTAGGCTCGTGA----GGACTGA--
B119Cmonilifera ------------------------------------GAGGGCCGGAAGGGAGGTAGGCTCGTGA----GGACTGA--
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Appendix 5. (Continued)

```
B318Cmonilifera ------------------------------------GAGGGCCGGAAGGGAGGTAGGCTCGTGA----GGACTGA--
B095CMONILIFERA ------------------------------------GAGGGCCGGAAGGGAGGTAGGCTCGTGA----GGACTGA--
B092Cmonilifera ------------------------------------GAGGGCCGGAAGGGAGGTAGGCTCGTGA----GGACTGA--
B159Csubfarcina ------------------------------------GACGGCCGGAAGGGAGGTAGGCTCGTGAAAAAGGACTGA--
A154Cscalaris   ------------------------------------GACGGCCGGAAGGGAGGTAGGCTCGTGAAAAAGGACTGA--
A292Cscalaris   ------------------------------------GACGGCCGGAAGGGAGGTAGGCTCGTGAAAAAGGACTGA--
A469Cscalaris   ------------------------------------GACGGCCGGAAGGGAGGTAGGCTCGTGAAAAAGGACTGA--
B178Cscalaris   ------------------------------------GACGGCCGGAAGGGAGGTAGGCTCGTGAAAAAGGACTGA--
B321Csp         ------------------------------------GACGGCCGGAAGGGAGGTAGGCTCGTGAAAAAGGACTGA--
B336Cdistenta   ------------------------------------GACGGCCGGAAGGGAGGTAGGCTCGTGAAAAAGGACTGA--
B337Cdistenta   ------------------------------------GACGGCCGGAAGGGAGGTAGGCTCGTGAAAAAGGACTGA--
B336Cscalaris   ------------------------------------GACGGCCGGAAGGGAGGTAGGCTCGTGAAAAAGGACTGA--
B104Cplatylobiu ------------------------------------GAGGGCCGGAAGGGAGGTAGGCTCGTGA----GGACTGT--
A168Cplatylobiu ------------------------------------GAGGGCCGGAAGGGAGGTAGGCTCGTGA----GGACTGT--
B149Cycongesta  ------------------------------------GACGGCCGGAAGGGAGGTAGGCTCGTGAAAAAGGACTGA--
B152Ccongesta   ------------------------------------GACGGCCGGAAGGGAGGTAGGCTCGTGAAAAAGGACTGA--
B101Cretroflexa ------------------------------------GACGGCCGGAAGGGAGGTAGGCTCGTGAAAAAGGACTGA--
A080Cretroflexa ------------------------------------GACGGCCGGAAGGGAGGTAGGCTCGTGAAAAAGGACTGA--
A196Cretroflexa ------------------------------------GACGGCCGGAAGGGAGGTAGGCTCGTGAAAAAGGACTGA--
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B092Cmonilifera   -CTTCTT-----------------------------AACGAACGACGACGCCATACCCCCGAT
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```

7.6 Appendix 6. Alignment of mitochondrial spacer sequences from *Cystophora retroflexa* and related species.

```
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Appendix 6. (Continued)

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B455Ccongesta   TTTGTGAAAGTAGTTTACCTACAGTATAAGAGAAATTGTTTTCACAAAAAGTTAAG
```

7.7 Appendix 7. Additional work.

During the period of work on this thesis I have also contributed to the following published papers:

ZUCCARELLO, G. C., BUCHANAN, J. & WEST, J. A. (2006). Increased sampling for inferring phylogeographic patterns in *Bostrychia radicans/B. moritziana* (Rhodomeleaceae, Rhodophyta) in the eastern USA. *Journal of Phycology, 42,* 1349–1352.

HODGE, F., BUCHANAN, J. & ZUCCARELLO, G. C. (2010). Hybridisation between the endemic brown algae *Carpophyllum maschalocarpum* and *Carpophyllum angustifolium* (Fucales): Genetic and morphological evidence. *Phycological Research, 58,* 239–247.

JONES (KAIN), J., BUCHANAN, J., BOO, S. M. & LEE, K. M. (2010). *Colpomenia bullosa* crust masquerading as *Ralfsia verrucosa* in southeast Australia. *Phycologia, 49,* 617–627.

ZUCCARELLO, G. C., BUCHANAN, J., WEST, J. A. & PEDROCHE, F. F. (2011). Genetic diversity of the mangrove associated alga *Bostrychia moritziana* (Ceramiales, Rhodophyta) from southern Central America. *Phycological Research*. doi: 10.1111/j.1440-1835.2010.00605.x

7.8 Appendix 8

Phylogeography of *Carpophyllum maschalocarpum*

Joe Buchanan & G. C. Zuccarello
School of Biological Sciences, Victoria University of Wellington

A highly variable 225 bp mitochondrial spacer region from 651 samples of the endemic fucoid alga *Carpophyllum maschalocarpum* was analysed by SSCP or sequencing. We identified 52 haplotypes, and found a further 15 haplotypes in the sister species *Carpophyllum angustifolium* and in hybrids between these species (Fig. 1).

1. **Forty-one *C. maschalocarpum* haplotypes were restricted to a single population. Most other haplotypes were restricted to regions.**
2. **Haplotypic diversity was low in southern populations.**
3. **High diversity in three far north populations arises from hybridisation with *C. angustifolium*.**
4. **Connectivity was strong between East Coast and Northland populations and between the mainland and the Chatham Islands.**
5. **High diversity was also found in the Chatham Islands.**

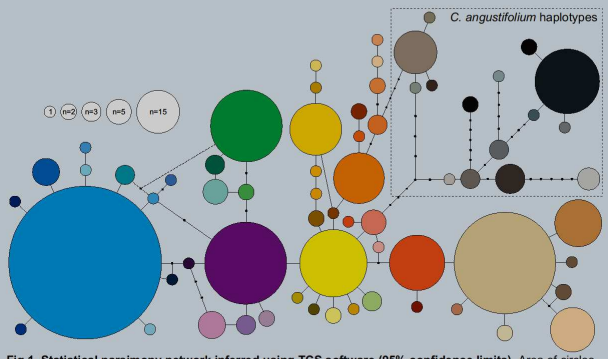


Fig 1. Statistical parsimony network inferred using TCS software (95% confidence limits). Area of circles is proportional to number of haplotypes sampled. Small black circles represent missing haplotypes.

1. Population differentiation and dispersal limitation

Population differentiation between populations with shared haplotypes was moderate to strong at distances over 50 kilometres (see Table 1 below), especially where there are habitat discontinuities. Despite large numbers of *C. maschalocarpum* thalli observed in drift (Kingsford 1992), gene flow is low. At several sites, all or most haplotypes sampled were restricted to that population.

Table 1. Pairwise Φst estimates from mtDNA spacer data (below diagonal) and minimum round coast distances (above diagonal) for Bay of Plenty populations of *Carpophyllum maschalocarpum*.

	Sailors Grave	Maketu	Opape	Maraehako Bay	Otanga
Sailors Grave	-	103 km	179 km	190 km	215 km
Maketu	0.54931**	-	87 km	117 km	151 km
Opape	0.46280**	0.84906**	-	48 km	86 km
Maraehako Bay	0.42232**	0.68024**	N. S.	-	38 km
Otanga	0.12573*	0.26357**	0.11334*	0.12548**	-

Significance shown as: ** = p <0.01 and * = p <0.05 after sequential Bonferroni correction. N. S. = No significant difference.

2. Low southern diversity

Haplotypic diversity was low in southern populations, and a single haplotype was sampled in the South Island. The southern limit of *C. maschalocarpum* is correlated with the 15°C sea surface temperature isotherm for the warmest month (Fig. 3 below). Northward movement of this isotherm during the Last Glacial Maximum (LGM) would have restricted *C. maschalocarpum* populations to the northern half of the North Island. Stepping stone recolonisation of southern New Zealand following the LGM would reduce genetic diversity through repeated founder effects.

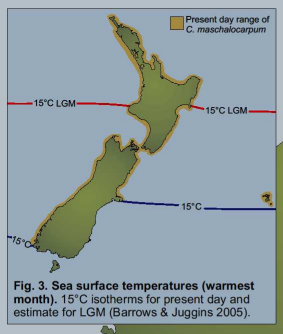


Fig. 3. Sea surface temperatures (warmest month). 15°C isotherms for present day and estimate for LGM (Barrows & Juggins 2005).

3. Hybridisation in northern populations

Morphologically intermediate hybrid specimens were found in the Bay of Plenty and Northland populations where *C. maschalocarpum* and *C. angustifolium* are sympatric. All specimens from Hooper Point, North Cape and Wekarua populations were hybrids, with either *C. angustifolium* and *C. maschalocarpum* haplotypes.

4. Northland and the East Coast connectivity

One haplotype was common in Northland (Cape Wiwiki and the Poor Knights) and the East Coast (Horoera and Kaiti Beach), but was absent from interjacent sites. The East Cape Current is a potential dispersal pathway connecting these populations. This current does not extend into the Bay of Plenty and the Hauraki Gulf. SAMOVA analysis (Dupanloup *et al.* 2002) grouped these populations together despite their spatial separation (Fig. 4 below).

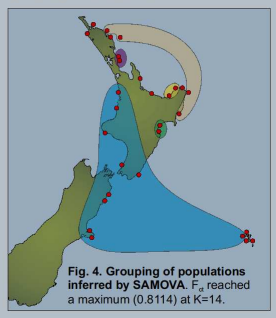


Fig. 4. Grouping of populations inferred by SAMOVA. F_{ct} reached a maximum (0.8114) at K=14.

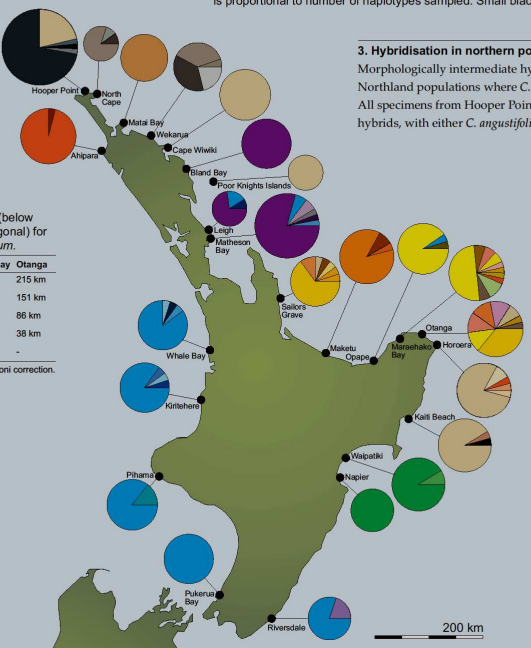
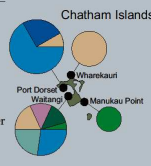


Fig 2. Distribution of mtDNA haplotypes of *C. maschalocarpum*

5. Chatham Islands diversity

Seven haplotypes were sampled in Chatham Islands populations, four were endemic. The presence of endemic haplotypes suggests populations survived LGM cooling. Paleoclimate studies indicate northward movement of the sub-tropical convergence during the LGM might have been constrained by the Chatham Rise (Nelson *et al.* 2000), maintaining relatively warm water temperatures near the Chatham Islands, with cooler temperatures around mainland New Zealand. Four haplotype lineages were present on the Chatham Islands, indicating at least four separate colonisation events, over a 650 km habitat discontinuity.



Conclusions

The population genetic structure of *C. maschalocarpum* reflects both contemporary processes (dispersal pathways, hybridisation) and historical events (climate change). Low connectivity between adjacent populations suggests dispersal limitation, but connections between Northland and the East Coast, and between mainland New Zealand and the Chatham Islands, show long distance dispersal by ocean currents.

References

Barrows TT, Juggins S (2005) Sea-surface temperatures around the Australian margin and Indian Ocean during the last glacial maximum, *Quaternary Science Review*, **24**, 1017–1047.

Dupanloup I, Schneider S, Excoffier L (2002) A simulated annealing approach to define the genetic structure of populations. *Molecular Ecology*, **11**, 2571–2581.

Kingsford MJ (1992) Drift algae and small fish in coastal waters of northeastern New Zealand. *Marine Ecology Progress Series*, **80**, 41–55.

Nelson CS, Hendy IL, Neil HL, Hendy CH, Weaver PPE (2000) Last glacial jetting of cold waters through the Subtropical Convergence zone in the Southwest Pacific off eastern New Zealand, and some geological implications. *Palaeogeography, Palaeoclimatology, Palaeoecology*, **156**, 103–121.

Acknowledgements

Thanks to Wendy Nelson, Kate Neil, Tracy Farr, Erasmo Macaya, Nick Shears and Peter Martin for assistance with collections and the Department of Conservation for support.

7.9 Appendix 9

Phycological Research

Phycological Research 2010; **58:** 239–247

Hybridization between the endemic brown algae *Carpophyllum maschalocarpum* and *Carpophyllum angustifolium* (Fucales): Genetic and morphological evidence

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SUMMARY

Hybridization is an important evolutionary process, which can have significant effects on biodiversity. While hybridization is well known in plants, less is known about the prevalence of hybridization in other kingdoms. Hybridization in the field has been confirmed in brown algae in a few cases, mainly in the northern hemisphere genus *Fucus*. Putative hybrids have been found in the New Zealand endemic species *Carpophyllum angustifolium* and *Carpophyllum maschalocarpum*. We used nuclear-encoded molecular data (ITS2) and morphometrics to confirm hybridization between *C. angustifolium* and *C. maschalocarpum*. Putative hybrid thalli were collected that had heterozygous ITS2, each copy corresponding to one of the parental species from that population. Morphological analysis also showed that the three classes (two parental species and hybrids) were easily distinguishable in these populations. It was found that the hybrids had an intermediate morphology to the parent species. Some individuals with *C. angustifolium* morphology had hybrid ITS2 ribotypes suggestive of backcrossing between the hybrids and *C. angustifolium*. Our data reveal another case of hybridization within the Fucales and suggests that further research on how these species remain separate is needed.

Key words: *Carpophyllum angustifolium*, *Carpophyllum maschalocarpum*, Fucales, hybrids, hybridization, ITS2, New Zealand, morphometrics, Phaeophyceae.

INTRODUCTION

Hybridization is an important evolutionary process that brings together diverged genetic lineages, through the crossing of two genetically distinct species. Hybridization challenges our species definitions and provides insight into speciation processes (Seehausen 2004). Often it also produces unique hybrid morphologies. Hybrids are generally expected to have intermediate morphologies reflecting their mixed genotypes, but may also have morphologies similar to, novel from, or more extreme than the parental species (Coyer *et al*. 2002a; Seehausen 2004; Lihova *et al*. 2007). Identification of hybrids based on morphology alone can be difficult due to the large degree of intra-specific variation and phenotypic plasticity present in many taxa (Rieseberg & Ellstrand 1993; Scott & Hardy 1994). Furthermore, morphological intermediacy does not always indicate hybrids and may reflect patterns of plasticity or environmental variation (Mathieson *et al*. 1981).

Introgression, the backcrossing of hybrids with parental species, can result in a continuum of morphologies between the parental species (Albert *et al*. 1997). Introgression is of great evolutionary significance as it can result in the transfer of adaptations between species (Seehausen 2004). Other potential outcomes of introgression include the strengthening of reproductive barriers and further divergence of the parental species, or the development of new hybrid lineages (Grant *et al*. 2004; Seehausen 2004).

The Phaeophyceae, brown algae, are morphologically diverse. Individuals range from microscopic filaments to the more familiar large conspicuous fleshy seaweeds (Graham & Wilcox 2000). Even within species there is great morphological variation (Mathieson *et al*. 1981). Despite this variability putative hybrids, especially in the morphologically complex Fucales, have been observed in wild populations since the 1850s (references within Thuret 1854; Mathieson *et al*. 1981).

New Zealand has a distinct brown algal flora with high levels of endemism (Nelson 1994). The dominance of Fucalean algae in the shallow subtidal is considered one of the more unusual aspects of the flora (Schiel 1990). Fucalean algae in the Northern Hemisphere dominate the intertidal, and consequently have

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Communicating editor: C. Katsaros.
Received 17 December 2009; accepted 26 March 2010.
doi: 10.1111/j.1440-1835.2010.00583.x

been relatively well studied (Chapman 1995). The vast majority of research into hybridization in Phaeophyceae is on the Northern Hemisphere fucalean genus *Fucus* (Mathieson *et al.* 1981; Chapman 1995; Wallace *et al.* 2004; Engel *et al.* 2005; Perrin *et al.* 2005; Billard *et al.* 2005b; Coyer *et al.* 2006b). Research into hybridization has revealed hybrids with intermediate morphologies, and in some cases evidence of introgression has been reported (Coyer *et al.* 2002a; Wallace *et al.* 2004; Billard *et al.* 2005b; Mathieson *et al.* 2006). Little work has been done on hybridization in Fucales outside of *Fucus,* despite the well documented abundance, diversity and ecological importance of this order in the Southern Hemisphere (Clayton 1984).

In north-eastern New Zealand two species of Fucales (Sargassaceae) form dominant stands in the high subtidal: *Carpophyllum maschalocarpum* (Turner) Greville and *Carpophyllum angustifolium* J. Agardh (Dromgoole 1973; Schiel 1988; Schiel 1990). Based on intermediate morphotypes it has been hypothesized that hybridization is occurring between the two species (Dromgoole 1973).

The internal-transcribed-spacer (ITS) is part of the nuclear ribosomal cistron, and is an often used sequence in plant evolutionary studies (Feliner & Rossello 2007). ITS is multi-copy, and homogenization of the multiple ITS sequences can occur (Álvarez & Wendel 2003). Coyer *et al.* (2002b) showed that artificially produced F_1 hybrids of two *Fucus* species with distinct ITS ribotypes were all heterozygous in ITS. Introgressed and F_2 individuals will not necessarily have heterozygous ITS ribotypes (Álvarez & Wendel 2003). Combining ITS and morphometric data can allow non-subjective identification of hybrid and parental individuals, and help determine the presence of introgression and F_2 hybrids within a population.

The present study using combined ITS2 identification and morphometric analysis confirmed hybridization between the non-*Fucus* fucalean algae, *C. angustifolium* and *C. maschalocarpum*. The expansion of the study of hybridization from the one northern hemisphere model, to a related but endemic Fucalean alga, will provide additional understanding of the importance of hybridization in this class.

MATERIALS AND METHODS

Sampling

Samples were collected from two sites on the East Cape of the North Island of New Zealand, where putative *C. angustifolium* × *C. maschalocarpum* hybrids were known to occur (Fig. 1). The two sites are both within the greater Whanarua Bay, which faces north-west and contains a number of smaller sandy bays separated by large rocky islands. The first site is located at the

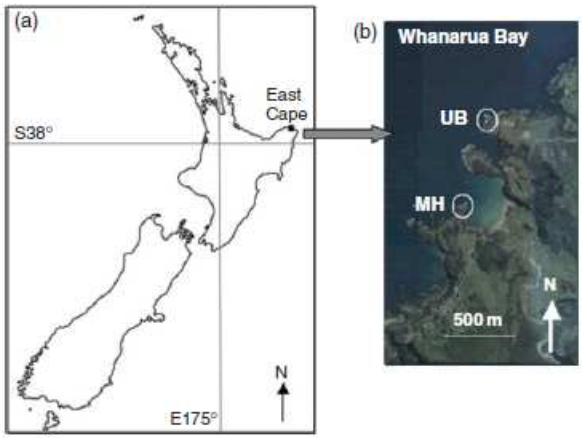

Fig. 1. The location of sampling (a) on the East Cape of the North Island of New Zealand, and (b) the two sites in the greater Whanarua Bay: Maraehako Bay (MH) and Uncles Bay (UB) (Google Earth 5.0).

south-eastern end of Maraehako Bay (S37°40.4′ E177°47.8′). The second site was located at Uncles Bay (S37°40.0′ E177°48.0′), which is the adjacent bay to the north-east. The two sites are approximately 500 m apart, and are separated by Motu Kaimeanui Island and two deep inlets. Sampling was conducted during the 2007–2008 summer.

Transect lines were laid along rock walls in the high subtidal, and quadrat locations were selected randomly from the top 0.5 m of *Carpophyllum* depth distribution. Quadrats were rejected if the rock face was not vertical, less than three individuals were present, or if the quadrat was less than 0.5 m from a previously sampled quadrat. All *Carpophyllum* individuals with holdfasts inside the 20 cm by 20 cm quadrats were collected. Sixty-nine quadrats were sampled in the two sites, with 280 *C. angustifolium*, 152 *C. maschalocarpum* and 32 hybrid adults collected in total (see Fig. 2 for typical morphologies). Only a subset that was characterized molecular was used in this study ($n = 80$). Collected material was frozen at –20°C within a week of collection, and thawed in seawater prior to morphometric analysis.

Representative herbarium specimens of *C. angustifolium*, *C. maschalocarpum* and (putative) hybrids collected in East Cape during this study have been deposited in the Museum of New Zealand – Te Papa Tongarewa herbarium under the WELT numbers A029625-29, A029635-38 and A029630-34, respectively.

Morphometrics

Adults longer than 20 cm were morphologically identified, cleared of epiphytes, and measured. Only those individuals with complete haptera were used in mor-

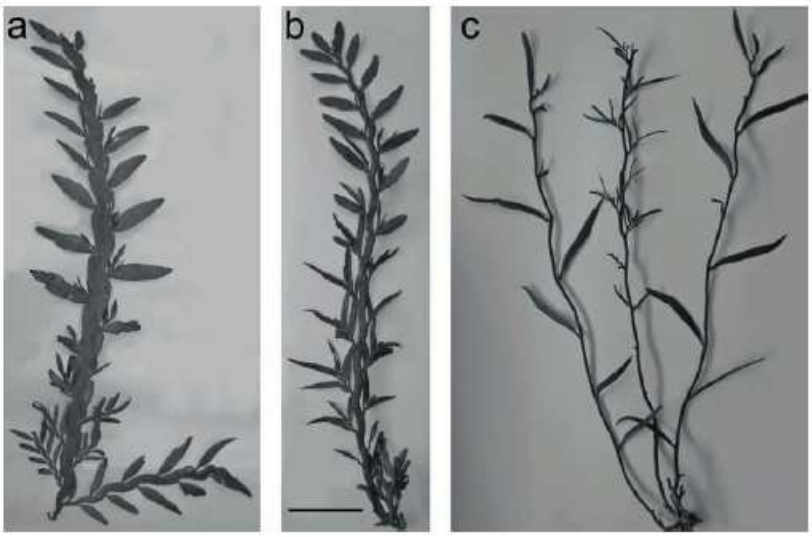

Fig. 2. Typical morphology of (a) *Carpophyllum maschalocarpum*, (b) putative *Carpophyllum angustifolium* × *C. maschalocarpum* hybrids, and (c) *C. angustifolium*. All at the same scale, scale bar in (a) shows 50 mm.

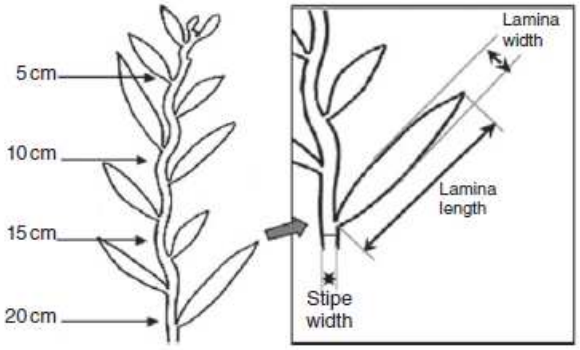

Fig. 3. The location of the measurements taken from each individual are shown with arrows on a stylized axis. Stipe width, lamina length and width are illustrated at 20 cm from the apex.

phometrics so as to avoid pseudo-replication (i.e. the doubling up of measurements from the same individual). Individuals were measured using digital callipers and a meter rule. A small section of lamina was removed from each individual after processing and placed into a labeled tube with silica gel for genetic analysis. The total length of the individual, presence of vesicles longer than 8 mm, and the presence of secondary branches longer than 20 cm were also recorded.

The morphometric characteristics selected for measurement were stipe width and thickness; and lamina length, width and thickness (Fig. 3). The present study focused on the stipe and lamina characteristics of the primary axis (defined as the dominant axis, which arises directly from the holdfast). The stipe and lamina measurements were taken from approximately 5, 10, 15, and 20 cm from the tip of the axis. Lamina length was measured from the edge of the stipe where the lamina forms to its tip. Lamina and stipe width were measured at the widest point, perpendicular to an imagined midline. Stipe and lamina thickness was measured at the thickest point. In some places herbivory had altered the morphology of the stipe and lamina; these could be detected by sudden and distinct changes in lamina or stipe outline. Measurements of stipe and lamina that were affected by herbivory were excluded, and if possible replaced by measurements from the nearest intact equivalent within 3 cm.

Digital images for the *C. angustifolium* (#2572, Botanical Museum Lund) and *C. maschalocarpum* (#000562635, The Natural History Museum, London Department of Botany) type specimens were obtained and measurements of lamina length, lamina width and stipe width were taken where possible.

Genetic analysis

Validity of the morphological identification for the hybrids and parental species were checked by genetically identifying 80 individuals. Individuals with unusual morphological characteristics were also checked genetically.

DNA was extracted from approximately 3 mg of dry tissue using a cetyltrimethylammonium bromide (CTAB) extraction protocol (Zuccarello & Lokhorst 2005). The ITS2 was amplified using a PTC-100 (Programmable Thermal Controller, MJ Research Inc. Waltham, MA, USA) polymerase chain reaction (PCR) machine, and the primers KG4 (CTTTTCCTCCGCT TAGTTATATG) and KP5 (ACAACGATGAAGAACGCAG) (Lane *et al*. 2006). The PCR conditions were an initial 94°C for 4 min, followed by five cycles of 1 min at 94°C, 1 min 59°C, 1 min 72°C with the annealing temperature decreased by 1°C each cycle. Then 30

cycles of 45 s 94°C, 45 s 53°C, 45 s 72°C, followed by 10 min at 72°C. Sequencing was done commercially (Macrogen Inc., Seoul, Korea). The chromatograms were visually examined and individuals identified as either a parental species or a heterozygous hybrid (where double peaks were present).

Cloning

One specimen from Maraehako Bay that was found to be heterozygotic for ITS2 in direct sequencing was selected for cloning to determine whether both parental ribotypes could be obtained. The complete ITS1-5.8S-ITS2 cistron was amplified using KP1 and KG4 primers (Lane *et al.* 2006). PCR product was cloned using the pGEM-T Easy vector kit (Promega Corporation, Madison, WI, USA) following the manufacturer's protocols. Transformed colonies were sub-cultured and plasmid preparations carried out by alkali lysis (Sambrook *et al.* 1989). Inserts were sequenced commercially using pUC M13 forward and reverse primers.

Morphometric data analysis

The morphometric data contained a large number of missing values. Scatterplots showed no difference between the character traits measurements at 5, 10, 15 and 20 cm from the apex, so the measurements from each individual were averaged. There were some missing data for lamina characteristics after this averaging, as many lamina were excluded from measurement due to herbivory damage. The missing values were not randomly distributed, and *C. maschalocarpum* averages were based on fewer data points than the others due to greater levels of herbivory and exposure damage. Randomly removing data points to an equivalent level in *C. angustifolium* and hybrid data made little or no change to overall group averages (0.002% to 0.03% change). This suggests no bias in accuracy due to variation in the number of data points averaged between the species.

Cluster analysis finds natural groupings or clusters within the data (Fielding 2007). These were conducted with genetically confirmed data and type specimens using the software NTSYSpc version 2.11 software (Rohlf 2000). The unweighted pair-groups method average (UPGMA) clustering method was used; this is a common and accepted method (Fielding 2007) for this type of data.

Boxplots were used to identify morphological traits that showed difference. These characters were incorporated into a distance matrix using Gower's Index in Le Progiciel R 4.0 software (Casgrain & Legendre 1999). This index was used as it can cope with missing values (Montanari & Mignani 1994; Podani 1999). It has also been used in other studies of macroalgae morphology (Fowler-Walker *et al.* 2006). Cophenetic values were calculated to test how well the cluster tree represents the data (Rohlf & Sokal 1981; Rohlf 2000).

ANOVA was used to compare the morphological groups found in the cluster analysis. Non-normal data were log transformed prior to analysis with ANOVA. Log transformed data that still did not meet ANOVA assumptions was analyzed using the non-parametric Kruskal–Wallis test. χ^2 tests were used to test for associations between wave exposure zones and the presence of vesicles and branches. Significance was determined at the 0.05 probability level.

RESULTS

ITS2 sequences and morphological identification

Only two ITS2 ribotypes were found, corresponding to the two morphologically identified parent species. The ribotypes differed by three different point mutations and one base pair indel (GenBank Numbers GU992939-GU992940). The point mutations appeared as double peaks in chromatograms from heterozygous individuals, and the indel appeared as a change from predominantly single peaks to all superimposed double peaks. This change occurred at the same base position in both forward and reverse sequences. All samples indentified a *priori* as hybrids had heterozygotic sequences (i.e. sequences of both *C. maschalocarpum* and *C. angustifolium*).

Twelve ITS2 sequences were obtained from cloned DNA from the heterozygotic specimen. Multiple copies of the two ribotypes were found, corresponding to those expected from direct sequencing. Some sequences showed single base changes (autoapomorphies) that were not previously detected by direct sequencing and were only present in a single clone from each specimen. We attribute these to copy errors during PCR with *Taq* polymerase.

Morphological identifications matched with genetic sequences in 93.8% of the individuals ($n = 80$). All *C. maschalocarpum* ($n = 25$) and hybrid ($n = 27$) morphological assignments were congruent with ITS2 sequences. 82.1% of the samples ($n = 28$) morphologically identified as *C. angustifolium* had matching *C. angustifolium* ITS2 sequences. The remaining 17.9% were five individuals with hybrid sequences; these will be described from here on as 'putative backcrosses'.

Cluster analysis

Stipe width, lamina length and lamina width were used in the cluster analyses. The cluster analysis tree of the genetically identified individuals ($n = 80$) and the type specimens is shown (Fig. 4). This had a cophenetic correlation of 0.84, indicating a 'good fit' (Rohlf 2000).

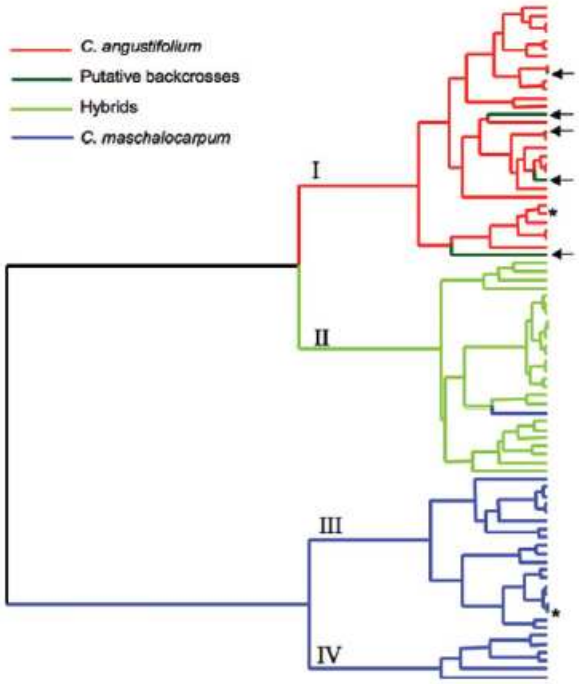

Fig. 4. Cluster analysis showing the morphology of a random sub-sample of genetically identified individuals, and the type specimens for the parental species (shown by asterisks). Individuals cluster into four morphological groups as labeled I to IV. Colors represent the identity of the individuals (see legend). Putative backcrosses are those morphologically identified as *Carpophyllum angustifolium* but with hybrid ITS2 sequences, as shown with arrows. The coenphetic value of this tree is r = 0.84486.

A cluster analysis of all individuals collected showed the groupings as seen for the genetically identified samples (Hodge 2009)

Genetically identified individuals clustered into four main morphological groups. Group I contained all of the genetically confirmed *C. angustifolium* ($n = 23$), two hybrids, and the putative backcrosses ($n = 5$). Group II included all of the other hybrids ($n = 25$), and one individual identified as *C. maschalocarpum*. Closer examination of this *C. maschalocarpum* individual reveals it was only just long enough to be considered 'adult' (>20 cm long) and therefore included in this study. Groups III and IV were composed of *C. maschalocarpum* only (III, $n = 19$; IV, $n = 6$). Hereafter Group I will be referred to as *C. angustifolium*, Group II as the hybrids, Groups III and IV combined as *C. maschalocarpum*. A major subdivision between the morphology of *C. angustifolium* and hybrid individuals, and the *C. maschalocarpum* individuals, was revealed in the cluster analysis. The hybrid cluster had a higher similarity coefficient with the *C. angustifolium* cluster (0.75) than with the *C. maschalocarpum* clusters (0.49).

A cluster analysis of all individuals collected, i.e. including specimens only morphologically identified, had a cophenetic correlation of greater than 0.90, indicating the cluster tree is a 'very good fit' of the data (Rohlf 2000). This cluster analysis showed the same group structure and membership of genetically identified individuals as the cluster tree of only genetically identified individuals (Hodge 2009). Consequently morphological comparisons were done between groups using data from both morphologically and genetically identified individuals.

Comparing morphologies

Comparisons of the morphologies of the *C. angustifolium* group (I), the hybrid group (II) and the pooled *C. maschalocarpum* groups (III and IV) found a range of differences between them (Table 1). Log stipe width ($P < 0.001$), lamina length ($P < 0.001$), and lamina width ($P < 0.001$) were different between the three groups (all pairwise Tukey Tests were $P < 0.05$) (Fig. 5). The pooled *C. maschalocarpum* group had a wider stipe, and a shorter and wider lamina than the *C. angustifolium* group. The hybrid group exhibited traits intermediate to the parents for each of these traits. There was no overlap in log stipe width for the three groups (Fig. 5a).

Stipe thickness was also different between the three groups ($P < 0.001$) with median stipe thickness being thickest for the *C. angustifolium* group (I), and thinnest for the hybrid group (II). Lamina thickness was also found to be non-uniform ($P < 0.01$). The *C. angustifolium* group (I) had significantly thicker lamina than the hybrids (II), and the pooled *C. maschalocarpum* group (III and IV) (Tukey tests: $P < 0.05$). The lamina thickness of the hybrid group (II) and the pooled *C. maschalocarpum* group (III and IV) were not significantly different (Tukey test: $P = 0.952$).

There were significant differences in total length, stipe width, stipe thickness, presence of vesicles between the two groups (III and IV) of *C. maschalocarpum* (Table 2). Group IV was generally longer in total length, had a wider and thicker stipe, and had a greater proportion of individuals with vesicles present than Group III. There were also non-significant trends ($0.05 < P < 0.10$) of Group IV having wider and thicker lamina, and more frequently having secondary branches. There was no difference between the lamina length of the two groups. There was no difference in the distributions between the two *C. maschalocarpum* groups between sites ($X_2 = 3.146$, d.f. = 1, $n = 146$, $P = 0.076$) or exposure zones.

DISCUSSION

Our molecular data confirms that *Carpophyllum angustifolium* and *C. maschalocarpum* hybridize in popula-

Table 1. Relative morphological differences between the three groups (tested with ANOVA and Kruskal–Wallis tests)

Character trait	Group I	Group II	Group III and IV	Statistics
Stipe width	Narrow	Intermediate	Wide	$F = 4843.291$, d.f. = 2, $n = 463$, $P < 0.001$
Lamina length	Long	Intermediate	Short	$F = 43.275$, d.f. = 2, $n = 240$, $P < 0.001$
Lamina width	Narrow	Intermediate	Wide	$F = 247.230$, d.f. = 2, $n = 240$, $P < 0.001$
Stipe thickness	Thick	Thin	Intermediate	$\chi^2 = 23.134$, d.f. = 2, $n = 463$, $P < 0.001$
Lamina thickness	Thick	Thin	Thin	$F = 21.22$, d.f. = 2, $n = 240$, $P < 0.001$

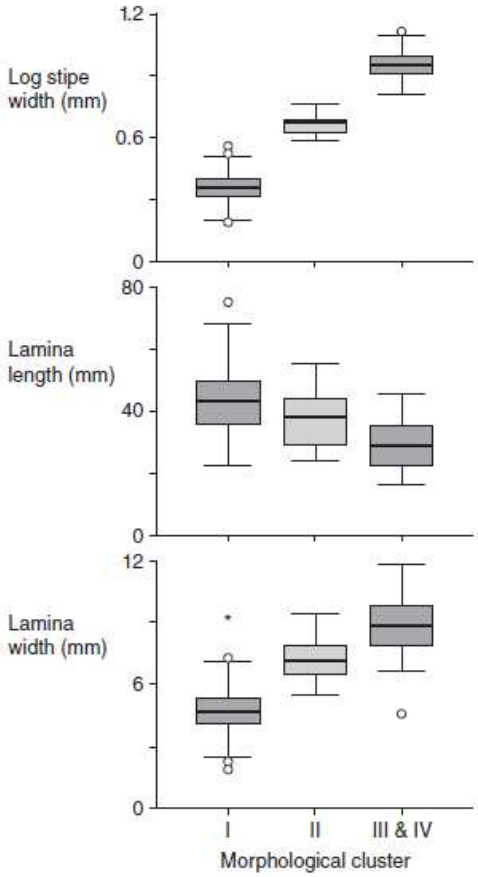

Fig. 5. The three groups had significantly different (a) log stipe widths, (b) lamina lengths and (c) lamina widths. The boxplots show the spread of data (line in box = mean, box = 75% of values measured, lines = 95% of values) with outliers marked by circles and extreme outliers marked with asterisks.

tions from East Cape. *Carpophyllum angustifolium, C. maschalocarpum* and their hybrids have distinct and distinguishable morphologies. Identifiable hybrid and parental morphotypes have been found within many *Fucus* hybrid species complexes; however, morphologically ambiguous individuals are often present (Scott & Hardy 1994; Coyer *et al.* 2002a; Kucera & Saunders 2008). No other detailed morphometric analysis of randomly sampled and molecularly identified individuals has been done in a Fucalean hybrid system. Subsequently the range and degree of overlap of parental and hybrid morphologies are unknown in other Fucalean hybrid systems.

Stipe width alone can distinguish between *C. angustifolium, C. maschalocarpum* and their hybrids, and provides a useful taxonomic character for field identification. Stipe width meets three requirements described for a useful macroalgal taxonomic character in macro-algae; it is constant, easy to observe and non-destructive (Mathieson *et al.* 1981). Stipe width of the parent species and hybrids was easy to measure and rarely affected by herbivory or exposure damage (pers. obs.). An investigation into the spatial variability of *F. spiralis* and *F. vesiculosus* (Coleman & Muhlin 2008) found stipe width is also a valuable character for identification of *Fucus* species.

Carpophyllum angustifolium × *C. maschalocarpum* hybrids generally had an intermediate morphology to the parent species. Hybrids had intermediate stipe width, lamina length, lamina width and vesicle presence. However, the thickness of hybrid lamina was novel, being thinner than both parents. Furthermore the proportion of hybrid individuals with secondary branching was indistinguishable from *C. maschalocarpum*. Intermediate hybrid morphologies are usually found in hybrid systems although not necessarily in all cases (Rieseberg & Ellstrand 1993; Seehausen 2004; Coyer *et al.* 2006b). Intermediate morphology is used to identify putative hybrid individuals for further investigation in *Fucus* (Scott & Hardy 1994; Coyer *et al.* 2002a). Despite this there are examples of morphological traits in hybrids being aligned to one of the parental species. For example, hybridization between the dioecious *Fucus vesiculosus* and the hermaphroditic *Fucus spiralis* results in hybrids with parental sexual phenotypes rather than intermediate sexual allocation (Billard *et al.* 2005b). In salt marsh habitats *F. vesiculosus* × *F. spiralis* hybrids are morphologically indistinguishable from polyploid *F. vesiculosus* individuals (Coyer *et al.* 2006b).

The presence of hybrid genotypes with *C. angustifolium* morphotypes suggests introgression might be occurring. Hybrid ITS genotypes with parental morphology were also found in the *F. vesiculosus* × *F. spiralis* species complex, and were also cautiously interpreted

Table 2. Relative morphological differences between the *Carpophyllum maschalocarpum* clusters III and IV

Significance	Morphological trait	Group III	Group IV	Statistics
Statistically significant at 5% level	Total length	Shorter	Longer	$F = 6.803$, d.f. = 1, $n = 146$, $P = 0.010$
	Stipe width	Narrower	Wider	$F = 250.906$, d.f. = 1, $n = 146$, $P < 0.001$
	Stipe thickness	Thinner	Thicker	$F = 14.188$, d.f. = 1, $n = 146$, $P < 0.001$
	Proportion with vesicles	Low	High	$\chi^2 = 11.026$, d.f. = 1, $n = 146$, $P = 0.001$
Trends in the data (significant at the 10% level)	Lamina width	Narrower	Wider	$F = 2.885$, d.f. = 1, $n = 49$, $P = 0.096$
	Lamina thickness	Thinner	Thicker	$F = 3.837$, d.f. = 1, $n = 49$, $P = 0.056$
	Proportion with secondary axes present	Low	High	$\chi^2 = 3.161$, d.f. = 1, $n = 146$, $P = 0.075$
Not significant and no trends.	Lamina length	Same	Same	$F = 1.517$, d.f. = 1, $n = 49$, $P = 0.224$

as indicating introgression (Kucera & Saunders 2008). Introgression is an important evolutionary process that has the potential to integrate genetic material from one species into another (Coyer *et al.* 2007). Introgression has been identified in *Fucus* hybrid complexes, and is considered a significant factor in the evolution of *Fucus* lineages (Wallace *et al.* 2004; Coyer *et al.* 2006a, 2007). Further investigation with nuclear markers lacking the unusual properties of ITS (Álvarez & Wendel 2003) are needed to confirm introgression and the degree of introgression.

Fucus species seem to have maintained their genetic integrity despite extensive hybridization. For example, hybrids made up 13% of *Fucus serratus* and *Fucus evanescens* in one population; however, nuclear DNA introgression was only 1.5% in the two species, and both species maintain different distributions and morphologies (Coyer *et al.* 2002a, 2007). Coyer *et al.* (2006a) suggested different parental mating systems might act as a barrier to extensive introgression, and protect the parental species integrity. Hybridization in *Fucus* appears to occur between dioecious (having separate male and female individuals) and hermaphroditic species (e.g. Wallace *et al.* 2004; Billard *et al.* 2005a; Mathieson *et al.* 2006; Coyer *et al.* 2007). All members of the genus *Carpophyllum* are dioecious (Lindauer *et al.* 1961). Extensive introgression, and the loss of parental species genetic integrity, could be more likely in *Carpophyllum* hybrid systems if Coyer *et al.* (2006a) hypothesis is correct.

The absence of individuals with *C. maschalocarpum* morphology and hybrid genotypes suggests introgression may be asymmetrical, with backcrossing only between hybrids and *C. angustifolium*. Asymmetries in angiosperm reproduction are thought to be common in a broad range of taxa (Tiffin *et al.* 2000). Asymmetrical hybridization and introgression has been found in *Fucus* spp. (Coyer *et al.* 2002a, 2007). Determining the symmetry of introgression is important for predicting its implications on parental species integrity (Coyer *et al.* 2007).

The morphometric analysis distinguished variation within *C. maschalocarpum*, which could be related to demography. The differences between the two *C. maschalocarpum* clusters (i.e. total length, stipe width, stipe thickness, vesicles) were characteristics thought to be associated with maturity (Dromgoole 1965). *Carpophyllum maschalocarpum* has pulsed peak recruitment, and has been shown to recruit in cohorts after bare rock is opened up by disturbance (Schiel 1980, 1988).

This work on *Carpophyllum* combining morphometric data with ITS2 identification found generally distinct and distinguishable parental and hybrid morphologies. Further investigation needs to be done using microsatellites and maternally inherited DNA in conjunction with morphology to confirm and determine the extent and symmetry of introgression, and identify backcrosses, F_2 and later hybrids.

ACKNOWLEDGMENTS

We thank Carlos Lehnebach for statistical help, and Tracey Taylor, Jen Van der Veur, and Aaron Packard for field assistance.

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