Population genetic studies of economically important *Gracilaria* and *Gracilariopsis* (Rhodophyta) in the south western Cape.

by

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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in the Department of Botany, Faculty of Science, University of Cape Town, South Africa.

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DECLARATION

I declare that this thesis is my own, unaided work. Experimental work discussed in this thesis was carried out under the supervision of Associate Prof. J.J Bolton of the Department of Botany and Dr. V Coyne of the Department of Cellular and Molecular Biology, University of Cape Town.

Material presented here is all original work by the author and has not been submitted in this or any other form to another university. Where use has been made of research of others, it has been duly acknowledged in the text.

signature removed

Kershini Govender
Department of Botany
March 2001

DEDICATION

For my Grandparents who have always encouraged me to study, the late Mr Kaiser Venketessa Moodley, Mrs Ruby Parvathi Moodley, Mr Bob Govender and my granny Mrs Aliamal Govender whom is 76 years of age and still enjoying good health.

OM SAI RAM

Contents

Preface

The following aspects of this thesis have been presented:

National conference presentation:

(i) **Govender, K., Coyne, V. and Bolton, J.J.** 2000. Population genetic studies of *Gracilaria gracilis* in the south western Cape as determined by various molecular techniques. 17th Congress of the Phycological Society of Southern Africa, Mtunzini **17**:16.

International conference presentation:

(i) **Govender, K., Coyne, V. and Bolton, J.J.** 2001. Population genetic studies of *Gracilaria gracilis* and *Gracilariopsis* sp. in the south western Cape as determined by AFLPs. XVIIth International Seaweed Symposium, Cape Town **17**:123.

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List of figures

List of tables

Abstract

Gracilaria (Rhodophyta) encompasses some of the world's most valuable agarophytes and has accordingly been the subject of much research in recent decades. *Gracilaria*, however, has presented a problem to taxonomists over the years in that extreme phenotypic variability in certain entities, and the occurrence of similar morphologies among different taxa (in both *Gracilaria* and *Gracilariopsis*), combine to impede species recognition. *Gracilaria* and *Gracilariopsis* occur naturally from the Eastern Cape Province, South Africa to northern Namibia and the genetic variation within this widespread species or species complex is unknown. In this study, samples were collected from various locations within the Langebaan Lagoon-Saldanha Bay system and the nearby St. Helena Bay. Their positions were accurately mapped with the aid of a Global Positioning System (GPS). Reproductive structures are of great taxonomic importance within the Gracilariaceae. The local utility of reproductive features is questionable however, as south western Cape material is very seldom fertile which presents a problem for identification. External morphology lacks taxonomic significance due to the isomorphic nature of terete taxa. It was found that one cannot differentiate between *Gracilaria gracilis* and *Gracilariopsis longissima* based on external morphology. Adding to this, it was found that under varying ecological conditions different external morphologies occur. Although these specimens appear different externally, molecular studies show that they belong to the same species. Various molecular methods have proven successful in differentiating within and between populations, species and genera in the Gracilariaceae. Sequencing results of a 299 bp variable region of the 18S rRNA gene reveal that *G. gracilis* predominates in the Langebaan Lagoon-Saldanha Bay system, whereas *Gp. longissima* predominates in the St. Helena Bay system. From the AFLP data one can infer that Saldanha

Bay isolates display moderate divergence, probably due to favourable ecological conditions in this locality. The levels of similarities within populations of *G. gracilis* in Langebaan Lagoon and *Gp. longissima* in St. Helena Bay would indicate that mostly vegetative propagation occurs within these populations. However, two populations of *Gp. longissima* were found to co-exist with Langebaan Lagoon *G. gracilis* populations. A single isolate from St. Helena Bay was identified as *G. gracilis*. Interestingly, all samples from the salt marsh in the St. Helena system formed a monophyletic clade within *Gp. longissima*, which was well supported by high bootstrap values. Future studies would involve analysing material from the same locations to determine genetic flow within these populations. Also, strains may be evaluated for their suitability for mariculture.

Chapter 1

LITERATURE REVIEW

1.1 RHODOPHYTA

Red algae, as the name suggests, are characterised primarily by a rosy, purplish or reddish brown colour, attributable to the presence of the bilipigments phycocyanin, phycoerythrin and allophycocyanin. The reddish hues vary considerably according to the amount of light and nutrients available to the plant, with shaded, nutrient-replete tissue being darker while well illuminated, nutrient-depleted fronds being lighter or greenish to yellowish. Several species of red algae on the South African south coast also display bluish iridescence when submerged. In a few taxa (for example, the freshwater genus *Batrachospermum*), the bilipigments may be present in such small quantity that chlorophyll predominates and the alga appears green. This phenomenon is also occasionally found in individuals of normally red species as a result of genetic mutation (Bird and McLachlan, 1992).

Red seaweeds show considerable structural diversity and various degrees of complexity between species. Most of the multicellular types are basically filamentous (Bird and McLachlan, 1992). Most red algae are marine, and species diversity is greatest in warmer oceans. Almost all are multicellular, ranging from delicate filaments to crustose, foliose or frondose forms. Red algae also have distinctive nutrient reserves and cell wall polysaccharides. Most red seaweeds are comprised (dry weight) of: 25-35% minerals, 30-60% carbohydrates (mostly sulphated galactans), 7-15% proteins, 1-5% lipids and 2-10% cellulose. In addition water forms 70-80% fresh weight (Jensen, 1993). The algal cells contain starch grains in the cytoplasm, which are referred to as floridean starch. These have been shown to

consist of amylopectin and/or amylose in a number of red algae and provide a polymeric storage compound for organic carbon and energy (Raven *et al.*, 1990).

1.2 TAXONOMY OF THE GRACILARIACEAE

Despite much effort in the past decade to resolve the taxonomy of algae in the Gracilariaceae, the family remains problematic. Generic concepts have become questionable as their diagnostic reproductive features have been reported as mixed in single species. Species concepts are often also uncertain since the full phenotypic range of a species is seldom known or appreciated. As more observations are made, features like spermatangial configuration and mode of gonimoblast nutrition, once considered characteristic of genera and sub-genera, are emerging as a continuum of types that may vary within species. Culture studies to establish the reproductive anatomy of a life history and to test crossabilities are invaluable to species concepts but also may yield false results due to clonal variability and intraspecific sterility. Characteristics of DNA have been effective in clarifying some aspects of gracilariacean taxonomy and phylogeny, and may be able to resolve the major inconsistencies if more widely applied (Bird, 1995).

Gracilaria gracilis (Stackhouse) Steentoft, Irvine *et* Farnham belongs to a commercially important genus of red macroalgae (Armisen, 1995; Steentoft *et al.*, 1995; Renn, 1997). *Gracilaria* species are notably difficult in their taxonomy owing to: (i) poorly understood species limits; (ii) large amount of variation in morphological features selected for taxonomy; (iii) large numbers of taxa mostly previously studied in narrow geographic range; and (iv) misapplication of species names due to the lack of reference to type specimens (Abbott, 1983).

In past years, *G. gracilis* was referred to as *G. confervoides* (Stackhouse) Greville in a number of countries (Steentoft *et al.*, 1995). According to Steentoft *et al.* (1995) *G. gracilis* and *Gracilariopsis longissima* (S. Gmelin) Steentoft, L. Irvine, and Farnham are superficially similar species that had long been confused under the name *G. verrucosa* (Hudson) Papenfuss. Similar specimens were also described in Italy and southern Africa as *G. verrucosa* (Bird and Kain, 1995).

Dawson (1949) separated *Gracilaria* from *Gracilariopsis* on the following basis:

(i) *Gracilaria*: gonimoblast irregular, consisting of a few, large vacuolated cells;
Gracilariopsis: dome-like gonimoblast consisting of many small, non-vacuolated cells;

(ii) *Gracilaria*: sparse protoplasm;
Gracilariopsis: dense protoplasm;

(iii) *Gracilaria*: carposporangia in clusters and short chains;
Gracilariopsis: carposporangia in well-marked radiating chains;

(iv) *Gracilaria*: nutritive tubular cells present;
Gracilariopsis: nutritive tubular cells absent.

Simons (1977) describes *Gracilaria* from Saldanha Bay, South Africa as consisting of "ramifying, stringy streamers and looks like branching, reddish-brown, bootlaces". This description also applies to many other members of the genus and to some species of *Gracilariopsis*. The morphological plasticity of the Gracilariaceae coupled with the fact that some populations rarely become fertile has proved a hindrance to algal taxonomists (Fredericq and Hommersand, 1989a; Fredericq and Hommersand, 1989b; Bird, 1995).

Different algal species were originally recognised and classified on the basis of their anatomical, morphological and reproductive organ features only. However, recent developments in molecular biology have provided new tools for aiding in the classification of these macroalgae. The electrophoresis of plastid DNA, digested with endonucleases, produces banding patterns that demonstrate the presence of highly conserved DNA sequences at the species and subspecies level. Goff and Coleman (1988) showed that plastid DNA restriction profiles differed not only between different macroalgal genera, but between three different *Gracilaria* species as well (*G. robusta* Setchell; *G. lemaneiformis* (Bory) Weber-van Bosse; *G. pacifica* Abbott), enabling them to distinguish between the three species. The validity of this protocol for aiding macroalgal systematics was confirmed by Bird and Rice (1990), who determined the profiles of eleven 'different' *G. verrucosa* strains from northern Europe. They showed that there were in fact only three different species, despite differences between all eleven strains in the traditional (morphological) classification system.

In addition to the above method, PCR-amplification of highly conserved DNA regions, and subsequent restriction fragment length polymorphism (RFLP) and/or sequence analysis of the amplified DNA, have aided in delineating many different *Gracilaria* species. Examples of the DNA regions are: *rbc*L (ribulose-1,5-biphosphate carboxylase); ITS (internal transcribed spacers) regions of the rRNA gene; 5.8S rRNA; and 18S rRNA (Bhattacharya *et al.*, 1990; Bird *et al.*, 1990; Scholfield *et al.*, 1991; Bird *et al.*, 1994a; Freshwater *et al.*, 1994; Goff *et al.*, 1994; Ragan *et al.*, 1994; Gonzalez *et al.*, 1996).

Plastino and Oliveira (1996) described the use of successful crossing between male and female specimens from isolated *Gracilaria* populations in Brazil to distinguish between different species and genera. This is based on the theory that specimens that can fertilise each other belong to the same species. Their results showed that the male reproductive structures and some features of the female reproductive structures are reliable taxonomic characters, whereas other features such as gross thallus morphology and branching pattern are not. All these results emphasise the need to combine a number of different approaches when classifying *Gracilaria* species.

Adding to the taxonomic confusion in the genus *Gracilaria* is the fact that sterile populations cannot be allocated to a species, since detailed information is required from the reproductive structures (female cystocarp and/or male spermatangia). In some species of *Gracilaria*, most plants become fertile at a given size-class, e.g. *G. verrucosa* in Britain (Jones, 1959) and *G. tikvahiae* from New Hampshire, USA (Penniman *et al.*, 1986). Whereas attached populations of *Gracilaria* frequently contain fertile individuals, most reports of free-living populations are entirely sterile, reproducing only by vegetative means. Farmed populations of *Gracilaria* often remain sterile and are similarly propagated indefinitely by fragmentation for crops in the Caribbean, Hawaii, Chile, Taiwan, China, and Namibia (McLachlan and Bird, 1986; Santelices and Doty, 1989).

Examination of material from the south coast of England, revealed that *Gp. longissima* had been confused with and misidentified as *G. gracilis* (Steentoft *et al.*, 1995). Misidentifications of other terete *Gracilariopsis* and *Gracilaria* species accounts to some extent for the very wide geographical distribution reported for *G. verrucosa*, and despite the description of some new species of *Gracilaria*, e.g. *G. pacifica*, much confusion remains.

Studies by Fredericq and Hommersand (1989a, 1989b), based on the characters identified by Dawson (1949) and several others, provide evidence that the genera *Gracilaria* and *Gracilariopsis* are separate and are both found in Britain. These studies clearly indicate that terete British gracilarioids (terete, stringy, highly branched members of the Gracilariaceae) have been regularly misidentified and that there has been some confusion in the application of the genera *Gracilaria* and *Gracilariopsis*.

Isaac (1956) studied the annual reproduction of *Gracilaria* in the Langebaan Lagoon-Saldanha Bay area. He concluded that cystocarpic plants are found throughout the year, tetrasporic plants occur for the greater part of the year and that the greatest proportion of the seaweeds was cystocarpic. Today *Gracilaria* in the Langebaan Lagoon-Saldanha Bay area is seldom fertile, reproducing almost exclusively by vegetative means. The reason for the change to an almost completely vegetative mode of reproduction is unknown. Interestingly, *Gracilaria* in Luderitz Bay, Namibia has never been observed to be fertile (Molloy, 1992).

1.3 GENERAL CHARACTERISTICS OF *GRACILARIA*

Plants of the genus *Gracilaria* grow attached or free-living from the eulittoral to the sublittoral zone (Oyieke and Kokwaro, 1995), such as *G. salicornia* (C. Agardh) Dawson (eulittoral) *and G. corticata* (J. Agardh) J. Agardh (lower eulittoral to sublittoral). *Gracilaria gracilis* normally occurs in the lower intertidal and upper subtidal regions of the coast. It is often attached to a solid surface by a holdfast, which is a crustose clump of 5-6 fused sporelings from which many erect thalli grow. However, South African material does not possess holdfasts. The alga is perennial and can regenerate readily from the holdfast as well as from small thallus pieces. Thalli are bushy and highly branched (Prescott, 1968), with commercial forms being less than 30 cm in length. Different species may be difficult to distinguish, as morphologies are similar (Critchley, 1993). The genus consists of over 100 described species and is widely distributed throughout the world (Oliveira and Plastino, 1994).

Although multicellular seaweeds lack the high degree of organisation and differentiation that are characteristic of higher plants, they exhibit specific patterns of construction. *Gracilaria* species have a pseudoparenchymatous cellular organisation, in which the thalli are formed by the aggregation of single branched filaments. A cross-section of these thalli shows large medullary cells positioned in the centre, with a gradual transition to smaller subcortical and cortical cells towards the outer cuticle layer (Oliveira and Plastino, 1994; Steentoft *et al.*, 1995). The cortex is usually two cells deep and densely pigmented. The medulla and subcortex consist of a total of four to nine cells in width and are unpigmented. The cortical cells contain phycobilisomes, on which pigmented phycobiliproteins such as phycoerythrin, phycocyanin and allophycocyanin are organised. These pigmented proteins

form part of the algal photosynthetic system. They extend the range of wavelengths of light absorbed by the alga and are responsible for conferring the reddish-brown colour of the thalli (Gantt, 1990; Grossman *et al.*, 1993). The outer surface of the thallus is referred to as cuticle. Although little is known about algal cuticles, it has been shown in some red algae that the cuticle is highly proteinaceous (Dixon, 1973; Craigie, 1990; Craigie *et al.*, 1992).

The chromosomes of *Gracilaria* species are extremely small and difficult to count. Of the species of *Gracilaria* investigated, most have a chromosome number (n) of 24, although a few have been observed to have 32 (Patwary and van der Meer, 1992; Kapraun *et al.*, 1993).

In the cell wall of *Gracilaria* species, the intercellular matrix in which the skeletal fibres are embedded is composed of flexible chains of differently sulphated galactans whose structure is based on alternating β-D-galactopyranose and α-L-galactopyranose residues (Knutsen *et al.*, 1994; Murano, 1995). This polymer is referred to as agar whilst the alga is referred to as an agarophyte. Agar polymers in cell walls of some *Gracilaria* species may be charged, suggesting that they play a minor role in controlling ion exchange between the medium and the cell cytoplast. However, the major biological function of agar is to help maintain the integrity of the algal cells and provide mechanical strength to the algal thalli (McLachlan, 1985).

1.4 LIFE CYCLE OF *GRACILARIA*

Gracilaria has a typical red algal, triphasic life history (*Polysiphonia*-type; McLachlan and Edelstein, 1977). There is an alternation of morphologically inseparable yet genetically distinct generations, with the sexes separated in the gametophyte phase (Fig. 1.1).

South African *Gracilaria* are seldom fertile and gametophytes have rarely been observed in the field. However, female thalli can be recognised, without the aid of a microscope, by the presence of cystocarps which appear as distinct, hemispherical lumps variously distributed along the thallus. The cystocarp is the manifestation of the successful fertilisation of male and female gametes. Non-motile spermatia are liberated in large numbers from the haploid male parent plant. The carpogonium is retained on the female thallus and fertilised *in situ*. A diploid zygote is the product of gametic union but remains attached to, and develops parasitically upon, the haploid female gametophyte plant (Critchley, 1993).

Post-fertilisation development of the zygote produces the diploid carposporophyte generation, which together with the protective outlayer is called a cystocarp. This diploid phase sporulates by mitotic cell division to produce a large number of genetic replicates of the zygote. In this multiplication process, many carpospores are released via an ostiole in the cystocarp wall. Each carpospore has the potential to germinate into the third phase of the life history, i.e. the tetrasporophyte generation (Critchley, 1993).

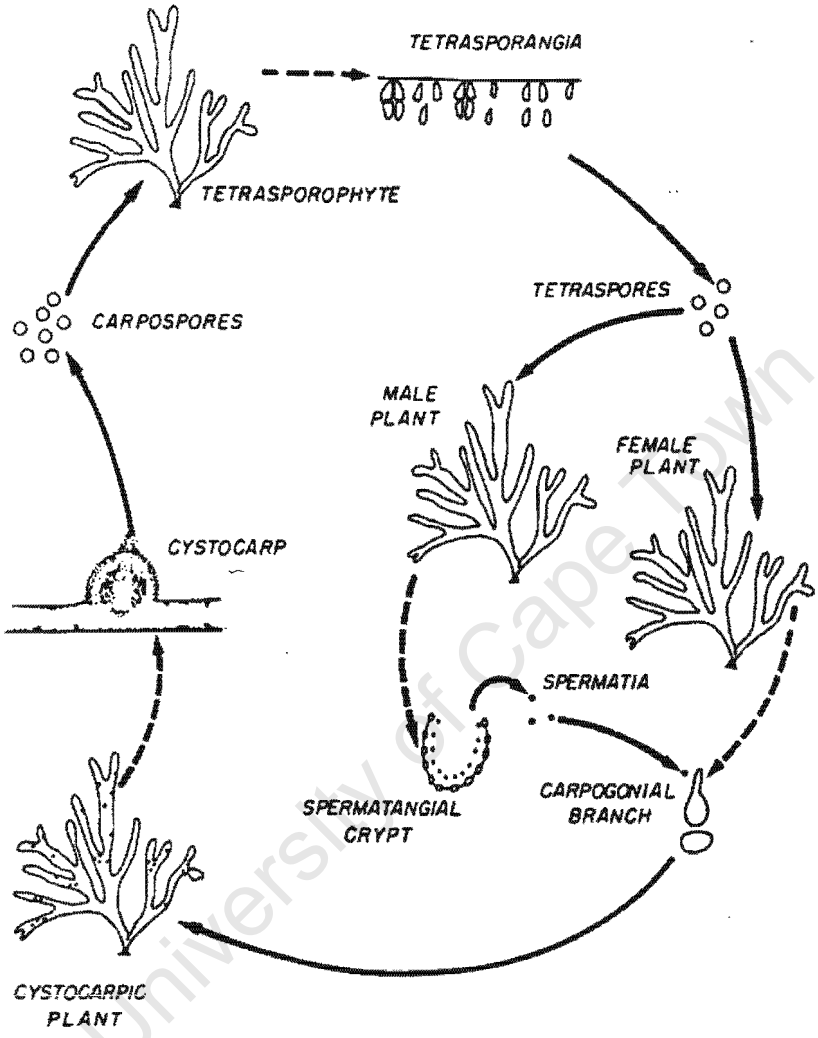

Fig. 1.1 A diagram of a *Polysiphonia*-type life history as reported in most species of *Gracilaria* (Oliveira and Plastino, 1994).

The diploid tetrasporophyte plants are morphologically similar to the haploid gametophyte plants. The tetrasporophyte generation completes the life cycle with the production of haploid tetraspores by meiotic sporogenesis within cortical sporangia. Likewise, each tetrasporophyte individual has the potential to produce a large number of progeny which are equally segregated into male and female gametophyte plants (Critchley, 1993).

1.5 COMMERCIAL IMPORTANCE OF *GRACILARIA*

Members of the red algal genus *Gracilaria* are among the most economically important seaweeds. They have a variety of uses, ranging from traditional foods and medicines to producers of agar for biological and industrial applications (Dawes 1987; Hurtado-Ponce and Umezaki, 1987; Ryan and Nelson, 1991; Murano *et al.*, 1992). The genus *Gracilaria* is of prime importance as a source of phycocolloids called agar. Agar is used in microbiological media for cultures, plant nutritional studies (Bornman and Barnard, 1993), and for food preparations. It is used in the food industry in baking jellies, meringues, pie fillings and various other types of confectionery. It is also used for the manufacture of dental impression media (Renn, 1997). Kain (1995) found that agar was the phycocolloid commanding the highest price on the world market. However, it is also consumed as a vegetable in the West Indies (Smith *et al.*, 1984), used as a consumable in tablet form in Japan (Armisen, 1995), and as a food source for abalone aquaculture (Chiang, 1981).

1.6 SIGNIFICANCE OF AGAR

Agar is mostly obtained from 5 genera (*Gelidium*, *Gelidiella*, *Pterocladia*, *Gracilaria* and *Gracilariopsis*) from 3 orders of red algae. Agar is a water soluble polysaccharide which constitutes the matrix component of cell walls of marine red algae known as agarophytes (Armisen, 1995).

Although several species of *Gelidium* have been and are continuing to be exploited because of the high quality of their agarocolloids, *Gracilaria* is growing in importance owing to its abundance in biomass and successful cultivation, particularly in Chile (Avila and Seguel, 1993; Critchley, 1993). Most of the world's agar production is based on *Gracilaria* (McHugh, 1991; Armisen, 1995). A number of local companies are currently showing interest in the cultivation of *G. gracilis* for agar, or as a possible feed for the more lucrative culture of abalone, and pilot mariculture studies are being carried out both in Saldanha Bay and Lüderitz, Namibia (Engledow and Bolton, 1992).

The most important properties of agars are that they form aqueous solutions at low concentrations, form thermo-reversible gels, are relatively inert, have a significant degree of hysteresis, retain moisture and resist hydrolysis by terrestrial microorganisms (Renn, 1997). There are three grades of agar available: bacteriological, sugar reactive, and food grade. Agar from *Gracilaria* species is normally only used as food grade agar because it has a higher gelling temperature than agars obtained from *Pterocladia* and *Gelidium* species.

Villanueva and Montaño (1999) found that the gel strength and syneresis index of extracted agar from *G. edulis* were considerably enhanced by the addition of sodium, potassium and calcium ions. The ion-driven gelation and peculiar sulphate position conferred the agar's similarity to κ-carrageenan. Studies by Martinez and Buschmann (1996) on agar yield and quality of *G. chilensis*, Bird, McLachlan *et* Oliveira, show that the highest agar yield (20-22%) was obtained when *Gracilaria* was cultivated with seawater as compared to fish effluents. The gel strength, gelling and melting point were higher in agar obtained from algae cultured with fish effluents. During spring, the gel strength, gelling and melting point increased in tanks with fish effluents and decreased in tanks with a supply of fresh water.

Agar prices have increased in the past years owing to worldwide shortages of wild resources of agarophytes, both *Gelidium* and *Gracilaria*, reflecting the need for controlled cultivation to ensure steady and increased supply of agarocolloids at stable prices (Critchley, 1993). A knowledge of the chemistry of agar for the appropriate selection of species more suitable for mariculture is needed. Nuclear magnetic resonance spectroscopy represents a powerful, non-destructive technique for rapid characterisation of the chemical structure of agars and hence for the prediction of the properties of the final product (Murano, 1995).

Gracilaria is of particular interest because of the large quantities of several species available in temperate and tropical regions and for the higher potential, with respect to *Gelidium*, for mariculture. In addition, *Gracilaria* is interesting in that some species yield good grade agarocolloids, whereas others produce agar or agaroids that have different, but unique, properties. With an understanding of the chemical structure and physical properties of agarocolloids, it might be that new uses will be found for these potentially valuable *Gracilaria* extracts (Murano, 1995).

1.7 MARKETING OF *GRACILARIA*

Market demands for gracilarioids have increased markedly in recent years. Japan is the most important producer of agars, but other countries such as Chile have started agar production factories, which has influenced *Gracilaria* imports into Japan (Armisen, 1995). In recent years Chile has become a major producer of agarophytes, and it is now regarded as the biggest producer in South America. More than half of the world agarophyte tonnage consists of *Gracilaria* and over half of this comes from Chile (McHugh, 1991). In 1991 the size and value of the global agar industry was estimated at 11 000 tonnes and US$160 million, obtained from 180,000 tonnes fresh weight raw material (Jensen, 1993). In 1995 the global phycocolloid market was estimated at 61 000 tonnes, valued at US$560 million, of which agar contributed 10 161 tonnes finished product, valued at US$203 million (Guiry, 1997).

The seaweed industry in the western Cape is small in international terms but nevertheless important locally. The seaweed industry earns between R10-15 million (currently US$1.3- 1.9 million) annually in foreign exchange. Annual yields of more than 1000 tonnes dry weight of *Gracilaria* were obtained from Saldanha Bay in the late 1960's and early 1970's (Anderson *et al.*, 1989). The building of an ore jetty (Fig. 1.2) during 1974 decreased beach-cast material to almost zero. By the mid 1980's the resource seemed to be recovering, and in 1988 four hundred dry tonnes beach-cast (Fig. 1.3) material was collected in the northern area of Saldanha Bay. Only this northern, enclosed part of the Bay (Inner Bay) has yielded commercially useful quantities of *Gracilaria* since 1974. However, the resource collapsed again when beach-casts ceased abruptly after December 1988 (Anderson *et al.*, 1992). This collapse reduced the biomass from more than 500 tonnes to less than 1 ton in 5 months (Anderson *et al.*, 1993). Beach-cast material was also noted for the first time in St. Helena Bay in 1991 and 1992 (Stegenga *et al.*, 1997).

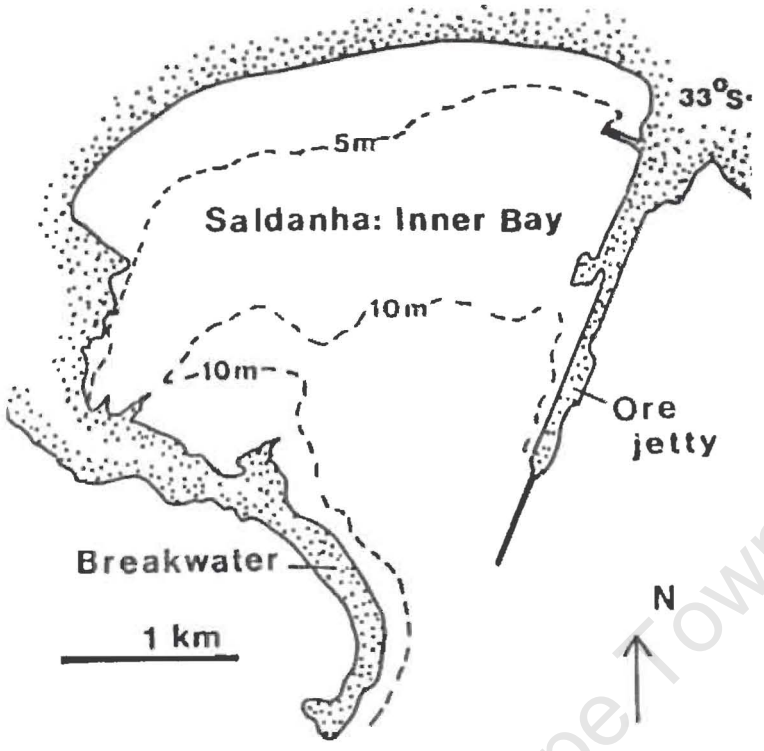

Fig. 1.2 Map of Saldanha Bay displaying depth contours (Anderson *et al.*, 1992).

Fig. 1.3 Collection of beach-cast material in Saldanha Bay (Anderson, pers. comm.).

Demand for agar in the world market is now declining, in part due to the replacement of the gel by other phycocolloids (e.g. carrageenans in tinned foods). The use of algal gels is also continually threatened by the production of an alternative polymer, which may have the same characteristics but can be produced cheaply with a consistent and reliable quality. Approximately 50% of the world supply of agar is utilised in Asian countries, principally Japan, in food and presumably this market is near saturation (Critchley, 1993).

1.8 COMMERCIAL CULTIVATION OF *GRACILARIA*

The cultivation of seaweeds generally is increasing very rapidly as natural stocks are unable to supply demand (Santelices and Doty, 1989). In addition users require very specific end products that may be obtained only from a particular strain (Hanson, 1984). The price of raw *Gracilaria* fluctuates according to supply and demand, species, origin, quality, quantity and currency exchange rates. To compete with the prices of natural *Gracilaria* supplies (in 1994 it ranged from US$ 0.8-1.4 kg^{-1}; Dawes, 1995), *Gracilaria* cultivation has to be productive and reliable or possess special properties such as better processing characteristics, increased agar yield and increased gel strength to compensate for overheads incurred in farming (McHugh, 1991).

The cultivation of *Gracilaria* is now of major importance in several parts of the world, such as Asia, South America, and southern Africa. Gracilarioids have been cultivated in the open sea using various ground planting methods such as direct insertion of thalli in soft substrata, or by ropes and/or nets hung horizontally or vertically in the water column (Fig. 1.4).

In all cases, *Gracilaria* can be cultivated from thalli or spores. Seeding surfaces with spores is a more attractive method as it produces a high biomass from small numbers of plants. The spore-settling technique consists of spores from selected fertile adult thalli being attached to lines (or nets) and subsequently developing into plantlets. After some time they are out-planted onto bigger rafts (Dawes, 1995). *Gracilaria* cultured in open waters using these methods produces high yields of good quality agar. There is a major drawback to this method, apart from it being expensive, spores may not always be available and the initial biomass is very small. Methods have also been investigated using various land-based techniques such as tanks and ponds.

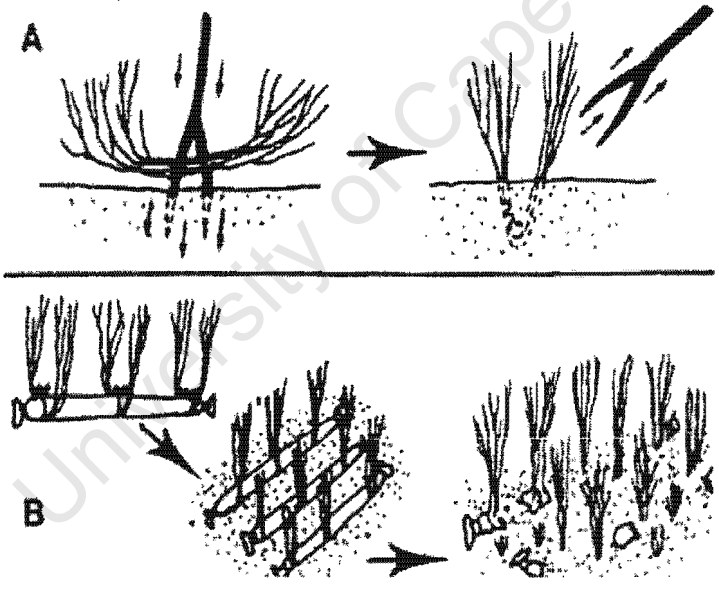

Fig.1.4 Illustration displaying ground planting methods used for cultivating gracilarioids in the open sea. A, Using Y-shaped tools to insert the *Gracilaria* thalli into the sand. B, Using elongated plastic bags filled with sand to anchor thalli of *Gracilaria* on the bottom (Santelices and Ugarte, 1987).

Commercial cultivation is done on a very large scale in several countries, such as Chile (Avila and Seguel, 1993), China (Ren *et al.*, 1984) and Taiwan (Chiang 1981). On a worldwide basis, the Chilean resource is reputed to be the *Gracilaria* crop with the highest

quality gels (Guiseley, 1970; McLachlan and Bird, 1983) and annually supplies 33-50% of the world demand for *Gracilaria*. Pilot scale cultivation is currently being carried out in medium sized farms mainly in Namibia, Venezuela and Malaysia (Guzmán-Urióstegui and Robledo, 1999).

In spite of the many species of *Gracilaria* found in Asia, only three [*G. verrucosa, G. tenuistipitata* Chang *et* Xia and *G. edulis* (S. Gmelin) P. Silva] are cultivated. There is clearly a large domestic market for agar in Malaysia, both in terms of food grade agar as well as high quality agarose for biotechnology research. *Gracilaria changii* (B. Xia and Abbott) Abbott, Zhang, and B. Xia, because of its good quality agar and adaptability to the harsh mangrove conditions, appears to be a good candidate for potential commercialisation (Phang *et al.*, 1996).

Tank or pond cultivation has been attempted with a view to commercial cultivation of *Gracilaria* in Namibia (Rotmann, 1987) and Chile (Edding *et al.*, 1987b) while in Taiwan pond cultivation is extensively practised on a commercial scale (Chiang, 1981). The yields and quality of *Gracilaria* cultured in ponds and tanks is often more difficult to predict (Santelices and Doty, 1989). Pond farming is more complicated in that salinity, which increases due to evaporation, needs to be regulated. For optimal cultivation of *Gracilaria*, efficient seeding, fast growth rate (Table 1.1), and genetic improvements are of prime importance to the farmer. According to Friedlander and Levy (1995), this cultivation may be divided into intensive (tank) and non-intensive (pond) cultivation systems. China and Taiwan are areas in which non-intensive pond cultivation is practised. The first countries to practise intensive tank cultivation were the U.S.A. and Canada, although the high cost of production was a disadvantage.

Table 1.1 Growth rate of *Gracilaria* under various cultivation methods and geographical locations (Molloy and Bolton, 1996).

Species	Substratum	Country	Growth (% d^{-1})
Gracilaria sp.	Rafts	China	8-11.9
G. chilensis		New Zealand	4-5
G. cornea	Ropes	Venezuela	1.4-2.4
G. gracilis	Ropes	Norway	8
G. gracilis		UK	2.6
G. gracilis	Ropes	South Africa	13.8
G. gracilis	Netting ropes	Namibia	6
G. gracilis	Ropes	Namibia	8-12

Some studies on the cultivation of *G. gracilis* in South Africa have indicated that tank cultivation is suitable in integrated abalone and *Gracilaria* cultivation systems where the seaweed is used to feed the animals (Smit, 1995). Other studies indicated that suspended raft cultivation in sheltered areas may be a more appropriate method for the mass cultivation of the species (Anderson *et al.*, 1992; Anderson *et al.*, 1996a).

Commercial interest in the seaweeds of southern Africa developed out of shortages imposed by World War II. Embargoes and disruption of shipping led to shortages in algal-derived colloids. This provided an impetus for a survey of gels from local seaweed stocks (Anderson *et al.*, 1989). The South African seaweed industry developed largely out of the collection of beach-cast material, with minor utilisation of fresh harvested plants. The seaweed industry at Luderitz depends on the beach-cast of *G. gracilis* for its survival. As total annual beach-cast is erratic, mariculture is needed to ensure a constant supply of material to the industry. Though seaweed stocks are small in Namibia, the seaweed industry has a great potential. As natural substrata are in short supply, artificial substrata will have to be used to take advantage of the very rich coastal waters of Namibia. So far only *G. gracilis* is being

cultivated, but other species of potential economic importance may also be considered in the future (Molloy and Bolton, 1992).

The only natural beds large enough to have produced commercial quantities of *G. gracilis* in South Africa occur in the Langebaan Lagoon-Saldanha Bay system on the west coast. Langebaan Lagoon is shallow and the *Gracilaria* occurs on sandbanks at depths of 0.5-1.5 m (Anderson *et al.*, 1992). However, there have never been significant commercial collections of beach-cast material from Langebaan Lagoon. During 1984 the West Coast National Park was created, incorporating most of the Langebaan Lagoon area and effectively ruling out commercial exploitation of these *Gracilaria* beds.

Although the results from experimental mariculture trials are encouraging, Saldanha Bay does not seem to be a good site for commercial gracilarioid farming. Apart from the poor summer growth, local eutrophication, herbivory, mussel fouling and dredging operations have affected growth and production of the local population of gracilarioids (Rotmann, 1990; Anderson *et al.*, 1989, Anderson *et al.*, 1993, Anderson *et al.*, 1996a). The water space and quality for farming gracilarioids is also questionable because there are plans to construct additional jetties for oil and cargo handling facilities. Conflicts are also expected from recreational activities (Smit, 1998). There is therefore a need to explore alternative sites for gracilarioid cultivation. The only other large, sheltered bay on the west coast of South Africa is St. Helena Bay.

Beach-cast material has also been reported from St. Helena Bay (Stegenga *et al.*, 1997. There is also a growing interest in mariculture of *G. gracilis* from local fishing and business communities in St. Helena Bay. Wakibia *et al.* (in press) investigated the suitability

of St. Helena Bay for the suspended cultivation of gracilarioids. They concluded that the high yields of gracilarioids grown in St. Helena Bay might be attributed to consistently high nutrient levels.

1.9 POPULATION GENETIC STUDIES

Molecular methods for the study of evolution and population genetics are becoming increasingly prevalent in biology (Avise, 1994; Hillis *et al.*, 1996), though their use in macroalgae is as yet limited. Population studies are important in community ecology research as aspects such as population variation, population subdivision, gene flow and recruitment can be addressed. The application of DNA-based molecular studies to the investigation of seaweeds has focused mostly on the use of DNA sequences to elucidate systematic and taxonomic problems.

Genetic studies on seaweeds that have determined variation at the population level, have relied mostly on protein electrophoresis (Pearson and Murray, 1997; Benzie *et al.*, 1997), although van Oppen *et al.* (1995b) were able to demonstrate the use of RAPD (randomly amplified polymorphic DNA) data in a study on the red alga *Phycodrys rubens* (L.) Batt. Microsatellite methods are ideal for population genetic studies due to the high variability of microsatellite loci (i.e. multiple alleles). The development, however, of single-locus microsatellite markers can be expensive and time consuming. Single-locus microsatellite markers have been used successfully in a study of the population genetics of *G. gracilis* (Wattier *et al.*, 1997). However, the microsatellite locus used by (Iyer, pers. comm.) was found to be conserved in southern African *G. gracilis* isolates and was not suitable for *Gracilariopsis* isolates. These studies all point to the need for a generally applicable PCR-based method that can assess variation within populations.

DNA derived from organelles, and especially mitochondrial DNA (mtDNA), has proved to be extremely useful in population studies and phylogenetic reconstructions in animals (Avise, 1994). The non-recombinant mode and rapid evolution of this genome often provides multiple haplotypes that can be ordered phylogenetically within a species and provide useful population, genetic and demographic data. PCR primers for the highly variable control region of the mitochondrial genome are readily available (Hillis *et al.*, 1996). Similar variable DNA regions are not known in organelles of plants or algae, with the usual method for scoring plastid haplotypes in plants being restriction fragment length polymorphism (RFLP) analysis (Avise, 1994; Hillis *et al.*, 1996). This procedure, however, usually involves elaborate extraction procedures and protocols.

Nuclear and plastid DNA markers have been used in a variety of studies on the evolution, biogeography and systematics of red algae. Molecular studies in red algae have helped resolve questions at various ranks, ranging from interordinal to intraspecific levels of investigation (Maggs *et al.*, 1992; Freshwater *et al.*, 1994; van Oppen *et al.*, 1995a; Pakker *et al.*, 1996; Lindstrom *et al.*, 1997; Saunders and Kraft, 1997; Zuccarello and West, 1997; Woolcott and King, 1998). Within the last few years, studies have focused on within-population differentiation using allozymes (Sosa and Garcia-Riena, 1993; Sosa *et al.*, 1996; Pearson & Murray, 1997), randomly amplified polymorphic DNA (van Oppen *et al.*, 1995b) and microsatellites (Wattier *et al.*, 1997). More recently, techniques for studying within-population variation using plastid markers have been available (Zuccarello *et al.*, 1999b). Nonetheless, there is still a need for single-locus DNA markers which require a minimum of optimisation, are of useful size and high variability, and are applicable at the population level in a variety of algal taxa.

1.10 AMPLIFIED FRAGMENT LENGTH POLYMORPHISMS

Amplified fragment length polymorphisms (AFLPs) produce information that appears useful for analyses from large biogeographic scales to smaller population-level investigations. In addition, AFLPs have been successful using as little as 0.1-1 ng of DNA (Rosendahl and Taylor, 1997). The AFLP methodology has been successfully applied to the molecular typing of bacteria (Lin *et al.*, 1996), the determination of genetic diversity among populations of the endangered plant *Astragalus cremnophylax* (Travis *et al.*, 1996), genetic analysis of single fungal spores (Rosendahl and Taylor, 1997), detection of diversity in fungal species (Majer *et al.*, 1996) and for assessment of diversity in potato cyst nematode populations (Folkertsma *et al.*, 1996).

The major advantage of the AFLP technique is the large number of polymorphisms that the method generates. Its ability to differentiate individuals in a population makes the technique useful for paternity analysis (Krauss, 1999), gene flow experiments, and also for Plant Variety registration (Law *et al.*, 1998). Other advantageous features of the AFLP technique are: (i) no sequence information is required; (ii) the PCR technique is fast; and (iii) simultaneous analysis of a number of loci is possible (Rafalski *et al.*, 1996). The lack of sequence information needed by the AFLP method is similar to that of the random amplified polymorphic DNA (RAPD) technique. This is contrary to restriction fragment length polymorphism's (RFLP's) and simple sequence repeats (SSRs) that need a high degree of characterisation of the target genome (Robinson and Harris, 1999). This advantage is diminished as more taxa are examined, and as the database of characterised organisms grow and "universal primers" are discovered. Since the AFLP technique is PCR-based it can provide high throughput; Krauss and Peakall (1998) suggest that, after the initial screening

period, up to 100 individuals for 100 polymorphic loci per week could be analysed. This makes AFLP ideal for large-scale population studies. Additionally, dried material can be used for the analysis (Russell *et al.*, 1999), since the method is DNA-based. This enables analysis of species that would be difficult to sample *ex situ* (Harris and Robinson, 1994).

The AFLP technique is based on the selective PCR amplification of restriction fragments from a total digest of genomic DNA (Fig. 1.5). This technique involves three steps: (i) restriction of DNA and ligation of oligonucleotide adapters; (ii) selective amplification of sets of restriction fragments; and (iii) gel analysis of the amplified fragments. PCR amplification of restriction fragments is achieved by using the adapter and restriction site sequence as target sites for primer annealing. Selective amplification is achieved by the use of primers that extend into the restriction endonuclease recognition site, amplifying only those fragments in which the primer extensions match the nucleotides flanking the recognition sites. Using this method, sets of restriction fragments may be visualised by PCR without knowledge of nucleotide sequence. The method allows the specific co-amplification of high numbers of restriction fragments. The number of fragments that can be analysed simultaneously, however, is dependent on the resolution of the detection system. Typically 50-100 restriction fragments are amplified and detected on denaturing polyacrylamide gels.

Population genetic studies have often been disappointing in marine macroalgae due to a combination of unreliable detection and/or low polymorphism (Sosa and Garcia-Reina, 1992; Williams and Di Fiori, 1996). The availability of more reliably detectable and highly polymorphic markers would therefore represent a major step forward for the development of algal genetics and especially population genetics.

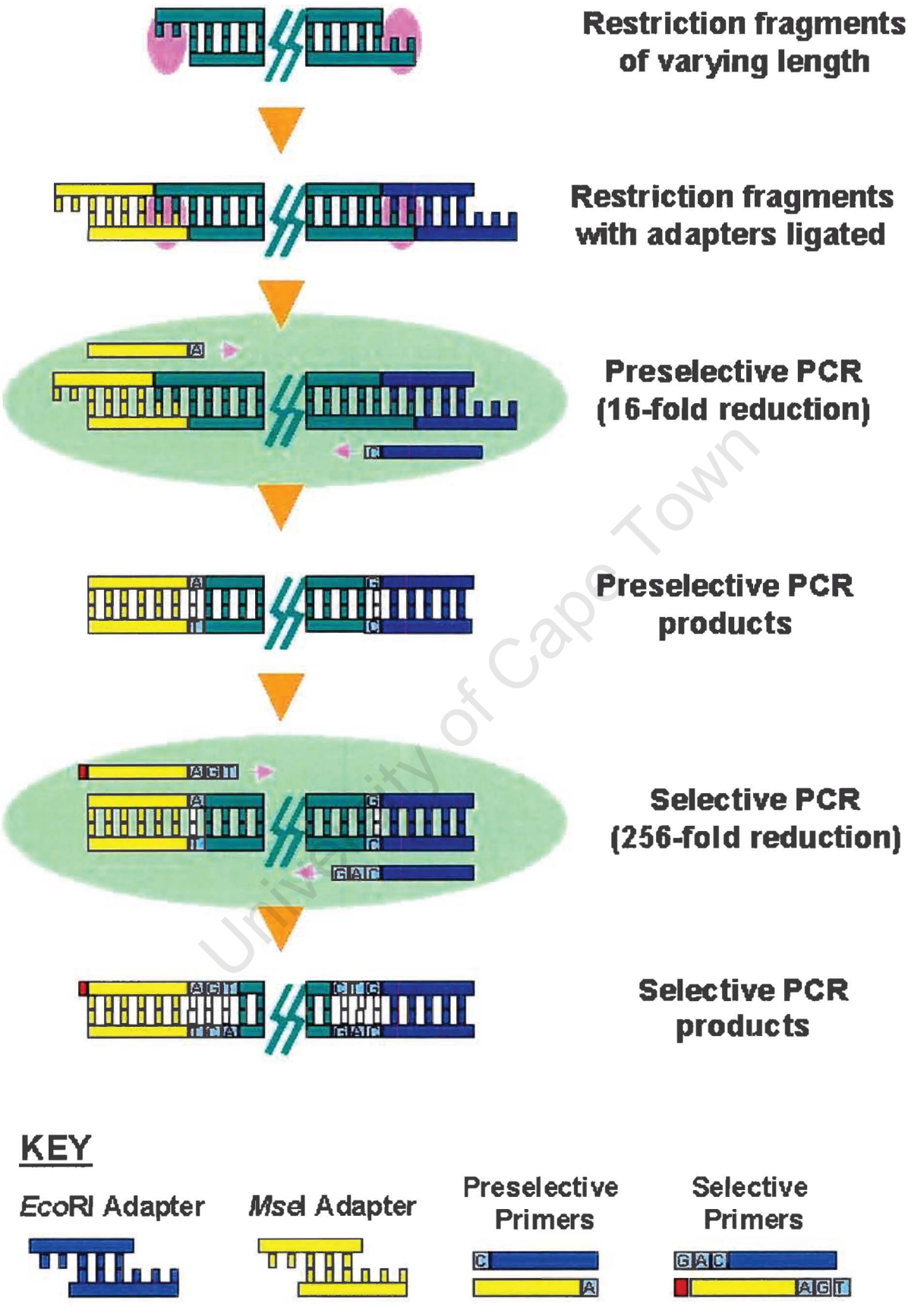

Fig. 1.5 Schematic illustration of the AFLP principle.

1.11 MOTIVATION FOR STUDY

Previous researchers have found the taxonomy of terete species of the Gracilariaceae to be complex. Gross morphological characters have been the main means of identification of commercial stocks and incorrect applications have led to a number of misidentifications. Unfortunately, many of the necessary taxonomic characters cannot be adequately determined in the field. As a result, almost all commercial resources which are generally terete, stringy thalli have been assigned to *G. verrucosa* (European and South African material now known as *G. gracilis*), regardless of locality collected (Critchley, 1993).

The discovery that plants from a number of regions, which were originally described as *Gracilaria* in fact belong to a different genus, *Gracilariopsis* (Bird and Rice, 1990), has presented difficulties with these two genera. Problems of identification arise mainly because these species are morphologically plastic, and populations in nature often reproduce entirely asexually. A key characteristic of *G. gracilis* is the possession of tubular nutritive cells connecting the inner gonimoblast cells of the pericarp or the floor of the cystocarp in the female fertile structures. These features are absent in *Gracilariopsis* material.

The last published report of fertile material from South African populations of *G. gracilis* was in 1956 (Stegenga *et al.*, 1997). However, Anderson (pers. comm.) obtained fertile material from Saldanha Bay and St. Helena Bay in the early 1990's. Based on the presence or absence of nutritive tubular cells, these were defined as *G. gracilis* and *Gracilariopsis* sp., respectively. Thus, it is possible that material from the Langebaan Lagoon-Saldanha Bay system is *G. gracilis,* whereas material from St. Helena Bay is *Gracilariopsis* sp. However, fertile plants are rare, and male plants have never been reported in the south western Cape. Hence, these taxonomic problems may only be solved by molecular or

biochemical systematic techniques, which are being carried out in a number of laboratories (Goff and Coleman, 1988; Bird and Rice, 1990; Candia *et al.*, 1999).

It is hypothesised that the use of molecular techniques will be useful in determining the taxonomic definition of material in the south western Cape and in identifying strains for selection in cultivation.

The following objectives were established:

(i) to determine whether what is known as *G. gracilis* in the south western Cape is a single species or a species complex;

(ii) to determine whether south western Cape material is correctly placed in *Gracilaria* or *Gracilariopsis*;

(iii) to determine whether the use of molecular techniques will be able to show how much genetic variation there is between sub-populations in the Langebaan Lagoon, Saldanha Bay and St. Helena Bay systems.

The seaweed industry on the west coast of South Africa mainly comprises the collection and drying of beach-cast material. A number of companies are planning to begin commercial mariculture of *Gracilaria* in Saldanha Bay and St. Helena Bay. In addition, *Gracilaria* is cultivated in pond culture in the Eastern Cape as a food source for cultured abalone. A National Research Foundation project that has been running for 4 years is concerned with providing scientific assistance to the fledging seaweed mariculture industry. This has suffered from lack of molecular expertise, causing difficulties in understanding genetic diversity in the resource.

It is known that *Gracilaria* in many parts of South Africa is seldom fertile, with clones spreading vegetatively. Strains of the species exhibit different characteristics under mariculture conditions, such as yield, agar content, growth rate, pigmentation, etc. (Engledow and Bolton, 1992; Wilson and Critchley, 1998). Although a number of these strains have slightly different appearances, there is no scientific basis available for selection of strains for cultivation. This is mainly due to the lack of knowledge as to whether the different morphotypes are based on genetic differences, or whether it is an adaptation to an ecological or environmental condition.

Growth studies on mariculture rafts in Saldanha and St. Helena Bays have revealed differences in economically important characteristics between isolates from Langebaan Lagoon, Saldanha Bay and St. Helena Bay (Anderson *et al.*, 1992; Wakibia *et al.*, in press). This study focuses on identifying gracilarioid taxa in the south western Cape and determining the level of genetic variability within these taxa in this region.

Chapter 2

SAMPLE COLLECTION AND ANATOMY

2.1 INTRODUCTION

The west coast is characterised by an open coastline which is exposed to a generally high energy wave regime. Two prominent coastal features are the Langebaan Lagoon-Saldanha Bay system and St. Helena Bay, in which more sheltered conditions prevail. Saldanha is the largest enclosed bay on the west coast of southern Africa.

Saldanha Bay is situated approximately 100 km north of Cape Town. The Bay is part of a larger complex known as the Langebaan Lagoon-Saldanha Bay system that opens to the sea through a wide mouth (Big Bay). This system falls within the Benguela upwelling region of the south east Atlantic ocean. The ecosystems associated with this system are largely controlled by the upwelling of the cold, nutrient rich Benguela current and the bay provides a diversity of habitats supporting marine floral and faunal communities. Approximately 100 km north of Saldanha Bay is St. Helena Bay. St. Helena Bay also falls within the Benguela upwelling system but differs from the Langebaan Lagoon-Saldanha Bay system by being a larger, open bay.

Natural populations of gracilarioids inhabit these cold south western Cape waters. These seaweed populations tend to thrive in shallower waters (<6 m). South African plants have in the past generally been assigned to *Gracilaria verrucosa* (now *G. gracilis*). The only evidence to support this taxonomic identification is based on molecular data of Namibian *G.*

gracilis material (Bird *et al.,* 1994a). In addition it is well known that terete gracilarioids, which span 2 genera, are morphologically similar.

For this study it was important to determine if these populations could be distinguished solely based on internal and external morphological features. Fertile cystocarps from Saldanha Bay were obtained and this initiated the laboratory cultivation of spores. It was also important to test the growth of vegetative thalli under experimental conditions. This information would be useful for future cultivation of gracilarioid material.

Since these seaweeds occur in different ecological systems, which differ in water temperature, salinity, pH and irradiance as well as harbouring different ecological communities, it is important to determine the taxonomic identities of these organisms.

2.2 MATERIALS AND METHODS

2.2.1 Sample collection

The positions of patches of gracilarioid material in Saldanha Bay, Langebaan Lagoon, and St. Helena Bay were determined and accurately mapped using a Geographical Positioning System (Magellan GPS Meridian, USA). Sampling in St. Helena Bay was carried out in subjectively defined sampling areas (radius approx. 25-40 m), as no distinct patches were observed, except for the salt marsh (designated as Patch E). Isolates representative of each patch in Saldanha Bay (Table 2.1), Langebaan Lagoon (Table 2.2) and St. Helena Bay (Table 2.3) were collected by divers (Figs. 2.1-2.3). The following nomenclature was used (e.g. Patch SalA3): (i) the first 3 letters refers to the location (e.g. 'Sal'- Saldanha Bay); (ii) the fourth letter refers to the patch (e.g. 'A'- Patch A); and (iii) the number refers to the isolate (e.g. '3'- isolate 3). The number of isolates collected per patch was dependent on patch size and level of contamination (Table 2.1-2.3). These isolates were stored in labelled plastic bags containing seawater (Fig. 2.4).

2.2.2 Sample processing

Fresh samples (Fig. 2.5) were processed in the laboratory: (i) The thallus (voucher specimen) was pressed onto blotting paper and covered with a liner to prevent movement. Pressed thalli were weighed down for 4 weeks at room temperature. (ii) A portion of the thallus was pickled in a 5% formalin (Fig. 2.6) solution and stored in the dark. (iii) Tips (c.a. 1cm) were excised from each thallus and placed in sterile crystalline dishes containing Provasoli Enriched Seawater medium (Provasoli, 1968; Appendix A) and incubated at 15°C. The culture media was supplemented with germanium dioxide 2.5 µg/l, which inhibits the growth of diatoms (Markham and Hagmeier, 1982). The sterile culture medium was

replenished weekly. (iv) A healthy portion of the thallus, free of epiphytes, was ground to a fine powder with a mortar and pestle (Fig. 2.7) using liquid nitrogen. The ground material (0.03-0.05 g) was aliquoted into 1.5 ml sterile Eppendorfs and stored at -20°C. This material was used for molecular studies in Chapters 3 and 4.

Mature cystocarps (Fig. 2.8) obtained from a Saldanha Bay specimen were cut into small pieces and inoculated in a Petri dish containing Pravosoli Enriched Seawater medium. Petri dishes were incubated at 15°C and 20°C and monitored for the development and growth of spores.

Table 2.1 Description of collection sites in Saldanha Bay.

Site	Isolates	Location	Depth (m)	Condition
Patch SalA	8	17° 58' 43" E 32° 59' 52" S	2.5	Healthy
Patch SalB	7	17° 58' 45" E 33° 00' 02" S	4.5	Healthy
Patch SalC	7	17° 57' 55" E 33° 00' 08" S	4.5	Healthy
Patch SalD	8	17° 57' 58" E 32° 59' 55" S	3	Healthy
Patch SalE	7	17° 57' 09" E 33° 00' 14" S	3	Slightly contaminated with *Ceramium diaphanum*
Patch SalF	8	17° 57' 25" E 33° 00' 07" S	3	Contaminated with *Ulva*, isopod eggs and *Ceramium diaphanum*
Patch SalG	2	17° 57' 26" E 33° 00' 23" S	5.5	Contaminated with *Ulva*, isopod eggs and *Ceramium diaphanum*

Table 2.2 Description of collection sites in Langebaan Lagoon.

Site	Isolates	Location	Depth (m)	Condition
Patch LanA	3	17° 58' 43" E 32° 59' 52" S	0.5	Healthy
Patch LanB	5	17° 58' 45" E 33° 00' 02" S	1.5	Healthy
Patch LanC	5	17° 57' 55" E 33° 00' 08" S	1.5	Healthy
Patch LanD	5	17° 57' 58" E 32° 59' 55" S	1.5	Healthy
Patch LanE	4	17° 57' 09" E 33° 00' 14" S	0.5	Healthy
Patch LanF	6	17° 57' 25" E 33° 00' 07" S	0.5	Healthy

Table 2.3 Description of collection sites in St. Helena Bay.

Site	Isolates	Location	Depth (m)	Condition
Patch HelA	5	18° 08' 28" E 32° 46' 12" S	2.5	Healthy
Patch HelB	2	18° 10' 22" E 32° 43' 34" S	3.5	Healthy
Patch HelC	5	18° 09' 36" E 32° 43' 48" S	4	Healthy
Patch HelD	3	18° 09' 07" E 32° 44' 09" S	4	Healthy
Patch HelE	5	18° 08' 57" E 32° 44' 30" S	<0.5	Healthy
Patch HelF	5	18° 01' 18" E 32° 45' 10" S	0.5	Healthy
Patch HelG	1	18° 01' 40" E 32° 46' 50" S	0.5	Healthy
Patch HelH	3	18° 02' 47" E 32° 46' 11" S	0.5	Slightly contaminated with *Ceramium diaphanum*
Patch HelI	1	18° 08' 39" E 32° 46' 22" S	0.5	Contaminated with *Enteromorpha* and isopod eggs

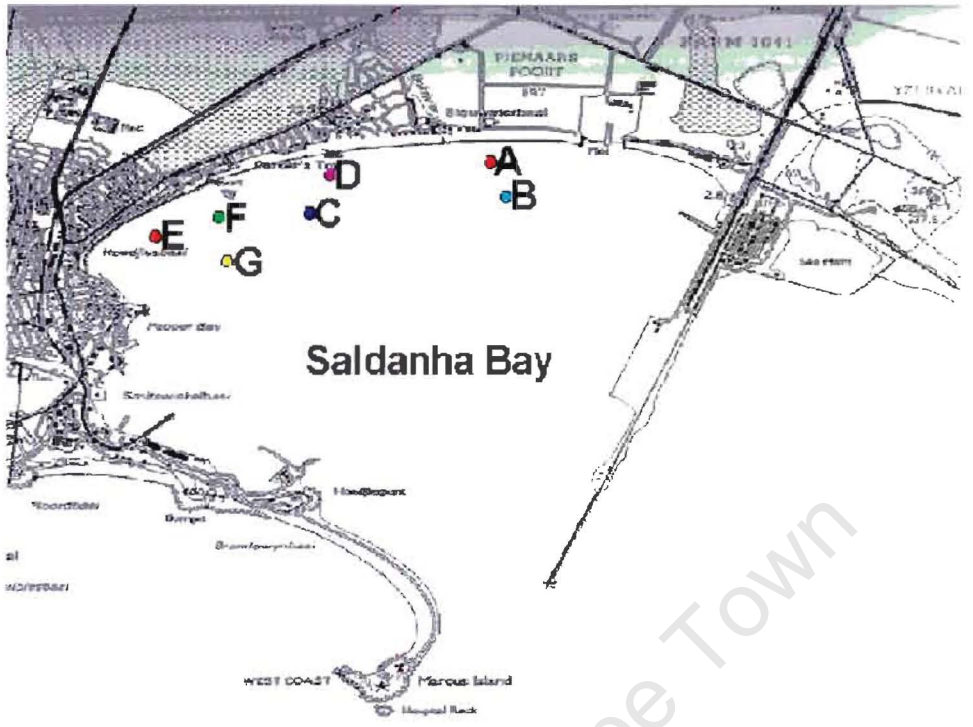

Fig. 2.1 Map of Saldanha Bay displaying location of patches.

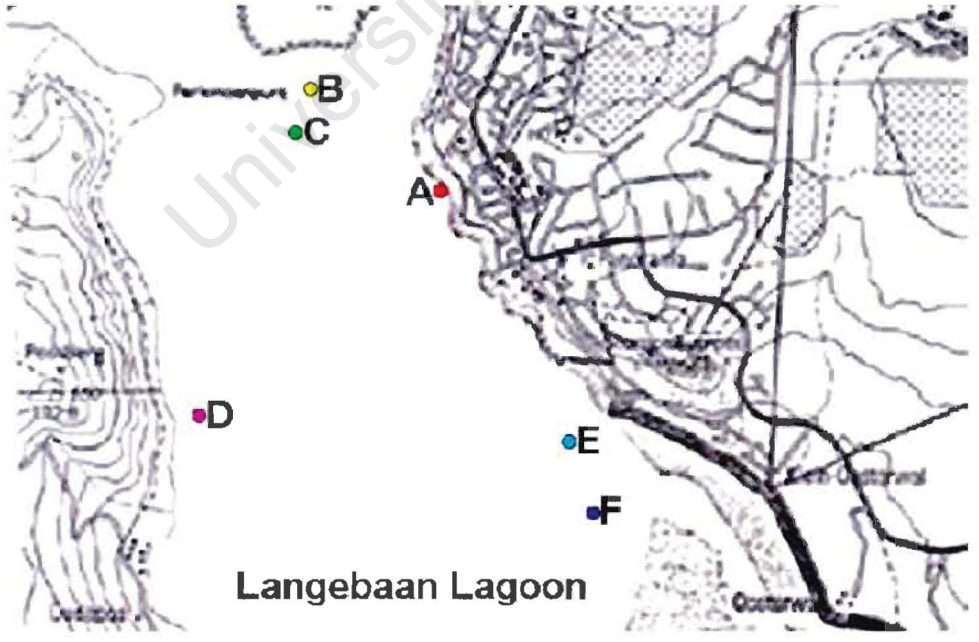

Fig. 2.2 Map of Langebaan Lagoon displaying location of patches.

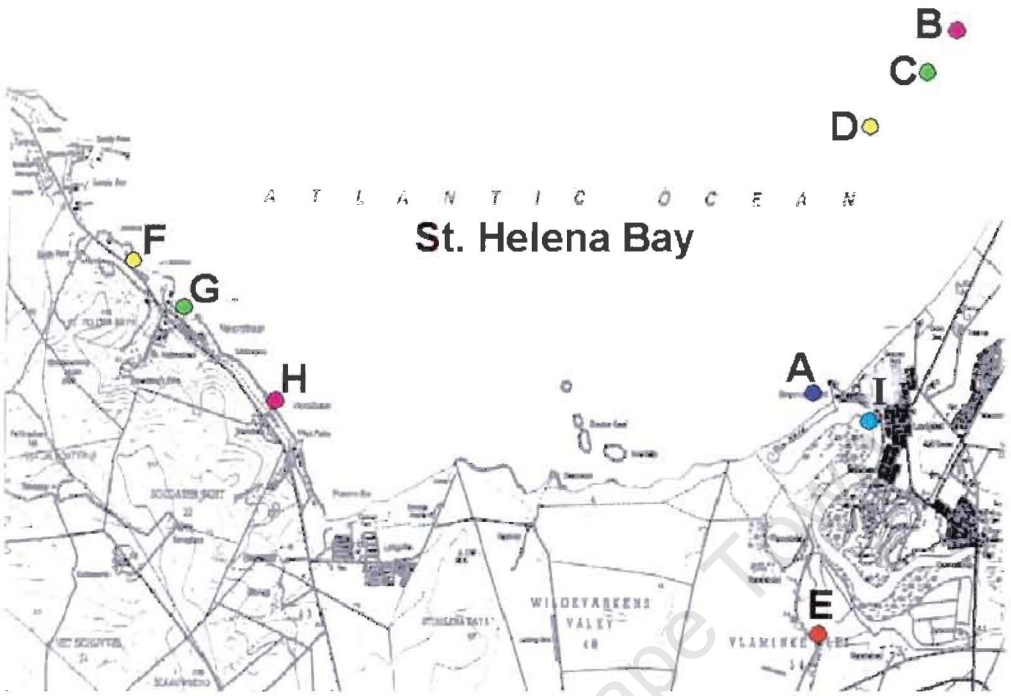

Fig. 2.3 Map of St. Helena Bay displaying location of patches.

Fig. 2.4 Sampling in Saldanha Bay. Isolates were stored in labelled plastic bags containing sea water.

Fig. 2.5 Freshly harvested gracilarioid material.

Fig. 2.6 Gracilarioid material pickled in a 5% formalin solution.

Fig. 2.7 Material ground in liquid nitrogen using a mortar and pestle.

Fig. 2.8 Fertile cystocarps (arrow) on material found in Saldanha Bay.

2.2.3 Sectioning

Sectioning was performed on formalin preserved samples using a freezing microtome (E. Leitz Wetzlar, Germany). Samples were embedded in Hamilton's freezing medium (Appendix A) prior to sectioning. Sections were 20-50 μm thick depending on the type of sample sectioned. Sections were stained for 2 minutes with aniline blue and mounted on slides. Slides were fixed with a 50% Karo solution to prevent drying of mounted samples. Sections were viewed using a compound microscope (Leitz Diaplan, Switzerland) and captured digitally using a Zeiss Axiocam (Germany) camera.

2.3 RESULTS

2.3.1 Sample collection

Seven distinct patches of gracilarioids were sampled in Saldanha Bay. The material could be described as mats of plants growing on and in the surface sand. Plants lacked holdfasts. The base of the thalli was generally buried within the surface sand. Depths of plants sampled ranged from 2.5-5.5 m (Table 2.1). Isolates from Patches F and G were contaminated with various molluscs, epiphytes and microbes. A significant level of fish factory waste was noted in this locality, offering a possible explanation for the high levels of contamination.

Six distinct patches of gracilarioids were sampled in Langebaan Lagoon. Material was also in the form of mats, although it was noticed that patches were much larger (approx. 100 cm^2). No holdfast was observed. Material was trapped within the surface sand as observed in Saldanha Bay material. Plants occurred at depths of 0.5-1.5 m (Table 2.2). Five samples from Patch F (LanF1-LanF5) displayed very fine morphologies. However, it was also noted that other plants from the same patch had more typical gracilarioid morphologies.

Nine sampling sites were chosen in St. Helena Bay. Sites were chosen based on the availability of material. Patches were defined as an area within which samples occurred. Patches within the intertidal had a radius of approx. 20 m, whereas patches within the Bay had a radius of approximately 100 m. The salt marsh and Berg river mouth were defined as Patches E and I, respectively.

Interestingly, samples in St. Helena Bay did not occur in mats. Samples were generally scattered, the exception being Patch E, which was a shallow salt marsh. Samples tended to grow in small clumps in this marsh. Samples occurred in a range of depths (0.5-4 m) and no holdfast was observed (Table 2.3). The isolate obtained from Patch I was contaminated with epiphytes and could not be used in this study.

2.3.2 Analysis of gross external morphology

Analysis of branching patterns of pressed samples revealed differences in morphologies. Most samples displayed a single central axis with scattered 2° (secondary) and 3° (tertiary) branching patterns. The apex of the main stem generally ended with several smaller branchlets. However, some variation to this pattern was observed: (i) shorter tertiary branches; (ii) short central axis with a y-shaped divergence at the apex of the main stem resulting in 2° branches (these 2° branches had several 3° branches arising from them); (iii) single main stem with 2° branches, but no 3° branches; and (iv) the most striking branching patterns were observed in samples that displayed a greatly thickened central stem with numerous short 2° branches arising from the central axis. None of these morphologies predominated within a single Bay system (Fig. 2.9, A-F; Fig. 2.10, A-D; Fig. 2.11, A-F), however it was noted that a single morphology generally predominated within a patch.

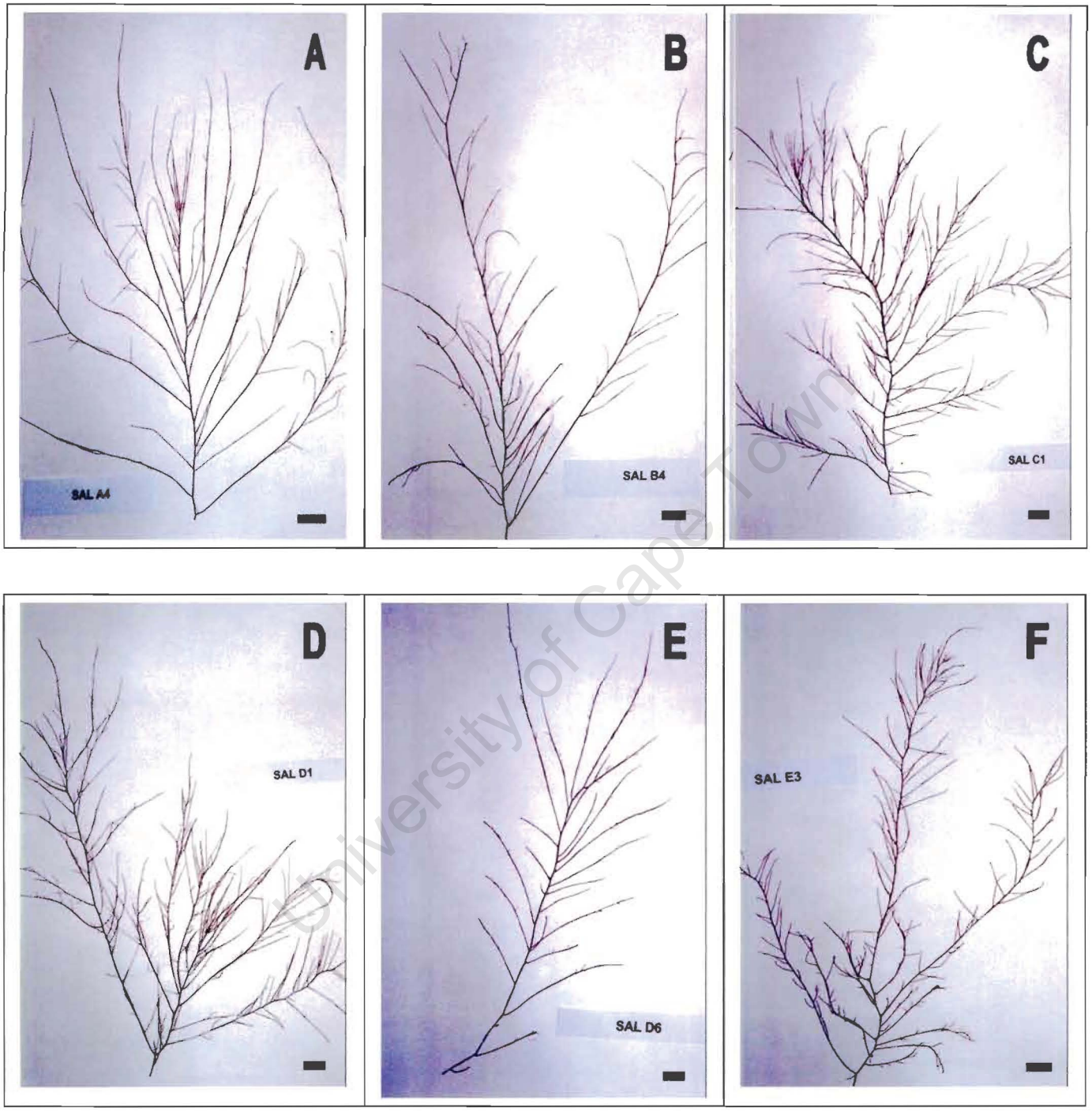

Fig. 2.9 Pressed samples of Saldanha Bay. A, SalA4; B, SalB4; C, SalC1; D, SalD1; E, SalD6; and F, SalE3. Scale bar represents 1 cm.

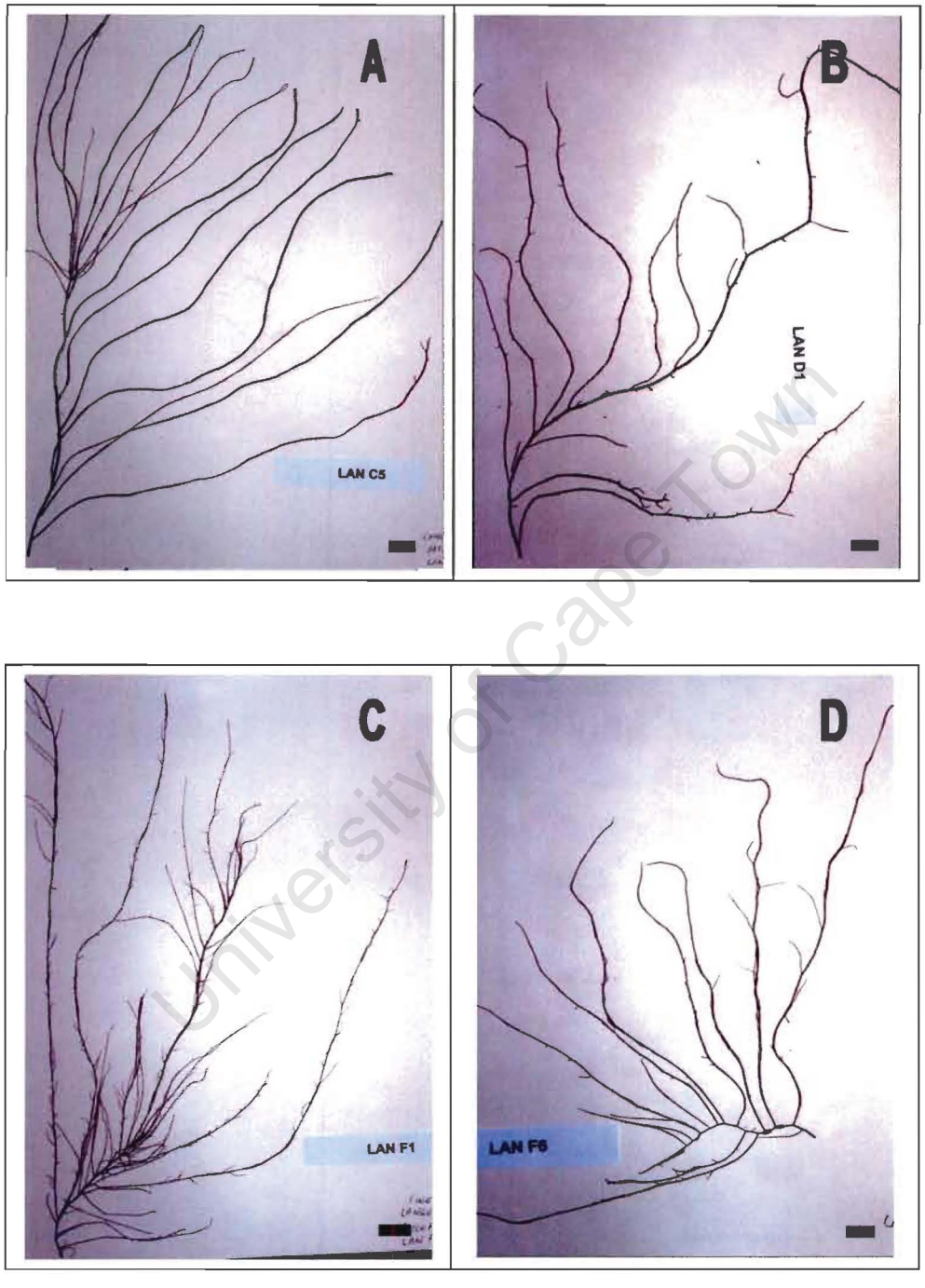

Fig. 2.10 Pressed samples of Langebaan Lagoon. A, LanC5; B, LanD1; C, LanF1; and D, LanF6. Scale bar represents 1 cm.

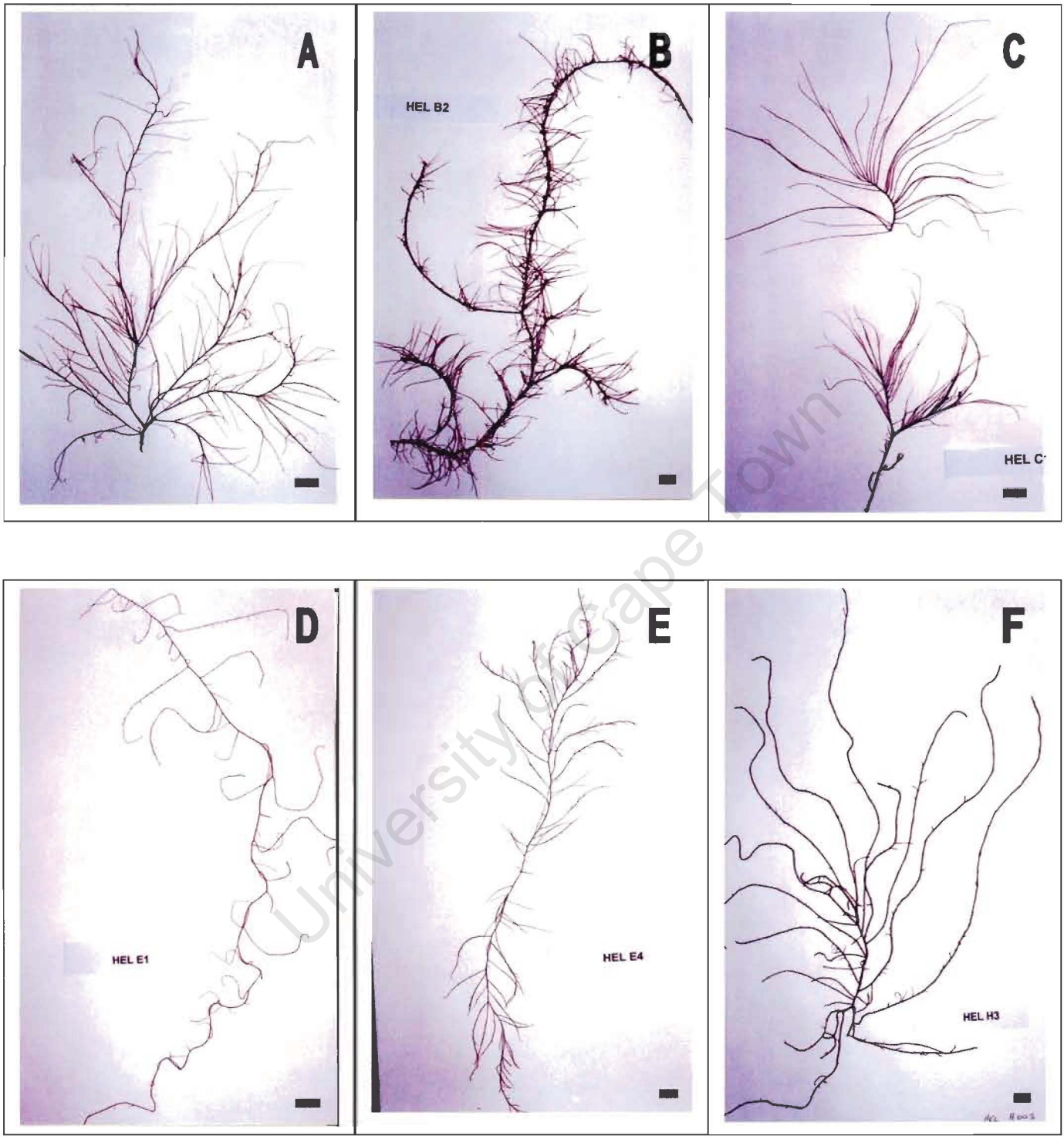

Fig. 2.11 Pressed samples of St. Helena Bay. A, HelB1; B, HelB2; C, HelC1; D, HelE1; E, HelE4; and F, HelH3. Scale bar represents 1 cm.

2.3.3. Cultivation

The excised 1 cm tips grew well during the first 6 months. However, it was observed that apices cultured in this way resulted in stunted growth (Fig. 2.12). This could probably be attributed to a lack of water movement. After 6 months of experimental cultivation, plants were approximately 5 cm in length with numerous side branches. However, at this point the plants started to bleach and eventually died. Experiments using 1 cm excised tips from the original plants were successful. The successful cultivation of these tips was only observed for a few months when the plants reached lengths of approximately 3-5 cm. After reaching this length these plants only grew by lateral branches resulting in stunted growth. Previous studies (Isaac, 1956) used sections of the thallus, whereby side branches were pruned indicating that the axis did not increase in length but new branches arose at intervals. A single isolate collected from Saldanha Bay had a green thallus (Fig. 2.13). DNA extraction of this isolate relied on successful laboratory cultivation, as the thallus was too small to yield sufficient DNA. This isolate unfortunately died after 3 months and hence could not be included in this study. Spores germinated best at 20°C, however these were contaminated with epiphytes very early in their development and died. Fertile cystocarps obtained became contaminated and died before sectioning could be performed.

2.3.4 Analysis of vegetative and reproductive morphology

General vegetative morphological characteristics observed in the Langebaan Lagoon-Saldanha Bay material were erect thalli ranging from 0.2-1.5 m in length, not attached by means of a holdfast, pseudoparenchymatous throughout and having up to three orders of

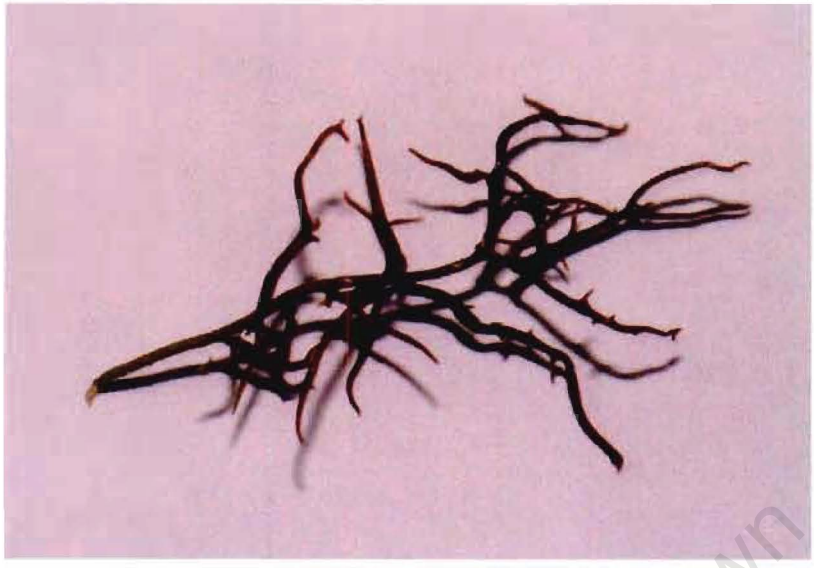

Fig. 2.12 Stunted growth of a laboratory cultivated gracilarioid.

Fig. 2.13 Green thallus of a gracilarioid cultivated in a Petri dish.

branches. Temperature, depth, salinity and irradiance within sampling areas played a major role in the length and growth of the thallus. These isolates were terete throughout and purplish in colour. Although a single main axis is sometimes distinguishable (Fig. 2.9, C), there are often few to several (Fig. 2.9, D) main axes arising from a shortened stem. Branching is irregularly subdichotomous (Fig 2.9, D) to unilateral (Fig 2.10, A). Second and third order branches taper toward their apices and are not constricted at the point of insertion.

Transverse sections through the apices of young branches of isolates from Saldanha Bay and Langebaan Lagoon display a loosely compacted inner cortex and thin walled medullary cells with large intercellular spaces (Fig. 2.14, A and B). The boundary between the cortex and medulla is indistinct except that cells in the outer two to three layers are conspicuously pigmented. The medulla is composed of enlarged, highly vacuolated, thick walled cells (Fig. 2.14, C-E). Outer cortical cells are typically longer than broad (Fig. 2.14, F and Fig. 2.15, A).

Cross sections of fertile material (Fig. 2.15, B) from the Langebaan Lagoon-Saldanha Bay system (Iyer, pers. comm.) reveal tubular nutritive cells connecting the inner gonimoblast cells with cells of the pericarp or the floor of the cystocarp. These features correspond with referenced data of *Gracilaria*. Thus it is likely that material from this area is predominantly *Gracilaria*.

Samples from St. Helena Bay show terete thalli, 0.3-0.5 m in length, brown to reddish purple, and consisting of few to several long, slender, terete, percurrent axes 0.5-3 mm wide. Thalli are not attached to substratum by means of a holdfast. Axes were found to taper toward the base and branch irregularly, especially in lower portions. Branching is either sparse, giving the habit a stringy appearance (Fig. 2.11, D), or dense, with few to many very short to long laterals that may or may not be constricted basally at points of insertion on the main axis.

Thalli are pseudoparenchymatous throughout. Transverse sections through a mature branch (Fig 2.15, C) reveal a sharp gradation from a small celled outer cortical zone to a large celled, thin walled central medulla. The cell walls of these medullary cells were collapsed (Fig. 2.15, D). This is probably due to staining with aniline blue. However the same was not observed for cross sections of the Langebaan Lagoon-Saldanha Bay system samples. Medullary cells are rich in cytoplasm, and stain darkly close to the tip of young branchlets (Fig 2.15, C). The cortex consists of one or two cell layers of pigmented, elongated outer cells (Fig 2.15, E). Outer layers of of the cortex are often arranged in a palisade (Fig 2.15, D and E).

Cross sections (Fig. 2.15, F) of fertile material from St. Helena Bay (Iyer, pers. comm.) reveal that nutritive tubular cells are absent. Gonimoblasts fill the entire cystocarpic cavity. These features correspond with referenced data of *Gracilariopsis* species. Thus it is likely that material from this area is predominantly *Gracilariopsis*.

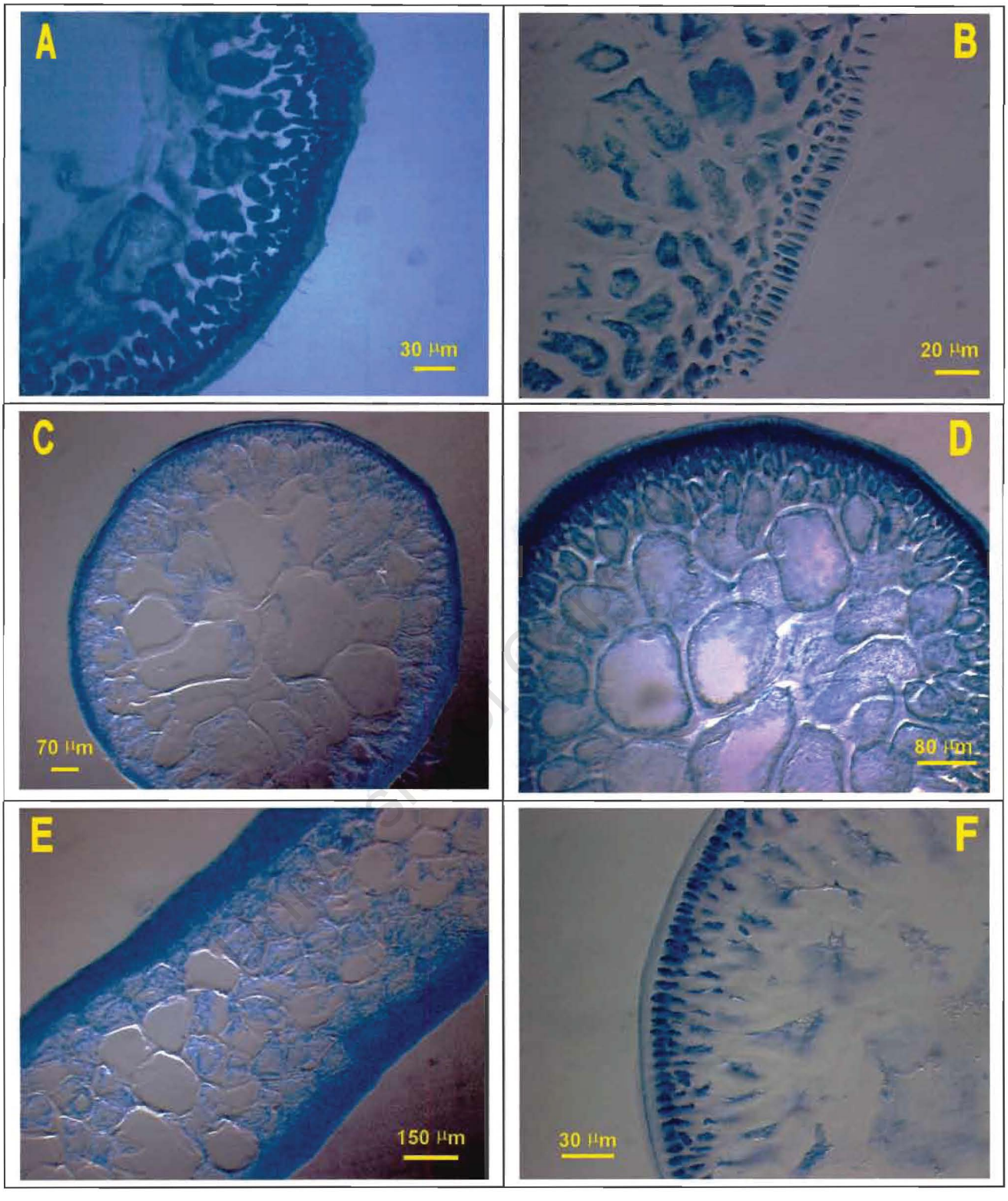

Fig. 2.14 A-F, Langebaan Lagoon-Saldanha Bay material. A-B, Cross and longitudinal section through the apices of young branches. C-E, Cross and longitudinal sections through the main stem of mature branches. F, Cross section of mature branch showing cortical cells.

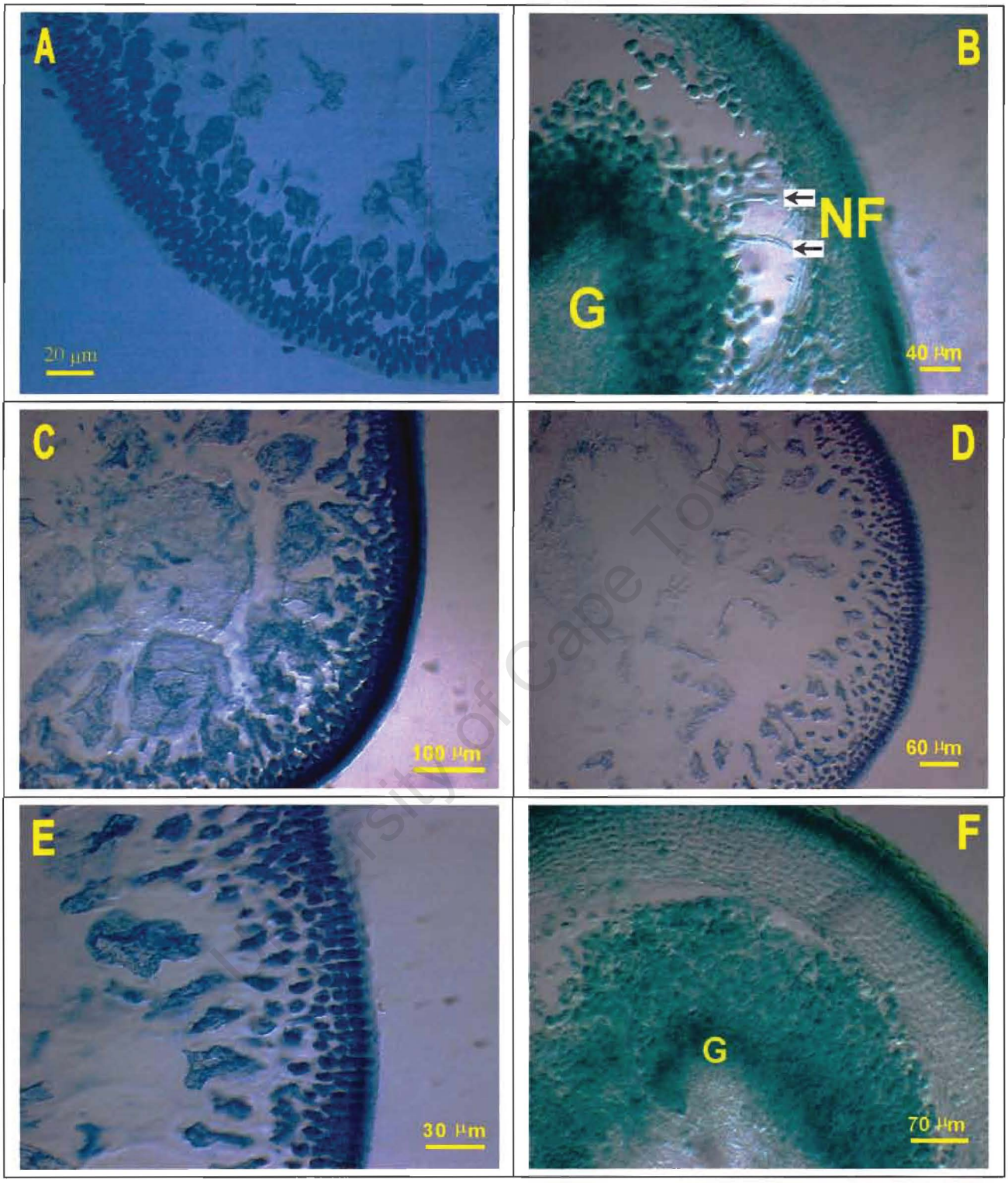

Fig. 2.15 A, Cross section through mature branch showing cortical cells of Saldanha Bay material. B, Fertile material from the Langebaan Lagoon-Saldanha Bay system (Iyer, pers. comm.) NF: nutritive filament, G: gonimoblast. C-F, St. Helena Bay material. C, Transverse section through a mature branch. D-E, Transverse section showing the outer layers of the cortex arranged in a palisade. F, Fertile cystocarps from St. Helena Bay (Iyer, pers. comm.), G: gonimoblast.

2.4 DISCUSSION

Eutrophication of marine waters, particularly bays, is a well known phenomenon which leads to various environmental problems (Vollenweider, 1992). The Langebaan Lagoon-Saldanha Bay system is the only deep, large embayment in the otherwise very exposed West Coast of South Africa. It is also the site of numerous and sometimes conflicting human activities. While the shallow southern portion (Langebaan Lagoon) forms part of a nature reserve (West Coast National Park), the deeper Saldanha Bay is divided into Small Bay and Big Bay by a quay which is used for ore and oil loading. Small Bay is used as a fishing harbour, for mariculture (it is the centre for mussel cultivation in SA), and various recreational activities. Small Bay, where most of these activities occur, also receives about 650 tons of nitrogen annually over and above natural fluxes, in the form of liquid fish-producing waste that is discharged from 2 factories on its west shore. This has measurable effects on benthic macrofauna (Christie and Moldan, 1977). Fish waste from the larger (pelagic processing) operation was also implicated in causing a problem bloom of the opportunistic green alga *Ulva lactuca* which reduced the benthic *Gracilaria* populations and fouled commercial beach casts of this economic agarophyte in 1993-1994 (Anderson *et al.*, 1996b).

The results of sampling of the Langebaan Lagoon-Saldanha Bay system points to the preference of *Gracilaria* for the shallower waters of Small Bay (Saldanha Bay) and Langebaan Lagoon. However, the larger biomass observed in Langebaan Lagoon of *Gracilaria* may be attributed to the high water flow due to tides which may improve nutrient and gas uptake. It is also possible that the shallow waters offer better light conditions.

It was reported that natural populations of *Gracilaria* in Saldanha Bay often carry heavy growth of *Ceramium* (Anderson *et al.*, 1992). They considered it likely that the numerous amphipods, isopods and possibly the small fish that are always present on *Gracilaria* may play a role in keeping it free from epiphytes. Brawley and Frei (1987) found that similar mesoherbivores (especially amphipods and caprellids) have a major effect in removing epiphytic algae from *Gracilaria* grown in a Chinese mariculture farm. Hence it is possible that *Ceramium* growth is inversely proportional to the biomass of mesoherbivores. Anderson *et al.* (1998) found that *Paridotea reticulata* (isopod) consumed 0,043±0.007 $mg_{(dry)}$ *Ceramium diaphanum* per $gram_{(dry)}$ of isopod per day. Consumption of *Gracilaria* was also observed when *Ceramium diaphanum* growth was low.

All the samples collected for this study had no holdfast. Interestingly Isaac (1956) stated that *G. confervoides* was found attached to a hard substratum by means of attaching discs (holdfast). He found these plants only on the concrete slipway of the South African Air Force crash station at Langebaan Lagoon where the plants are fairly common in shallow water below low water of spring tides. It has also been reported that attached populations of *Gracilaria* frequently contain fertile individuals (Critchley, 1993). This association between the presence of holdfasts on fertile plants could explain why present material propagates vegetatively.

Cross sections of fertile cystocarps from the Langebaan Lagoon-Saldanha Bay system and St. Helena Bay show similarities to previously referenced *Gracilaria* and *Gracilariopsis*, respectively. From these results it can be inferred that Langebaan Lagoon-Saldanha Bay

isolates are *Gracilaria* and those of St. Helena Bay are *Gracilariopsis*. This is confirmed by 18S rDNA sequences in Chapter 3.

St. Helena Bay seems to favour the growth of *Gracilariopsis*. However abiotic conditions in the salt marsh (Patch E) and river mouth (Patch I) are similar to that in the Langebaan Lagoon-Saldanha Bay system where *Gracilaria* thrives. It could be that *Gracilaria* was never introduced to these ecosystems which are relatively sheltered from the rest of St. Helena Bay. It is known that *Gracilaria* from Saldanha Bay has been introduced in the vicinity of St. Helena Bay on experimental rafts. However, Veldrift (where the river mouth and salt marsh are located) is quite distant from the town of St. Helena and it is improbable that the seaweed would have spread within such a relatively short time period.

External morphological characteristics differed within samples occurring in various locations. For example, Fig. 2.10, C and Fig 2.11, A which are pressed samples of *Gracilariopsis* from Langebaan Lagoon and St. Helena Bay (Chapter 3), respectively, (likewise Fig. 2.9, D and Fig 2.10, D are pressed samples of *Gracilaria* samples of Saldanha Bay and Langebaan Lagoon [Chapter 3], respectively), reveal different external morphologies. This could be attributable to the state of growth of plants.

Spore cultivation has a two fold significance: (i) it is necessary for studying the life cycle, and (ii) plants generated are useful for taxonomic purposes. Tetrasporophytes generated from carpospores release tetraspores which result in male and female gametophytes. However, these plants are not encountered frequently in natural populations, hence the need for spore

cultivation. The presence of epiphytes such as *Ulva* and *Sporocladopsis*, on spores is a shortfall of this method and must be addressed before successful cultivation is obtained.

Although *Gracilaria* in culture should have a typical *Polysiphonia*-type life history, abnormalities have been occasionally reported (van der Meer, 1981). Kim (1970) discovered that *G. verrucosa* from the Tubul River (Chile) formed tetrasporangia on female thalli. Later Kim and Candia (1977) found that tetrasporophytes were able to produce tetraspores which developed into tetrasporophytes. Experiments performed by Edding *et al.* (1987a) found that isolated plants were able to develop cystocarps, suggesting that the formation of the gonimoblast was apomictic. This observation was said to explain their inability to find male plants in La Herradura Bay.

Sectioning of vegetative thalli of *Gracilaria* has revealed prominent medullary cell walls, which are less prominent in *Gracilariopsis*. Cross sections of fertile material from the Langebaan Lagoon-Saldanha Bay system have confirmed the identity of this taxon as *Gracilaria*. However, fertile material from St. Helena Bay indicates that this taxon does not belong to the genus *Gracilaria* but is in fact a *Gracilariopsis*.

One can conclude that based solely on internal and external vegetative morphological characteristics the genera *Gracilaria* and *Gracilariopsis* cannot be differentiated. Fertile material, which provides the best distinction between these genera, is seldom encountered. Therefore there is a need for molecular based techniques to distinguish between these genera.

Chapter 3

MOLECULAR IDENTIFICATION OF TAXA

3.1 INTRODUCTION

Investigations at the DNA level have provided useful taxonomic tools for distinguishing organisms difficult to identify by more traditional means. Within the Gracilariaceae, some species share a common but variable morphology and are, consequently, often misidentified.

Attempts have been made (Hansen *et al.*, 2000; Nam *et al.*, 2000; Pueschel *et al.*, 2000; Rousseau *et al.*, 2000) to infer phylogenetic relationships of algae from morphological, anatomical, ultrastructural, life history, and chemical characters. Molecular sequences, particularly of nuclear genes encoding small subunit rRNA (18S rRNA) have proven useful in resolving phylogenetic relationships within other problematic algal groups (Linton *et al.*, 2000; Phillips, 2000; Pueschel *et al.*, 2000). These sequences are characterised by conserved regions common to all eukaryotes, and variable regions that reflect finer details of phylogenetic descent.

Nucleotide sequence data from intergenic spacer regions have also been used to discriminate species in various algal genera (Coleman and Mai, 1997), including *Gracilaria* (Goff *et al.*, 1994). Goff *et al.* (1994) determined other sequences of the nuclear ribosomal RNA cistron, the two internal transcribed spacers and intervening 5.8S rRNA gene. The total sequence, 850-1050 bp long, was similarly useful in distinguishing some congeners and was also sufficiently variable to infer relationships among very closely related taxa such as

verrucosa-type *Gracilaria* species. However, the variability was too great to allow unambiguous alignment of sequences from all congeners examined or from different genera, and so the sequences could not be used for determining phylogenetic relationships in the family as a whole. Plastid DNA RFLP (restriction fragment length polymorphism's) patterns have served to identify geographically distant conspecific taxa, as well as to differentiate morphologically similar taxa (Rice and Bird, 1990).

Nucleotide sequences of DNA vary widely among the Rhodophyta in general (Freshwater *et al.*, 1994; Ragan *et al.*, 1994), and are sufficiently divergent within the Gracilariaceae to be taxonomically useful at intrageneric levels. For the 18S rDNA there is greater sequence divergence within genera of this family than occurs between families of the Palmariales (Saunders *et al.*, 1995) and the Laminariales (Saunders *et al.*, 1993). Analyses of 18S rDNA sequences from non-parasitic genera of the Gracilariaceae (Bird *et al.*, 1992, Bird *et al.*, 1994b), revealed differences of 49-60 bp among species of *Gracilariopsis*, larger differences than observed in *Gracilaria* and capable of distinguishing the several species.

This study was undertaken to determine the identity of isolates from Saldanha Bay, Langebaan Lagoon and St. Helena Bay. A variable region of the 18S rRNA gene was employed to determine the identity.

3.2 MATERIALS AND METHOD

3.2.1 DNA extraction

DNA was extracted using the method described in the AFLP™ Analysis System II (GibcoBRL) instruction manual. However, the method was modified to allow mini-scale extraction, and additional purification steps were included.

DNA extractions were performed on 94 isolates. Forty milligrams of ground material were resuspended in 500 µl of lysis buffer (Appendix A). Two microlitres mercaptoethanol and 3 µl proteinase K (20 mg/ml) were added, followed by gentle inversion and incubation for 1 hr at 37°C. The lysis step was completed at this temperature rather than 50-65°C because algal polysaccharides are routinely extracted at temperatures above 50°C. Residual proteins were removed using an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). The organic and aqueous phases were separated by centrifugation for 5 min at 15 800 x *g*. The DNA-containing aqueous phase was transferred to a fresh Eppendorf tube. An equal volume of chloroform/isoamyl alcohol (24:1) was added, followed by centrifugation for 5 min. The aqueous phase was removed and transferred to a fresh tube. One microlitre RNase A (Appendix A) was added to the aqueous phase and incubated for 5 min at 37°C. DNA was recovered from the aqueous phase by the addition of 2.5 volumes of 95% ethanol and the tubes inverted a few times. The precipitate was pelleted by centrifugation for 5 min, washed once with 70% ethanol, and dried briefly. The DNA pellet was resuspended in 50 µl TE buffer (Appendix A). The concentration of genomic DNA extracted was estimated spectrophotometrically.

3.2.2 18S rDNA primer construction

Specific primers (Appendix B) were designed (based on sequences of Bird *et al.*, 1992) to amplify a 1.3 kb fragment of the 18S rRNA gene (Fig. 3.1). Melting temperatures and complementarity to target DNA were determined using DNAMAN (Lynnon Biosoft). Thermal and hybridisation temperatures for both primers are described in Table 3.1.

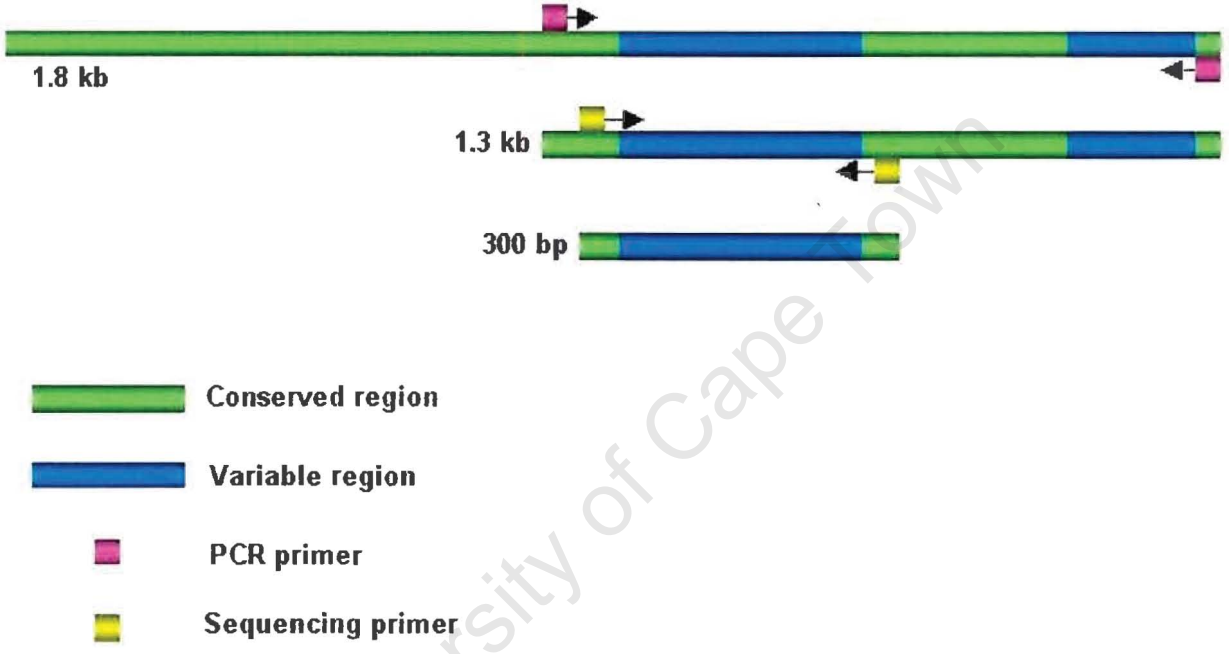

Fig. 3.1 Schematic illustration of the PCR strategy for amplifying a 1.3 kb portion of the 18S rRNA gene, using primer pair K18SF and K18SR. The 299 bp variable region was sequenced using a pair of nested sequencing primers.

Table 3.1 Characteristics of synthetic oligonucleotide primers for amplifying the 18S rRNA gene. Melting temperatures obtained by the thermodynamic and hybridisation methods are listed.

18S Primer	Thermodynamic Tm	Hybridisation Tm
K18SF	68.0°C	57.1°C
K18SR	69.9°C	57.4°C

3.2.3 Amplification of 18S rDNA

PCR was performed using a Biometra thermocycler (cycle profile 1; Appendix B). For each amplification, 50 µl reactions were set up using conditions and concentrations summarised in Table 3.2. Supertherm *Taq* DNA polymerase, PCR buffer, and $MgCl_2$ used for the amplification process were supplied by SR products. PCR products were electrophoresed on a 1% agarose gel (Appendix C) to determine whether or not DNA amplification was successful and to determine the size of the product.

Table 3.2 PCR conditions for amplification of the 18S rRNA gene.

Reactants	Final concentrations
Primer concentration	200 pM
DNTP mixture (Boehringer Mannheim)	40 µM
$MgCl_2$	1 mM
Taq polymerase buffer	1X
Template DNA	2 ng/µl
Supertherm *Taq* polymerase	0.02 U/µl

3.2.4 Purification of amplified products

Amplified DNA was purified using the High Pure PCR Product Purification Kit (Boehringer Mannheim) prior to sequencing. Two hundred and fifty microlitres binding buffer were added to a 50 µl PCR reaction and mixed well. The High Pure filter and collection tubes were combined and the sample pippetted into the upper reservoir. The sample was centrifuged for 30 s at 15 800 x *g* in a microcentrifuge. The flow through was discarded and the filter tube combined again with the same collection tube. Five hundred microlitres wash buffer were added to the upper reservoir and centrifuged for 30 s. The flow through was discarded and the filter tube again combined with the collection tube. Two hundred microlitres of wash buffer

were added and the sample centrifuged and recovered as described above. The collection tube was discarded and the filter tube inserted into a clean 1.5 ml reaction tube. DNA was eluted using 100 µl elution buffer (pH 8-8.5). The elution buffer was added to the filter tube and centrifuged for 30 s. The concentration of purified DNA was estimated spectrophotometrically.

3.2.5 Sequencing of 18S rRNA gene

Nested primers (sscpF and sscpR; Appendix B) were designed to sequence a 299 bp variable region of the 18S rRNA gene. A single sample of each patch from Saldanha Bay (Table 2.1; Fig. 2.1), Langebaan Lagoon (Table 2.2; Fig. 2.2) and St. Helena Bay (Table 2.3; Fig. 2.3) were sequenced. However, 2 isolates from Patch F (Langebaan Lagoon) were sequenced since significant morphological variation was observed among isolates from this patch (Figs. 2.10, C and 2.10, D). Samples were sequenced using an ALFexpress™ DNA Automated Sequencer (Alfwin Version 2.1; Pharmacia Biotech). All cycle sequencing reactions were performed according to the manufacturer's instruction.

3.2.6 Data analysis

Sequence data were aligned using DNAMAN and DNASIS (version 2.1; Hitachi Software Engineering). A strict consensus parsimony (MP) tree was inferred using Phylip (version 3.5c; Felsenstein, 1993). A neighbour-joining (NJ) tree was constructed using DNAMAN. Parsimony analysis was performed using DNAPARS (Phylip) which utilised heuristic algorithms. Trees were rooted with *Chondrus crispus* Stackhouse and *Palmaria palmata* (Linnaeus) Kuntz, the former sequence being relatively close phylogenetically to the Gracilariales and the latter rather more distant. Reference sequences are listed in Table 3.3.

The robustness of the MP and NJ phylogenetic hypotheses was tested by bootstrapping with 100 replicates of the data. Most parsimonious trees were determined by randomising the input order 7 times.

Table 3.3 Algal species included in the study, GenBank accession numbers and bibliographic references.

Taxon	Reference	Accession number
Gracilaria tikvahiae (Nova Scotia, Canada)	Bird *et al.*, 1990	M33640
G. cornea (St. Lucia, West Indies)	Bird *et al.*, 1992	L26212
G. chilensis (Wellington, New Zealand)	Bird *et al.*, 1992	L26217
G. gracilis (Cap Gris-Nez, France)	Bird *et al.*, 1992	L26211
Gracilariopsis sp. (De Mond, SA)	Iyer, pers. comm.	-
Gp. lemaneiformis (Vancouver, Canada)	Bird *et al.*, 1992	L26214
Gp. longissima (Plymouth, England)	Iyer, pers. comm.	-
Gp. longissima (Knysna, SA)	Iyer, pers. comm.	-
Curdiea flabellata (Wellington, New Zealand)	Bird *et al.*, 1992	L26207
Melanthalia obtusata (Victoria, Australia)	Bird *et al.*, 1992	L26215
Chondrus crispus (Unspecified locality)	Ragan *et al.*, 1994	Z14140
Palmaria palmata (Unspecified locality)	Ragan *et al.*, 1994	Z14142

3.3 RESULTS

3.3.1 DNA extraction

During this study, several DNA extraction methods were tested. Some protocols caused the initial crude DNA to be contaminated with RNA and polysaccharides (polysaccharides impede the electrophoresis of the DNA into the gel), resulting in the accumulation of ethidium bromide in gel wells. Most extraction protocols resulted in the accumulation of an agar pellet during the elution steps, within which the DNA was trapped. Attempts to extract this DNA from the agar via electroelution and various kits failed. However, a quick, easy and modified DNA extraction method from the AFLP manual resulted in DNA suitable for molecular manipulations.

DNA extractions from four Saldanha Bay (SalD2-D4, D6, E7; refer to section 2.2.1 for nomenclature of isolates), one Langebaan Lagoon (LanD5) and four St. Helena Bay (HelB2, C3, C5, E4) isolates were unsuccessful after multiple attempts. These samples were omitted from this study. DNA was successfully extracted from 84 isolates with concentrations ranging from 5-230 ng/µl. This DNA was slightly sheared (Fig. 3.2). The DNA obtained from these extractions, however, was suitable for PCR and restriction analysis.

3.3.2 Amplification of 18S rDNA

The 1.3 kb fragment (Fig. 3.3) of the 18S rDNA was successfully amplified from 20 isolates. No non-specific amplification or size differences were observed.

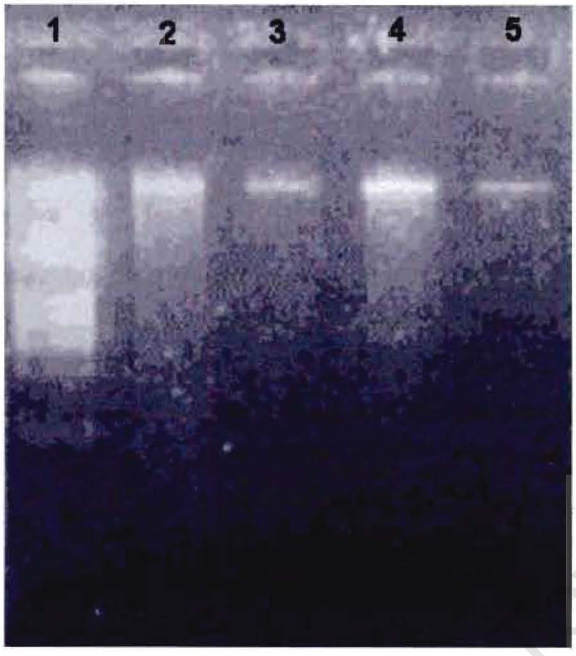

Fig. 3.2 Genomic DNA extracted from isolates. Lane 1, *Pst*I restricted λ DNA; lanes 2-5, genomic DNA.

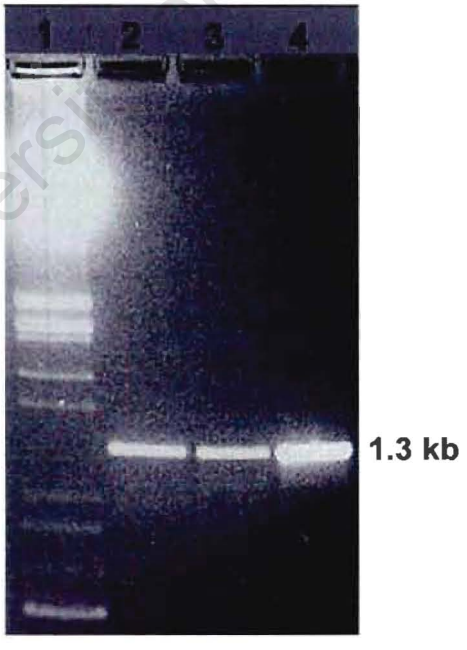

Fig. 3.3 Electrophoresis of amplified products of the 18S rRNA gene. Lane 1, *Bst*EII restricted λ DNA, lanes 2-4, amplified products.

3.3.3 Sequencing and data analysis

Multiple alignments of 18S rDNA sequences of isolates differed in 30 nucleotide positions (Fig. 3.4). Parsimony analysis resolved seven hundred and forty five trees. Differences between trees (Figs. 3.5 and 3.6) obtained by NJ and MP analysis concern only poorly supported basal nodes. Phylogenetic analysis resolved isolates from this study into 2 clades with significant bootstrap support. Langebaan Lagoon and Saldanha Bay isolates formed a monophyletic group with a known *Gracilaria gracilis* specimen from France (Bird *et al.*, 1992). The exception was a single Langebaan Lagoon isolate (LanF1). Based on this data it was inferred that most of the Saldanha Bay and Langebaan Lagoon isolates are *G. gracilis*.

All St. Helena Bay isolates and LanF1 formed a separate monophyletic group with a known *Gracilariopsis longissima* specimen from England. Based on this data it was inferred that all St. Helena Bay and some Langebaan Lagoon isolates belong to *Gp. longissima* (Figs. 3.5 and 3.6). The maximum sequence divergence within the Gracilariaceae was 12.1% (Table 3.4).

Fig. 3.4 Alignment matrix for 18S rDNA sequences. *Gp. longissima* (England) and *G. gracilis* (France) sequences were included for reference. Differences observed at 30 nucleotide positions are illustrated in colour and lower case.

Fig. 3.4 continued…

HelA1	CCCTGAATACATTAGCATGGAATAATAGAATAGGACCCGGTt CTATTTTGTTGGTTTGTTTGAATCaGGTAATGATTAAc	240
HelB1	CCCTGAATACATTAGCATGGAATAATAGAATAGGACCCGGTt CTATTTTGTTGGTTTGTTTGAATCaGGTAATGATTAAc	240
HelC1	CCCTGAATACATTAGCATGGAATAATAGAATAGGACCCGGTt CTATTTTGTTGGTTTGTTTGAATCaGGTAATGATTAAc	240
HelD1	CCCTGAATACATTAGCATGGAATAATAGAATAGGACCCGGTt CTATTTTGTTGGTTTGTTTGAATCaGGTAATGATTAAc	240
HelE1	CCCTGAATACATTAGCATGGAATAATAGAATAGGACCCGGTt CTATTTTGTTGGTTTGTTTGAATCaGGTAATGATTAAc	240
HelF1	CCCTGAATACATTAGCATGGAATAATAGAATAGGACCCGGTt CTATTTTGTTGGTTTGTTTGAATCaGGTAATGATTAAc	240
HelG1	CCCTGAATACATTAGCATGGAATAATAGAATAGGACCCGGTt CTATTTTGTTGGTTTGTTTGAATCaGGTAATGATTAAc	240
HelH1	CCCTGAATACATTAGCATGGAATAATAGAATAGGACCCGGTt CTATTTTGTTGGTTTGTTTGAATCaGGTAATGATTAAc	240
LanA1	CCCTGAATACATTAGCATGGAATAATAGAATAGGACCCGGTcCTATTTTGTTGGTTTGTTTGAATCgGGTAATGATTAAg	240
LanB1	CCCTGAATACATTAGCATGGAATAATAGAATAGGACCCGGTcCTATTTTGTTGGTTTGTTTGAATCgGGTAATGATTAAg	239
LanC1	CCCTGAATACATTAGCATGGAATAATAGAATAGGACCCGGTcCTATTTTGTTGGTTTGTTTGAATCgGGTAATGATTAAg	239
LanD1	CCCTGAATACATTAGCATGGAATAATAGAATAGGACCCGGTcCTATTTTGTTGGTTTGTTTGAATCgGGTAATGATTAAg	239
LanE1	CCCTGAATACATTAGCATGGAATAATAGAATAGGACCCGGTcCTATTTTGTTGGTTTGTTTGAATCgGGTAATGATTAAg	239
LanF1	CCCTGAATACATTAGCATGGAATAATAGAATAGGACCCGGTt CTATTTTGTTGGTTTGTTTGAATCaGGTAATGATTAAc	239
LanF6	CCCTGAATACATTAGCATGGAATAATAGAATAGGACCCGGTcCTATTTTGTTGGTTTGTTTGAATCgGGTAATGATTAAg	240
SalA1	CCCTGAATACATTAGCATGGAATAATAGAATAGGACCCGGTcCTATTTTGTTGGTTTGTTTGAATCgGGTAATGATTAAg	239
SalB1	CCCTGAATACATTAGCATGGAATAATAGAATAGGACCCGGTcCTATTTTGTTGGTTTGTTTGAATCgGGTAATGATTAAg	239
SalC1	CCCTGAATACATTAGCATGGAATAATAGAATAGGACCCGGTcCTATTTTGTTGGTTTGTTTGAATCgGGTAATGATTAAg	239
SalD1	CCCTGAATACATTAGCATGGAATAATAGAATAGGACCCGGTcCTATTTTGTTGGTTTGTTTGAATCgGGTAATGATTAAg	239
SalE1	CCCTGAATACATTAGCATGGAATAATAGAATAGGACCCGGTcCTATTTTGTTGGTTTGTTTGAATCgGGTAATGATTAAg	239
Gp. longissima	CCCTGAATACATTAGCATGGAATAATAGAATAGGACCCGGTt CTATTTTGTTGGTTTGTTTGAATCaGGTAATGATTAAc	240
G. gracilis	CCCTGAATACATTAGCATGGAATAATAGAATAGGACCCGGTcCTATTTTGTTGGTTTGTTTGAATCgGGTAATGATTAAg	239

Fig. 3.4 continued…

HelA1	AGGGACGGTTGGGGGCATTCGTATTCCGgCGctAGAGGTGAAATTCTTGGATTGTCGG	299
HelB1	AGGGACGGTTGGGGGCATTCGTATTCCGgCGctAGAGGTGAAATTCTTGGATTGTCGG	299
HelC1	AGGGACGGTTGGGGGCATTCGTATTCCGgCGctAGAGGTGAAATTCTTGGATTGTCGG	299
HelD1	AGGGACGGTTGGGGGCATTCGTATTCCGgCGctAGAGGTGAAATTCTTGGATTGTCGG	299
HelE1	AGGGACGGTTGGGGGCATTCGTATTCCGgCGctAGAGGTGAAATTCTTGGATTGTCGG	299
HelF1	AGGGACGGTTGGGGGCATTCGTATTCCGgCGctAGAGGTGAAATTCTTGGATTGTCGG	299
HelG1	AGGGACGGTTGGGGGCATTCGTATTCCGgCGctAGAGGTGAAATTCTTGGATTGTCGG	299
HelH1	AGGGACGGTTGGGGGCATTCGTATTCCGgCGctAGAGGTGAAATTCTTGGATTGTCGG	298
LanA1	AGGGACGGTTGGGGGCATTCGTATTCCGaCGtcAGAGGTGAAATTCTTGGATTGTCGG	298
LanB1	AGGGACGGTTGGGGGCATTCGTATTCCGaCGtcAGAGGTGAAATTCTTGGATTGTCGG	298
LanC1	AGGGACGGTTGGGGGCATTCGTATTCCGaCGtcAGAGGTGAAATTCTTGGATTGTCGG	298
LanD1	AGGGACGGTTGGGGGCATTCGTATTCCGaCGtcAGAGGTGAAATTCTTGGATTGTCGG	298
LanE1	AGGGACGGTTGGGGGCATTCGTATTCCGaCGtcAGAGGTGAAATTCTTGGATTGTCGG	298
LanF1	AGGGACGGTTGGGGGCATTCGTATTCCGgCGctAGAGGTGAAATTCTTGGATTGTCGG	299
LanF6	AGGGACGGTTGGGGGCATTCGTATTCCGaCGtcAGAGGTGAAATTCTTGGATTGTCGG	298
SalA1	AGGGACGGTTGGGGGCATTCGTATTCCGaCGtcAGAGGTGAAATTCTTGGATTGTCGG	298
SalB1	AGGGACGGTTGGGGGCATTCGTATTCCGaCGtcAGAGGTGAAATTCTTGGATTGTCGG	298
SalC1	AGGGACGGTTGGGGGCATTCGTATTCCGaCGtcAGAGGTGAAATTCTTGGATTGTCGG	298
SalD1	AGGGACGGTTGGGGGCATTCGTATTCCGaCGtcAGAGGTGAAATTCTTGGATTGTCGG	298
SalE1	AGGGACGGTTGGGGGCATTCGTATTCCGaCGtcAGAGGTGAAATTCTTGGATTGTCGG	298
Gp. longissima	AGGGACGGTTGGGGGCATTCGTATTCCGgCGctAGAGGTGAAATTCTTGGATTGTCGG	299
G. gracilis	AGGGACGGTTGGGGGCATTCGTATTCCGaCGtcAGAGGTGAAATTCTTGGATTGTCGG	298

Fig. 3.4 continued.

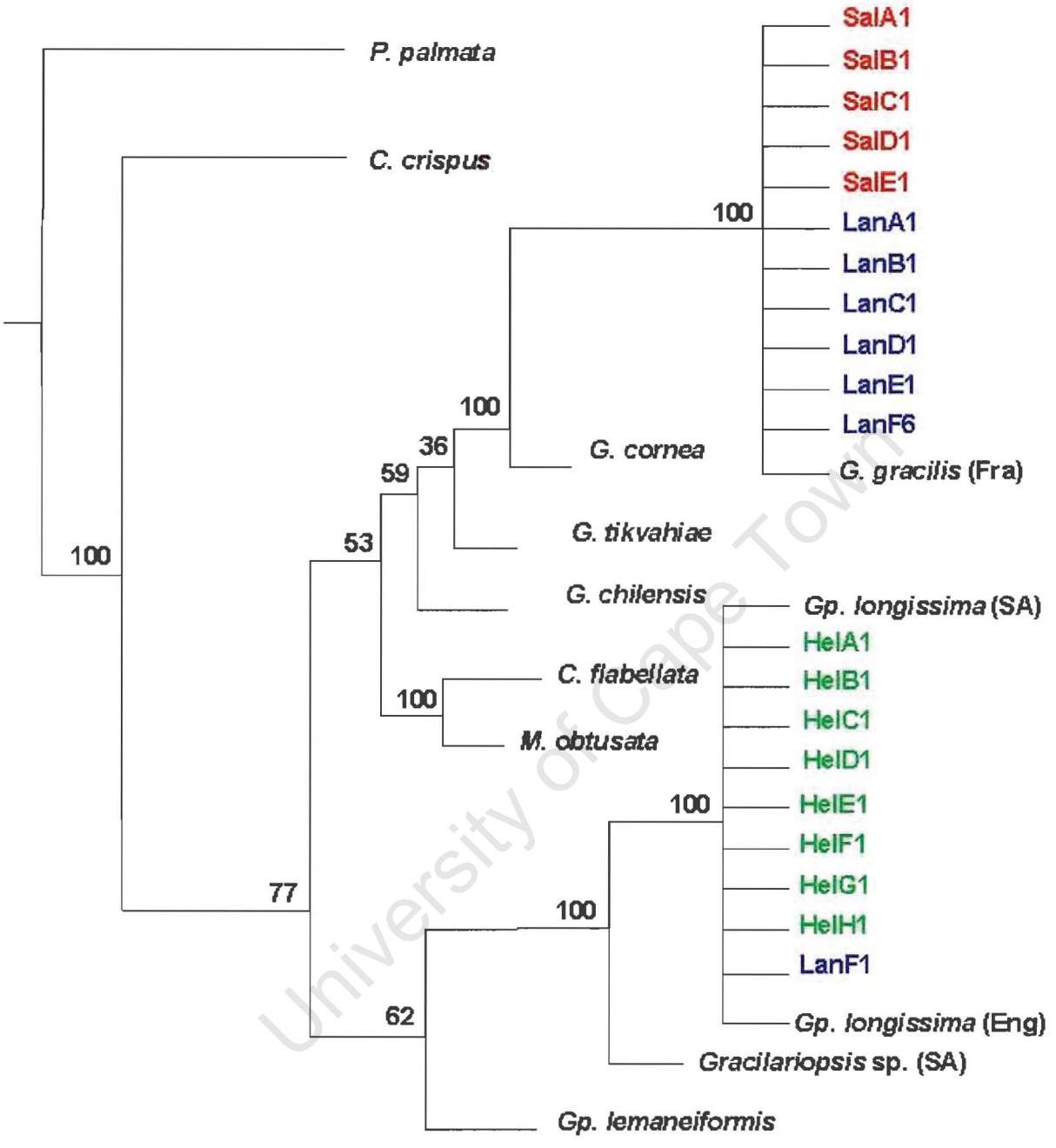

Fig. 3.5 Phylogenetic tree inferred from neighbour joining analysis of the 18S rDNA sequence data. Numbers above the branches indicate bootstrap proportions (percentage of 100 replicates).

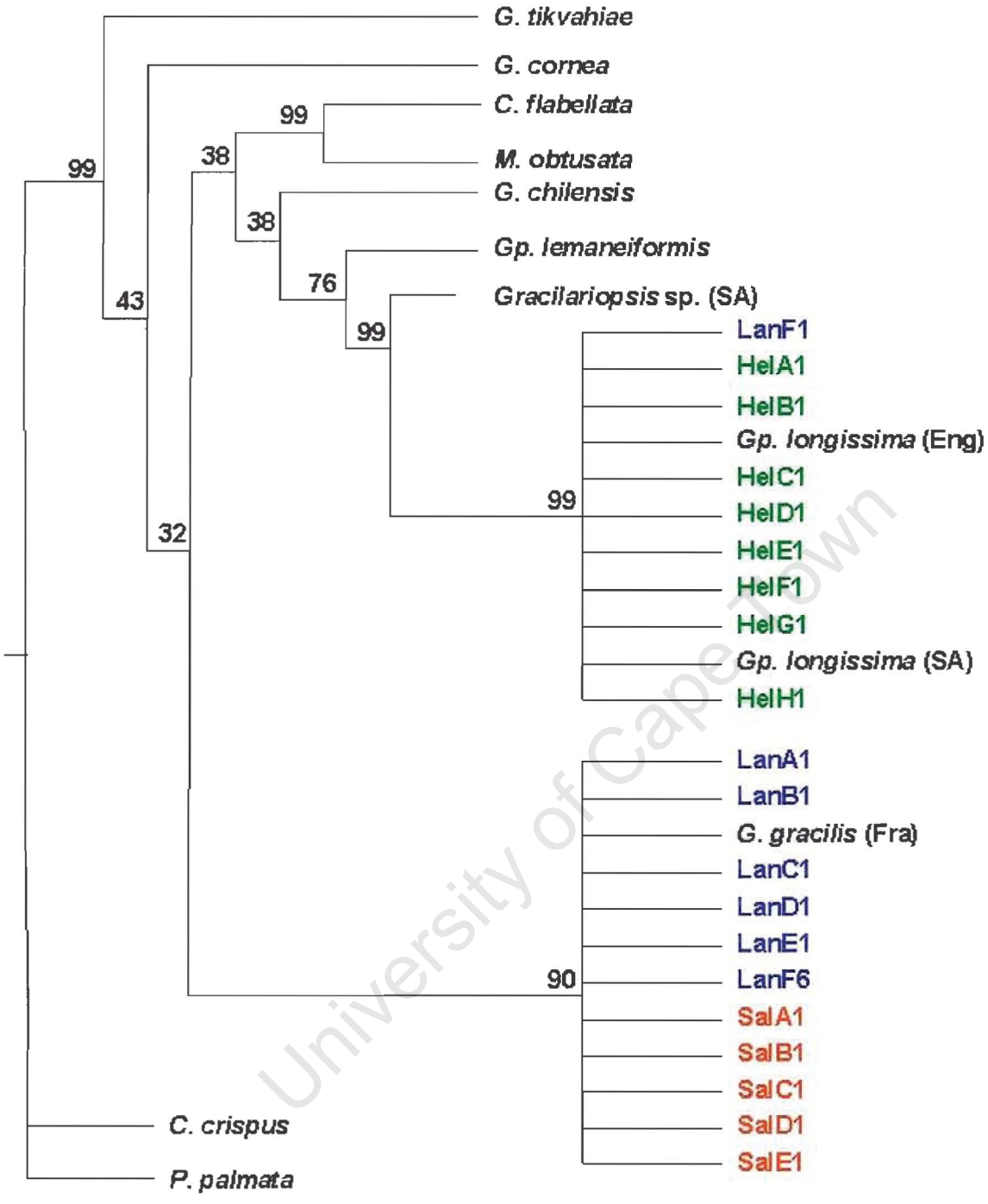

Fig. 3.6 Strict consensus of most-parsimonious trees inferred from 18S rDNA sequence data. Numbers above the branches indicate bootstrap proportions (percentage of 100 replicates).

Table 3.4 Distance divergence (%) within the Gracilariaceae based on 18S rDNA sequences.

	Gpl	Ggr	Ccr	Cfl	Gch	Gco	GgrF	Gti	Gple	GplE	GplS	GpD	Mob	Ppa
Gpl	0													
Ggr	9.4	0												
Ccr	18.1	15.1	0											
Cfl	12.1	6	16.8	0										
Gch	9.4	2.7	15.1	5.4	0									
Gco	9.1	2.7	15.1	6.7	3.4	0								
GgrF	9.4	0	15.1	6	2.7	2.7	0							
Gti	9.1	2.3	13.4	6.4	3	3	2.3	0						
Gple	7	5	15.1	6.7	3.7	5.7	5	5.4	0					
GplE	0	9.4	18.1	12.1	9.4	9.1	9.4	9.1	7	0				
GplS	0	9.4	18.1	12.1	9.4	9.1	9.4	9.1	7	0	0			
GpD	2.3	8.4	17.1	11.1	7.7	7.4	8.4	8.1	5.4	2.3	2.3	0		
Mob	11.7	5.7	16.8	1.7	5	5.7	5.7	5.4	6.4	11.7	11.7	10.7	0	
Ppa	22.9	21.9	21	23.6	22.6	22.2	21.9	21.2	22.2	22.9	22.9	22.6	23.9	0

Gpl *Gracilariopsis longissima*
Ggr *Gracilaria gracilis*
Ccr *Chondrus crispus*
Cfl *Curdiea flabellata*
Gch *Gracilaria chilensis*
Gco *Gracilaria cornea*
GgrF *Gracilaria gracilis* (Fra)
Gti *Gracilaria tikvahiae*
Gple *Gracilariopsis lemaneiformis*
GplE *Gracilariopsis longissima* (Eng)
GplS *Gracilariopsis longissima* (SA)
GpD *Gracilariopsis* sp. (SA)
Mob *Melanthalia obtusata*
Ppa *Palmaria palmata*

3.4 DISCUSSION

Molecular biological investigations of marine macroalgae have been hindered by considerable difficulties in acquiring usable DNA from these organisms (Mayes *et al.*, 1992). In particular, the copious storage and structural polysaccharides of phaeophycean and rhodophycean taxa have presented researchers with a formidable obstacle to DNA preparations. DNA extraction from marine macroalgae is difficult because polysaccharides copurify with the DNA and interfere with enzymatic treatments.

Most DNA extraction techniques are suitable for use with fresh plant material. Field botanists very often work far from the laboratory, and many rely on silica dried plant samples (Chase and Hillis, 1991). Polysaccharide accumulation during DNA extraction is quite common and therefore working with temperatures below 50°C greatly reduces contamination. The extraction protocol utilised in this study proved successful for both fresh and silica dried samples. The DNA was suitable for PCR amplification, restriction analysis and AFLPs although it was slightly sheared and of low concentration.

Amplification of the 299 bp variable region usually resulted in non-specific banding and at times no amplification. This was due to primers that lacked specificity to the area of amplification. Primers constructed to amplify the 1.3 kb fragment were successful and reproducibly amplified. Nested primers designed to sequence the 299 bp product resulted in high quality sequence data which lacked background interference.

Delineation of species in *Gracilaria* and *Gracilariopsis* has proven extremely difficult using available morphological characteristics. Both genera have similar external morphologies i.e. terete, slender and freely branched and are difficult to differentiate. Molecular systematic studies of these genera (Goff *et al.*, 1994) reveal that there is as much variation among individuals of a population as there is between individuals of geographically separate populations.

Nucleotide sequences of the nuclear genes encoding small-subunit ribosomal RNA's (18S rDNA's) are being used increasingly as criteria for proposing phylogenies of organisms (Bird *et al.*, 1992). These sequences are characterised by conserved regions common to all eukaryotes, and variable regions that reflect finer details of phylogenetic descent. Several such sequences have been reported for members of the Rhodophyta (Bhattacharya *et al.*, 1990; Bird *et al.*, 1990; Hendriks *et al.*, 1991) and have been used to determine the position of these algae in wider phylogenetic schemes.

Investigations of the phylogeny of the Gracilariaceae based on the nucleotide sequence of the small-subunit rRNA gene (Bird *et al.*, 1992; Ragan *et al.*, 1994) have indicated that *Gracilariopsis sensu stricto* is strongly separated phylogenetically from *Gracilaria* and all other nonparasitic genera of the family, as the earliest divergence in the Gracilariaceae. This observation has been confirmed by Freshwater *et al.* (1994) using the *rbc*L gene sequence. Scholfield *et al.* (1991) employed PCR to amplify the 18S rDNA of several *Gracilaria* and *Gracilariopsis* species and examined restriction patterns generated using restriction endonucleases that recognise 4 bp sequences. They concluded that this method may be useful

in determining species affinities within these taxa; however, this method is not useful for examining species interrelationships.

Sequence data have not been widely employed to determine the identity of specimens. This is probably due to the limited number of algal sequences present in databases. The utility of sequence data for specimen identification and confirmation has been demonstrated in this study (Figs. 3.5 and 3.6). Isolates were conclusively identified as either *G. gracilis* or *Gp. longissima* based on sequence analysis. The sequence divergence between *G. gracilis* and *Gp. longissima* was 9.4%, which was relatively high compared to the maximum sequence divergence within the Gracilariaceae which was 12.1% (Table 3.4).

From this study it can be concluded that *G. gracilis* predominates within the Langebaan Lagoon-Saldanha Bay system, with a single population of *Gp. longissima* among those sampled in Langebaan Lagoon. All isolates in St. Helena Bay have been identified as *Gp. longissima* (Figs. 3.5 and 3.6). These data correlate with anatomical studies (Chapter 2) which pointed to the presence of *Gracilaria* sp. in the Langebaan Lagoon-Saldanha Bay system and *Gracilariopsis* sp. in St. Helena Bay based on cystocarpic structure. Comparisons of 18S rDNA sequences show that the single Langebaan Lagoon isolate and the St. Helena Bay isolates are identical to the reference *Gp. longissima* sequence. Thus the species identity of St. Helena Bay isolates based on 18S rDNA sequences is *Gp. longissima*.

Three hypotheses have been put forward to explain the presence of a single isolated patch of *Gp. longissima* in Langebaan Lagoon. (i) Fragmented material may have been transferred by boats from another nearby locality, possibly St. Helena Bay. (ii) Fragmented

material may have been spread from the experimental rafts in Small Bay or from the mariculture rafts in Big Bay to discrete locations, which may not have been discovered in this study. However this would only be possible if rafts were seeded with material occurring outside of the Langebaan Lagoon-Saldanha Bay system. (iii) The introduction of *Gp. longissima* to Langebaan Lagoon is not recent, i.e. the material may be localised in this area due to favourable conditions within that particular niche and was unable to spread due to adverse ecological conditions in surrounding areas.

The utility of the 18S rDNA gene sequence for the accurate identification of isolates against known sequences has been displayed in this study. Three characteristics of this identification system make it feasible for routine identification. (i) The method provides a more accurate means of identification, relative to routine morphological methods, especially in the absence of reproductive structures. However, differentiation between closely related species has not been demonstated in this study and needs further investigations. (ii) The use of a small 299 bp variable gene sequence, ensures that costs are minimal, as sequencing is accomplished in just two reactions. (iii) Greater accuracy is obtained by utilising nested primers. Only target DNA strands are sequenced, decreasing the amount of background interference.

Chapter 4

POPULATION GENETIC STUDIES

4.1 INTRODUCTION

Molecular biology has provided systematic biologists with new and powerful tools to examine variation in the genomes of individuals, populations and species. A commonly used method for analysis of genomic DNA is restriction fragment length polymorphisms (RFLPs). RFLP analysis is informative and reproducible, but labour intensive and requires large quantities (10 μg) of DNA (Karp *et al.*, 1996). A more recent innovation, the random amplified polymorphic DNA (RAPD) technique, offers advantages over RFLP because previous knowledge of the genome is not necessary and only a small amount of DNA is required (10-25 ng). The RAPD technique however, is sensitive to slight variations in reaction conditions and therefore reproducibility is a concern (Karp *et al.*, 1996; Malyshev and Kartel, 1997). Patwary *et al.* (1993) and van Oppen *et al.* (1996) argued that RAPDs are more useful at larger biogeographic scales than at finer population levels.

Methods for screening DNA variation in polymerase chain reaction (PCR)-amplified DNA from large sample sizes are well known, although not yet applied in algal research (Lessa and Applebaum, 1993). Single stranded conformation polymorphisms (SSCPs) were first described by Orita *et al.* (1989). The ability of the SSCP method to distinguish between mutant DNA fragments relies on the principle that the molecular conformation of single-stranded DNA is nucleotide-sequence specific, that this conformation is altered by point substitutions, insertions and deletions, and that the altered conformation affects the mobility of the molecule to migrate through polyacrylamide gel. Like RFLPs, SSCPs were found to be

allelic variants of true Mendelian traits, and therefore they should be useful genetic markers. SSCP analysis has the advantage over RFLP analysis that it can detect DNA polymorphisms and point mutations at a variety of positions in DNA fragments. This method has been reported to reveal up to 100% of the mutations in fragments of 100-400 bps (Zuccarello *et al.*, 1999b).

Whole genome fingerprinting by determination of amplified fragment length polymorphisms (AFLPs) is a high resolution genotyping method that has been widely applied to both eukaryotic and prokaryotic organisms (Savelkoul *et al.*, 1999). The technique is very powerful because it produces a dense but reliable banding pattern without the necessity of probe development. Although the technique has not reached its full potential yet, it is already widely used in genetic studies in plants (Meksem *et al.*, 1995; Van Eck *et al.*, 1995), animals (Otsen *et al.*, 1996), fungi (Majer *et al.*, 1996), nematodes (Folkertsma *et al.*, 1996), bacteria (Keim *et al.*, 1997) and more recently algae (Donaldson *et al.*, 1998).

Donaldson *et al.* (1998) reported that their preliminary AFLP results suggest that plants of the red seaweed *Chondrus* (Gigartinaceae) in the southern Gulf of St. Lawrence and the Bay of Fundy are more similar to each other, with the plants from the Atlantic coast of Nova Scotia being most divergent. Recent data by Donaldson *et al.* (2000) found that AFLP may not be appropriate for population level investigations in *Chondrus* and other methods must be tried. This lack of useful molecular markers from AFLP may extend to other seaweed species in the field.

This study was undertaken to determine the levels of genetic variation between isolates of *Gracilaria gracilis* and *Gracilariopsis longissima*. Three questions are posed: (i) Are populations within the respective systems clonal (i.e. entire populations generate from a single individual by vegetative propagation)? (ii) How is genetic variation distributed among and within populations? (iii) Is there a relation between population size (observed biomass) and genetic variation?

4.2 MATERIALS AND METHOD

4.2.1 Agarose gel electrophoresis

Agarose gel electrophoresis was performed in 1X TAE running buffer (Appendix A) as described by Sambrook *et al.* (1989).

4.2.2 Single-stranded conformation polymorphism (SSCP) protocol

SSCP was employed to determine genetic variation within populations of *G. gracilis* and *Gp. longissima* prior to sequencing of the 18S rDNA region. A 299 bp variable region of the 18S rRNA gene was amplified using a labelled forward primer. Shifts in mobility of single-stranded DNA due to conformational changes formed the basis of determining genetic variability.

4.2.2.1 18S rDNA primer design

Specific primers (Appendix B) were designed to amplify a 299 bp region of the 18S rRNA gene. Initial primers constructed resulted in non-specific banding therefore a second set was designed with higher thermal and hybridisation temperatures. Melting temperatures and complementarity to target DNA were determined using DNAMAN. Thermal and hybridisation temperatures for both primers are described in Table 4.1.

Table 4.1 Characteristics of synthetic oligonucleotide primers for amplifying the 18S rRNA gene for SSCP analysis. Melting temperatures obtained by the thermodynamic and hybridisation methods are listed.

SSCP Primer	Thermodynamic Tm	Hybridisation Tm
sscpF	71.8°C	58.7°C
sscpR	69.7°C	56.7°C

4.2.2.2 End-labelling reaction

The forward primer sscpF was end labelled. The labelling reaction (Table 4.2) was carried out in a total volume of 25 µl.

Table 4.2 Labelling reaction components.

Components	Final concentration
sscpF primer	44 ng/µl
T4 polynucleotide kinase buffer	1X
T4 polynucleotide kinase	0.2 U/µl
[γ-^{32}P]-ATP	100 µCi

Following incubation for 30 min at 37°C, the labelling mixture was heat inactivated for 15 min at 65°C. DNA was precipitated with 25 µl of 4 M ammonium acetate and 250 µl ethanol (100%) with overnight incubation. Precipitated DNA was pelleted by centrifugation for 15 min and washed with ethanol (70%); this was followed by centrifugation for a further 5 min and the pellet air dried. The above precipitation method was repeated. Labelled DNA was resuspended in 100 µl distilled water and incubated overnight at 4°C.

4.2.2.3 PCR amplification of genomic DNA using an end labelled primer

Samples were amplified using forward primer sscpF (labelled) and reverse primer sscpR. A range of temperatures was tested for optimal amplification. For each amplification, 20 µl reactions were set up using conditions and concentrations set out in Chapter 3 (Table 3.2). PCR cycle profile 1 (Appendix B) was followed. PCR products were electrophoresed on 1% agarose gels (Appendix C) to determine whether primer labelling and DNA amplification were successful.

4.2.2.4 Electrophoresis of single-stranded DNA

Amplified DNA (5 µl) was made single-stranded by the addition of 10 µl denaturing solution (Appendix A), with gentle inversion and centrifugation for 5 min. Eppendorf tubes were sealed with parafilm and incubated for 5 min at 95°C on a heating block. At the end of the incubation period tubes were placed directly on ice. Five microlitres of this solution was loaded onto a 3% non-denaturing polyacrylamide gel (Appendix A), and electrophoresed for 6 hrs at 30 W in 1X TBE buffer (Appendix A). Following electrophoresis, the gel was transferred onto Whatman 3 MM chromatography paper and dried for 45 min at 60°C on a slab gel dryer (Hoefer Scientific Instruments). The gel was transferred to an X-ray film cassette. Autoradiographic film (3 M type XDA) was placed on the gel and exposed for 5 days at room temperature. The film was developed using an automatic film developer (Okamoto X2).

4.2.3 AFLP protocol

The AFLP protocol used was based on the method described by Bachem *et al.* (1996).

4.2.3.1 Restriction endonuclease digestion of genomic DNA

Two pairs of restriction endonucleases (*Bcl*I-*Taq*I and *Eco*RI-*Mse*I) were analysed for optimal digestion of genomic DNA. Restriction reactions were carried out according to the manufacturers instructions. A typical reaction (20 µl) contained 150-200 ng DNA, 1X restriction buffer and 5 U restriction endonucleases. Reaction mixtures were incubated for 2 hrs on a heating block at the temperature required for optimal enzyme activity. The first restriction set used was *Bcl*I (Promega) and *Taq*I (Boehringer Mannheim). The second set included *Mse*I (Biolabs) and *Eco*RI (Boehringer Mannheim). The reaction mix was adjusted with NaCl and Tris (during the *Eco*RI digestion) to a final concentration of 100 mM and 50 mM, respectively.

4.2.3.2 Annealing of complementary primers to form adaptors

Two pairs of adaptor oligonucleotides were synthesised (Oligo 1-Oligo 2 and Oligo 3-Oligo 4; Appendix B) such that the oligonucleotides of each pair were complementary to each other, and when annealed would generate either *Eco*RI (rare cutter) or *Mse*I (frequent cutter) sticky ends. Equal amounts of complementary primers were annealed to each other as described in Appendix C.

4.2.3.3 Ligation of adaptors to restricted DNA

Ten microlitres of ligation mix was prepared consisting of 5 pmol of *Eco*RI adaptor, 50 pmol of *Mse*I, 1 U of T4 DNA ligase (Boehringer Mannheim), ligase buffer and sterile water. Five microlitres of ligation mix was added to 20 µl of *Eco*RI-*Mse*I-digested genomic DNA and incubated for 3 hrs at 37°C to anneal the adaptors to the sticky ends of the DNA fragments. The adaptor-ligated restriction fragments were stored in 5 µl aliquots at -20°C.

4.2.3.4 Pre-selective PCR amplification of adaptor-ligated fragments

Adaptor-ligated fragments were amplified under low-stringency conditions of PCR using cycle profile 2 (Appendix B). Primer 5 and primer 6 (Appendix B) were designed to correspond to sequences at the *Mse*I and *Eco*RI ends of the adaptors, respectively. Twenty microlitre PCR reaction mixes were prepared (Table 4.3). Small amounts (5 µl) of the amplified products were electrophoresed in a 1.5% agarose gel (Appendix C) at 100 V in 1X TAE running buffer to verify the presence of DNA smears representing a range of fragment sizes.

Table 4.3 Reaction components for pre-selective PCR. Final concentrations are included.

Reaction Constituents	Final concentration
Primer 5	2.5 ng/µl
Primer 6	2.5 ng/µl
Adaptor ligated DNA fragments	37.5-50 ng/µl
Supertherm *Taq* polymerase	0.05 U/µl
$MgCl_2$	1.5 mM
Taq polymerase buffer	1X
dNTPs	0.5 mM

4.2.3.5 Selective PCR using pre-amplified DNA fragments

A second round of high-stringency step down PCR amplification (cycle profile 3; Appendix B) was performed, using primer 7 and primer 8 (Appendix B) which have a two base pair degeneracy at their 3' ends. Primer 7 (complementary to the *Mse*I-digested end of the DNA) was Cy5 end labelled (Pharmacia Biotech). Twenty microlitre PCR reaction mixes were prepared (Table 4.4).

Table 4.4 Reaction components for selective PCR. Final concentrations are included.

Reaction Constituents	Final concentration
Primer 8	1.5 ng/µl
Unlabelled primer 7	1.25 ng/µl
Cy5 labelled primer 7	11.25 ng/µl
Pre-amplified DNA product	22.5-30 ng/µl
Supertherm *Taq* polymerase	0.05 U/µl
$MgCl_2$	1.5 mM
Taq polymerase buffer	1X
dNTPs	0.5 mM

Following the above amplification reaction, 5 µl of PCR products were mixed with an equal volume of formamide dye (Epicentre Technologies), denatured for 3 min at 95°C, and chilled on ice.

4.2.3.6 Electrophoresis of amplified products

Samples were prepared in duplicate. Five microlitres of the selective PCR product was loaded onto a 7% denaturing gel (Reprogel Long Read; APBiotech) and electrophoresed on an Alfexpress DNA automated sequencer (Pharmacia Biotech) for 5 hrs (1000 V; 60 mA) at 55°C.

4.2.3.7 Analysis of AFLP profiles

AFLP profiles were exported to Fragment Manager version 1.2 (Pharmacia Biotech) and displayed as electropherograms. AFLP peaks that could be scored unambiguously by eye within the molecular size range 50-300 bps were included in the analysis. A presence/absence data matrix from the AFLP samples was constructed based on a diploid/dominant marker set. The presence of a band was assumed to represent the dominant genotype. Allele frequencies were estimated based on: (i) the square root of the frequency of the null (recessive) genotype (Weir, 1990); and (ii) Taylor expansion (Lynch and Milligan, 1994). Genotype frequencies were assumed to be in Hardy-Weinberg equilibrium (Lynch and Milligan, 1994). Associations among individuals were revealed by cluster analysis with the un-weighted pair group method using arithmetric averages (UPGMA). Distance was calculated based on Nei's (1978) unbiased distance. The phylogenetic hypotheses were tested by bootstrapping 100 replicates of the data.

4.3 RESULTS

4.3.1 SSCP analysis

Since SSCP's was performed prior to 18S rRNA sequencing, no sequence information was available at the time. Therefore it was not known that the 18S sequences of *G. gracilis* and *Gp. longissima* samples were 100% homologous. For SSCP analysis, non-specific banding patterns (Fig. 4.1) were observed on X-ray film after amplification using the initial SSCP primers. These primers had low thermal and hybridisation temperatures which could have led to the non-specific banding. The presence of primer dimers and amplified products served as confirmation of successful labelling of the forward primer. However, SSCPs performed on samples were unsuccessful (gel not shown). The second set of primers, sscpF and sscpR, resulted in a single distinct 299 bp product (Fig. 4.2). However, no bands were observed on the non-denaturing polyacrylamide gel following denaturation and electrophoresis. This could be due to a number of factors such as: (i) gel concentration, (ii) electrophoresis run time, (iii) denaturation of strands, (iv) radioactive reagent used, and (v) electrophoresis of gels at room temperature. Loading of samples at different time points still resulted in no bands.

SSCP has been reported to reveal up to 100% of the mutations in fragments of 150-400 bps. The fragment used in this study was approximately 299 bps in size. This size falls within the reported range. The highest reading recorded on the Geiger counter (Mini Instruments) was obtained from the wells. This would indicate that the labelled product did not migrate out of the wells. Although different gel percentages were utilised, no bands were observed.

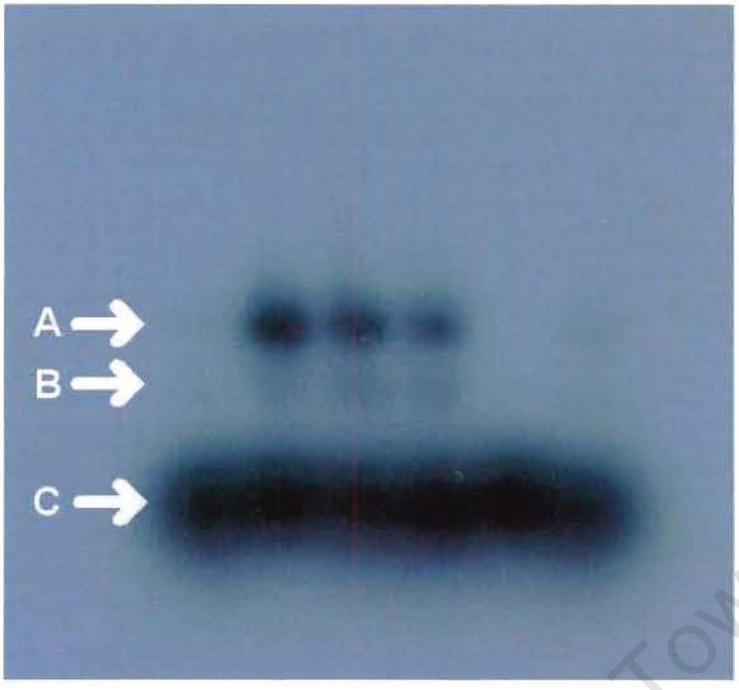

Fig. 4.1 Autoradiograph displaying amplified products (A). SSCP primers were used in the PCR reaction. Non-specific products (B) and primer dimers (C) are also visible on the autoradiograph.

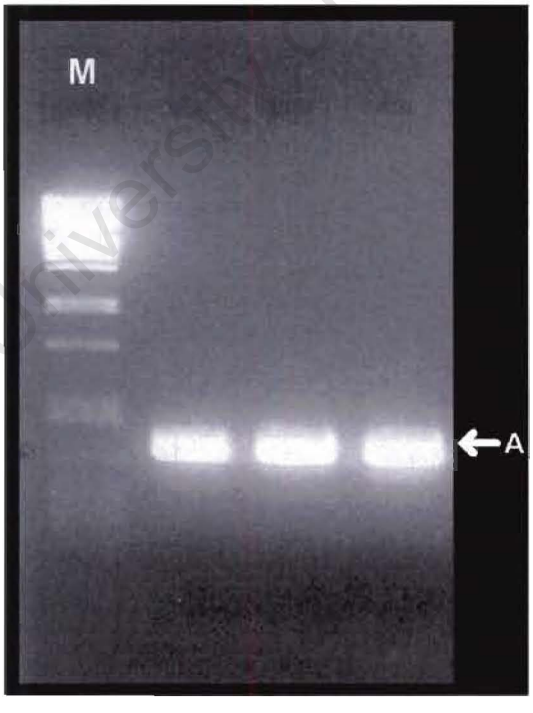

Fig. 4.2 Amplified products (A; 299 bp) and *Pst*I restricted λ DNA (M; marker) following electrophoresis through 1% agarose gel. Primers sscpF and sscpR were used in the PCR reaction.

4.3.2 Digestion of DNA with restriction endonucleases for AFLP analysis

Digestion of *G. gracilis* and *Gp. longissima* genomic DNA by *Taq*I-*Bcl*I and *Mse*I-*Eco*RI restriction endonuclease pairs could only be evaluated after pre-selective PCR when amplification products could be visualised. DNA was digested to completion using both enzyme sets. However, results for the *Taq*I-*Bcl*I restriction pair were not reproducible as partial or no digestion often occurred. Digestion of DNA using the restriction endonuclease pair *Mse*I-*Eco*RI was successful as pre-selective PCR resulted in the presence of DNA smears representing a range of fragment sizes. The use of this restriction endonuclease pair resulted in total digestion of DNA and was reproducible.

4.3.3 Processing of AFLP data

Fragments in the range of 40-400 bps were resolved for the time period specified (5 hrs). Fragments smaller than 50 bps were not selected as these could be a result of primer dimers or trimers. Since some gels were electrophoresed slightly longer than others, for consistency of analysis, the range selected for the AFLP fragments was between 50-300 bps (Fig. 4.3). Sequencing markers were used as references for alignment of multiple gels. Significantly, distinct differences were observed in profiles between samples of *G. gracilis* (Saldanha Bay isolates; Fig. 4.3) and *Gp. longissima* (St Helena Bay isolates; Fig. 4.3).

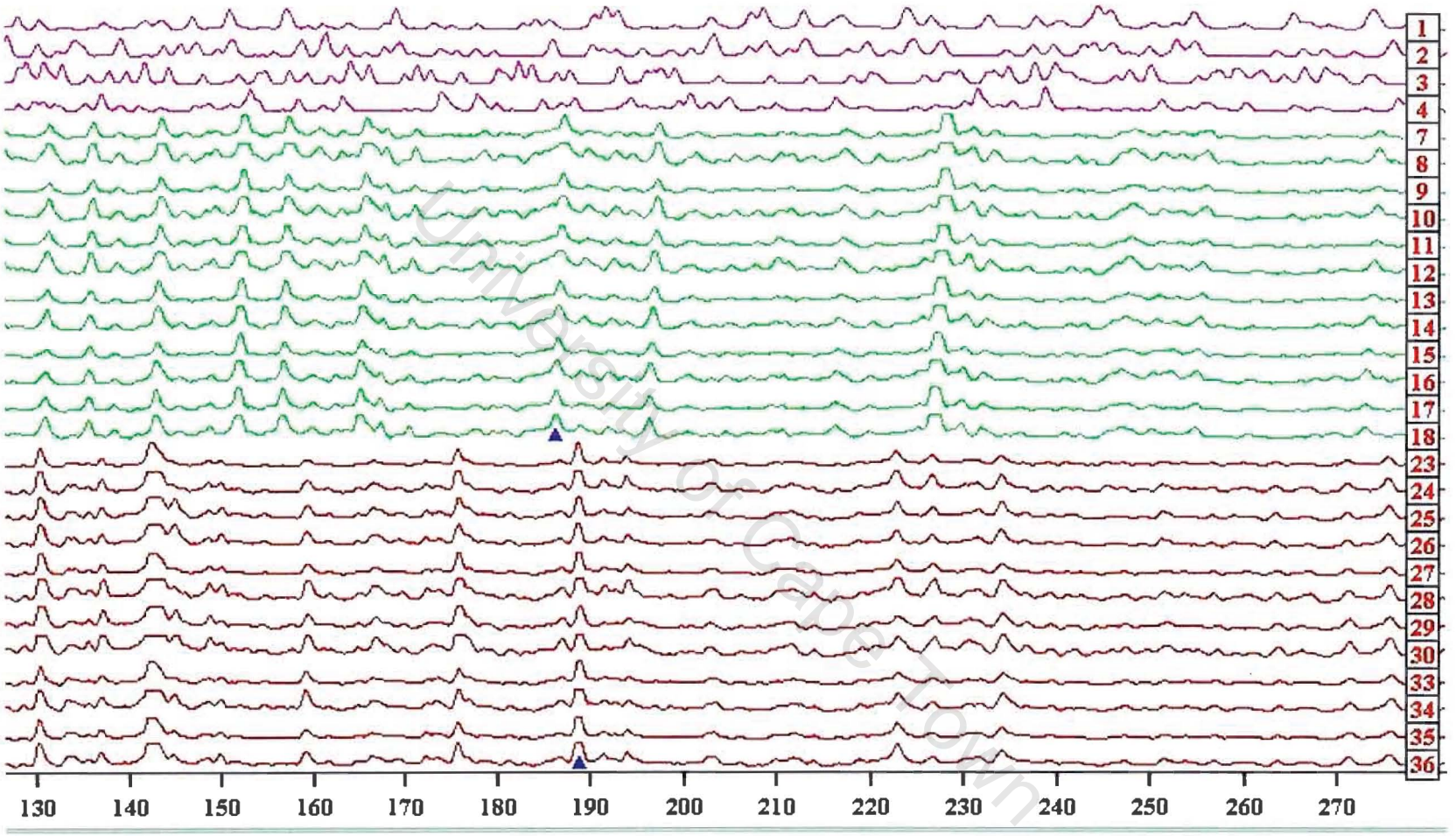

Fig. 4.3 Electropherogram displaying peaks of 6 St. Helena Bay (green; lanes 7-18) and 6 Saldanha Bay (red; lanes 23-36) isolates in duplicate. Sequencing markers were used as standards (purple; lanes 1, A; 2, C; 3, G; 4, T). Distinct peaks (σ) which could be unambiguously scored by eye were chosen as loci.

4.3.4 Analysis of AFLP profiles

Separate sets of loci were scored for *G. gracilis* (18 loci; Tables 4.5 and 4.7) and *Gp. longissima* (19 loci; Table 4.6 and 4.7). However, three loci (1, 7, 14; Table 4.7) were common to *G. gracilis* and *Gp. longissima*. The set of loci used for analysing a particular system depended on the species which predominated. Phenograms were constructed by TFPGA (tools for population genetic analyses; Miller, 1997) based on UPGMA. Both methods (refer to section 4.2.3.7) of determining allele frequency generated identical phenograms (only one shown). This would indicate that the phenograms are well supported by the available methods. These phenograms discriminated between *G. gracilis* and *Gp. longissima*. Hence, a certain amount of phylogenetic information may be inferred from the trees. Terminal nodes within the Saldanha Bay (Fig. 4.4) phenogram were poorly supported, whereas basal nodes had high bootstrap support. The terminal nodes may therefore be collapsed. Bootstrap support for nodes was significant (>50%) for Langebaan Lagoon (Fig. 4.5) and St. Helena Bay (Fig. 4.6) trees.

4.3.4.1 Saldanha Bay clustering analysis

All Saldanha Bay isolates clustered together (Fig. 4.4) and could easily be differentiated from LanF1 (*Gp. longissima*; refer to section 2.2.1 for nomenclature of isolates). This correlates with sequencing results (Chapter 3) that showed that all Saldanha Bay isolates analysed are *G. gracilis*. The distance between LanF1 and the *G. gracilis* clade was 0.94 (c.a. 5.6% similarity). The distance from the most basal node of the *G. gracilis* clade was 0.45 (c.a. 55% similarity). Bootstrap support was very low (< 50%) for the majority of nodes within the *G. gracilis* clade. HelD3 was included in the Saldanha Bay analysis as its

electropherogram profile appeared similar to that of Saldanha Bay isolates. This isolate clustered within the *G. gracilis* clade indicating that it is a *G. gracilis* specimen.

4.3.4.2 Langebaan Lagoon clustering analysis

The majority of Langebaan Lagoon isolates clustered together in a *G. gracilis* clade (Fig. 4.5) The exceptions were LanA3, LanF1, LanF2, LanF3, LanF4 and LanF5 which clustered together in a separate clade. This correlates with sequencing results (Chapter 3) which showed LanF1 to be a *Gp. longissima* specimen. The distance between the two clades was 1.37 (c.a. 2.5% similarity). The distance from the most basal node of the *G. gracilis* clade was 0.31 (c.a. 78% similarity). Bootstrap support was high (> 50%) for the majority of nodes.

4.3.4.3 St. Helena Bay clustering analysis

All St. Helena Bay isolates clustered together with the exception of HelD3 (Fig. 4.6). This clade could easily be differentiated from HelD3 (*G. gracilis*). This correlates with sequencing results (Chapter 3) which showed St. Helena Bay isolates to be *Gp. longissima*. The distance between the two clades was 1.79 (c.a. 5.6% similarity). The distance from the most basal node of the *Gp. longissima* clade was 0.18 (c.a. 90.5% similarity). Bootstrap support was high (> 50%) for the majority of nodes within the *Gp. longissima* clade. Isolates obtained from the salt marsh at Vlaminke Vlei (Fig. 2.3; Patch E) clustered together. HelE4 was not analysed in this study (refer to section 3.3.1).

4.3.4.4 Combined Langebaan Lagoon-Saldanha Bay clustering analysis

A combined Langebaan Lagoon-Saldanha Bay phenogram (Fig. 4.7) showed that Langebaan isolates were generally genetically distinct from Saldanha Bay isolates with most of the Langebaan Lagoon isolates clustering separately from Saldanha Bay isolates. From Fig. 4.7 it can also be inferred that LanA1 is more similar to Saldanha Bay samples whereas isolates SalE3-E5 are more similar to Langebaan Lagoon samples. It was also evident that HelD3 clustered together with Saldanha Bay isolates. The distance between the *G. gracilis* and *Gp. longissima* clade was 1.16. The distance from the most basal node of the *G. gracilis* clade was 0.41. Bootstrap support was low (< 50%) for the majority of the clades.

Table 4.5 Genotype information[a] for *G. gracilis* isolates.

Isolate	Locus																	
	1	2	4	5	7	8	9	10	11	14	18	20	21	23	25	26	30	34
SalA1	1	1	2	2	1	1	1	1	2	1	1	1	1	1	2	1	1	1
SalA2	1	1	1	2	1	1	1	1	2	1	1	1	2	2	2	1	1	2
SalA3	1	1	1	1	1	1	1	1	2	1	1	1	2	2	1	1	1	2
SalA4	1	1	1	1	1	1	1	1	2	1	1	1	1	1	2	1	1	1
SalA5	1	1	1	2	1	1	1	1	2	1	1	1	2	2	2	1	1	2
SalA6	1	1	1	1	1	1	1	1	2	1	1	1	1	2	2	1	1	1
SalA7	1	1	1	1	1	2	1	1	2	1	1	1	1	1	2	1	1	1
SalA8	1	2	2	1	1	2	1	1	2	1	1	1	1	1	2	1	1	2
SalB1	1	1	2	2	1	1	1	2	1	1	1	1	1	2	2	1	1	2
SalB2	1	1	1	1	1	1	1	2	1	1	1	1	1	1	1	1	1	2
SalB3	1	1	1	1	1	1	1	1	2	1	1	1	1	1	1	1	1	2
SalB4	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2
SalB5	1	2	2	1	1	1	1	1	2	1	1	1	1	1	1	1	1	2
SalB6	1	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2
SalB7	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2
SalC1	1	1	1	1	1	1	1	1	2	1	1	1	1	1	1	1	1	2
SalC2	1	2	2	2	1	1	2	1	2	1	1	1	2	1	1	2	1	2
SalC3	1	1	1	1	1	1	1	1	2	1	1	1	1	2	1	2	1	2
SalC4	1	1	2	1	1	1	1	1	2	1	1	1	1	1	1	2	1	2
SalC5	1	1	2	1	1	1	1	1	2	1	1	1	1	1	1	1	1	2
SalC6	1	1	1	1	1	1	1	1	2	1	1	1	1	1	1	1	1	2
SalC7	1	1	2	1	1	1	1	1	2	1	1	1	1	1	1	1	1	2
SalD1	1	1	1	1	1	1	1	1	2	1	1	1	1	2	1	1	1	2
SalD5	1	1	1	1	1	2	1	2	2	1	1	1	2	2	2	2	1	2
SalD7	1	1	2	1	1	1	1	1	2	1	1	1	1	1	1	2	1	1
SalD8	1	1	2	1	1	1	1	1	2	1	1	1	1	1	1	2	1	1
SalE1	1	1	2	1	1	2	2	1	2	1	1	1	1	1	1	1	1	1
SalE2	1	1	1	1	1	2	1	1	2	1	1	1	1	1	1	1	1	1
SalE3	1	1	1	1	1	1	1	1	2	1	1	1	1	1	1	1	1	1
SalE4	1	1	1	1	1	1	1	1	2	1	1	1	1	1	1	1	1	1
SalE5	1	1	1	1	1	1	1	1	2	1	1	1	1	1	1	1	1	1
SalE6	1	1	2	1	1	2	1	1	2	1	1	1	1	1	1	1	1	2
HelD3	1	1	1	1	1	1	1	1	1	1	1	1	1	2	1	2	1	2
LanA1	1	2	2	2	1	1	2	1	2	1	1	1	1	1	1	1	1	2
LanA2	1	1	2	1	1	1	1	1	2	1	1	1	1	1	1	1	1	1
LanB1	1	1	2	1	1	1	1	1	2	1	1	1	1	1	1	1	1	1
LanB2	1	1	2	1	1	1	1	1	2	1	1	1	1	1	1	1	1	1
LanB3	1	1	2	1	1	1	1	1	2	1	1	1	1	1	1	1	1	1
LanB4	1	1	1	1	1	1	1	1	2	1	1	1	1	1	1	1	1	1
LanB5	1	1	1	1	1	1	1	1	2	1	1	1	1	1	1	1	1	1
LanC1	1	1	1	1	1	1	1	1	2	1	1	1	1	1	1	1	1	1
LanC2	1	1	1	1	1	1	1	1	2	1	1	1	1	1	1	1	1	1
LanC3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
LanC4	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
LanC5	1	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
LanD1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
LanD2	1	1	2	1	1	1	1	1	2	1	1	1	1	1	1	1	1	1
LanD3	1	1	2	1	1	1	1	1	2	1	1	1	1	1	1	1	1	1
LanD4	1	1	2	1	1	1	1	1	2	1	1	1	1	1	1	1	1	1
LanE1	1	1	1	1	1	1	1	1	2	1	1	1	1	1	1	1	1	1
LanE2	1	1	1	1	1	1	1	1	2	1	1	1	1	1	1	1	1	1
LanE3	1	1	2	1	1	1	1	1	2	1	1	1	1	1	1	1	1	1
LanE4	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
LanF6	1	1	1	1	1	1	1	1	2	1	1	1	1	1	1	1	1	1

[a]1 represents the presence of a band.
[a]2 represents the absence of a band.

Table 4.6 Genotype information for *Gp. longissima* isolates.

Isolate	Locus																		
	1	3	6	7	12	13	14	15	16	17	19	22	24	27	28	29	31	32	33
HelA1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
HelA2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
HelA3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
HelA4	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
HelA5	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
HelB1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
HelC1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
HelC2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
HelC4	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
HelD1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
HelD2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
HelE1	2	1	1	1	1	2	1	1	1	1	1	1	1	1	1	1	2	1	1
HelE2	2	1	1	1	1	2	1	1	1	1	1	1	1	1	1	1	2	1	1
HelE3	2	1	1	1	1	2	1	1	1	1	1	1	1	1	1	1	2	1	1
HelE5	2	1	1	1	1	2	1	1	1	1	1	1	1	1	1	1	2	1	1
HelF1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
HelF2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
HelF3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
HelF4	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
HelF5	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
HelG1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
HelH1	1	1	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
HelH2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	1
HelH3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	1
LanF1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
LanF2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
LanF3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
LanF4	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
LanF5	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
LanA3	1	2	2	1	2	2	1	2	2	2	2	2	2	2	2	2	2	2	2

[a]1 represents the presence of a band.
[a]2 represents the absence of a band.

Table 4.7 Genotype ratios of *G. gracilis* and *Gp. longissima* isolates.

		G. gracilis		*Gp. longissima*	
Locus	Estimated time[a] (min)	Ratio	(%)	Ratio	(%)
1	124	54/54	100	26/30	87
2	127	50/54	93	0	0
3	128	0	0	30/30	100
4	129	31/54	57	0	0
5	130	48/54	89	0	0
6	132	0	0	30/30	100
7	134	54/54	100	29/30	97
8	135	48/54	89	0	0
9	137	51/54	94	0	0
10	138	51/54	94	0	0
11	139	11/54	20	0	0
12	141	0	0	30/30	100
13	144	0	0	26/30	87
14	146	54/54	100	30/30	100
15	150	0	0	30/30	100
16	152	0	0	30/30	100
17	154	0	0	30/30	100
18	159	54/54	100	0	0
19	169	0	0	30/30	100
20	171	54/54	100	0	0
21	175	49/54	91	0	0
22	177	0	0	30/30	100
23	184	45/54	83	0	0
24	195	0	0	30/30	100
25	198	45/54	83	0	0
26	200	47/54	87	0	0
27	201	0	0	30/30	100
28	202	0	0	30/30	100
29	204	0	0	30/30	100
30	210	54/54	100	0	0
31	224	0	0	26/30	87
32	230	0	0	28/30	93
33	242	0	0	30/30	100
34	245	31/54	57	0	0

Ratio Number of isolates displaying allele/ total number of isolates sampled.
(%) Percentage of samples displaying the respective allele.
0 Absence of allele in all isolates sampled.
[a] Time at which respective allele was observed on electropherogram.

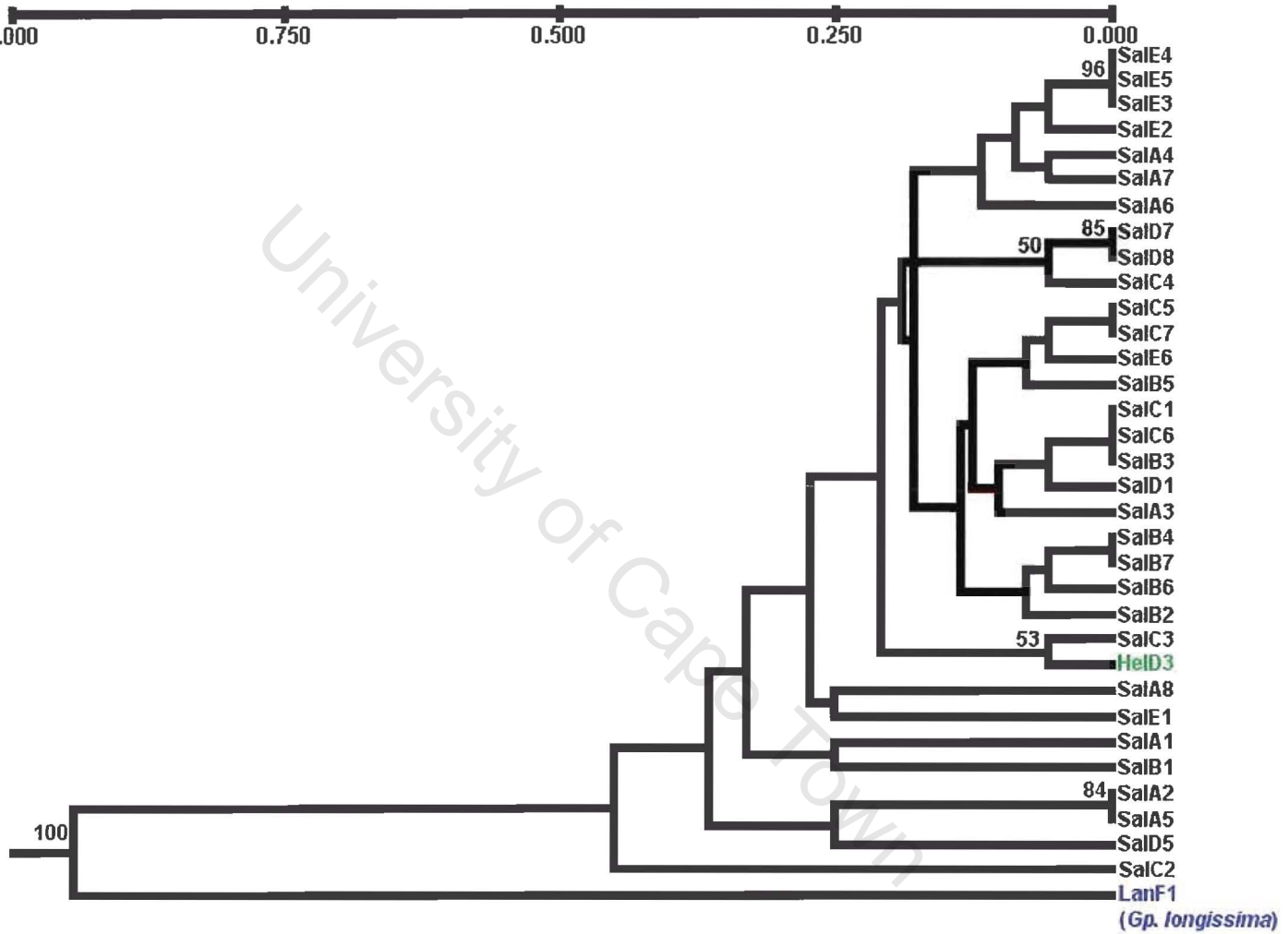

Fig. 4.4 Phenogram of Saldanha Bay isolates (HelD3 also included; refer to section 4.3.4.1). The tree is rooted with *Gp. longissima* (LanF1) as the outgroup. Numbers above the branches indicate bootstrap values (percentage of 100 replicates; values below 50% are not included). Scale is based on Nei's unbiased (1978) distance measures. Refer to section 2.2.1 for nomenclature of isolates.

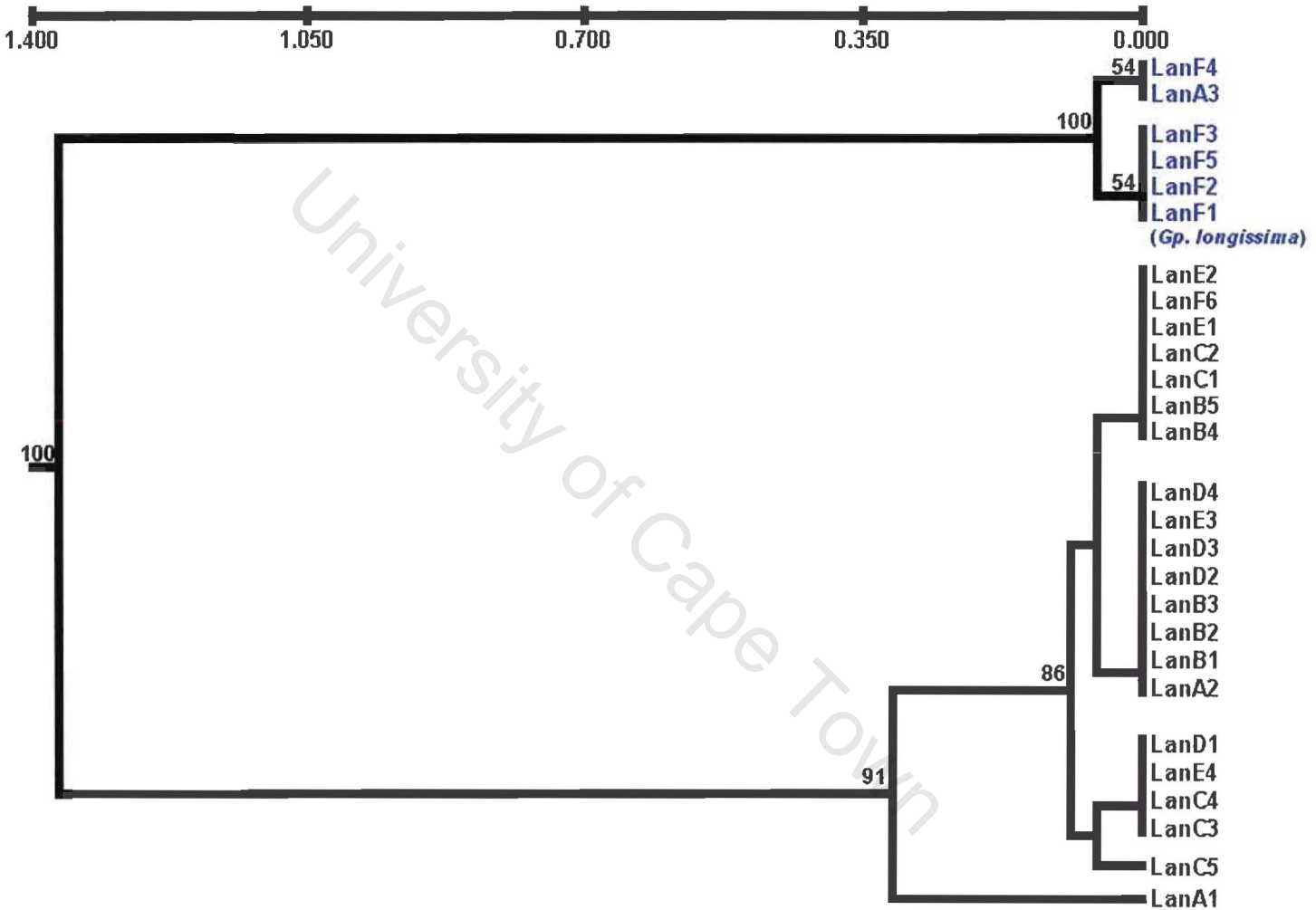

Fig. 4.5 Phenogram of Langebaan Lagoon isolates. The tree is rooted with *Gp. longissima* (LanF1) as the outgroup. Numbers above the branches indicate bootstrap values (percentage of 100 replicates; values below 50% are not included). Scale is based on Nei's unbiased (1978) distance measures. Refer to section 2.2.1 for nomenclature of isolates.

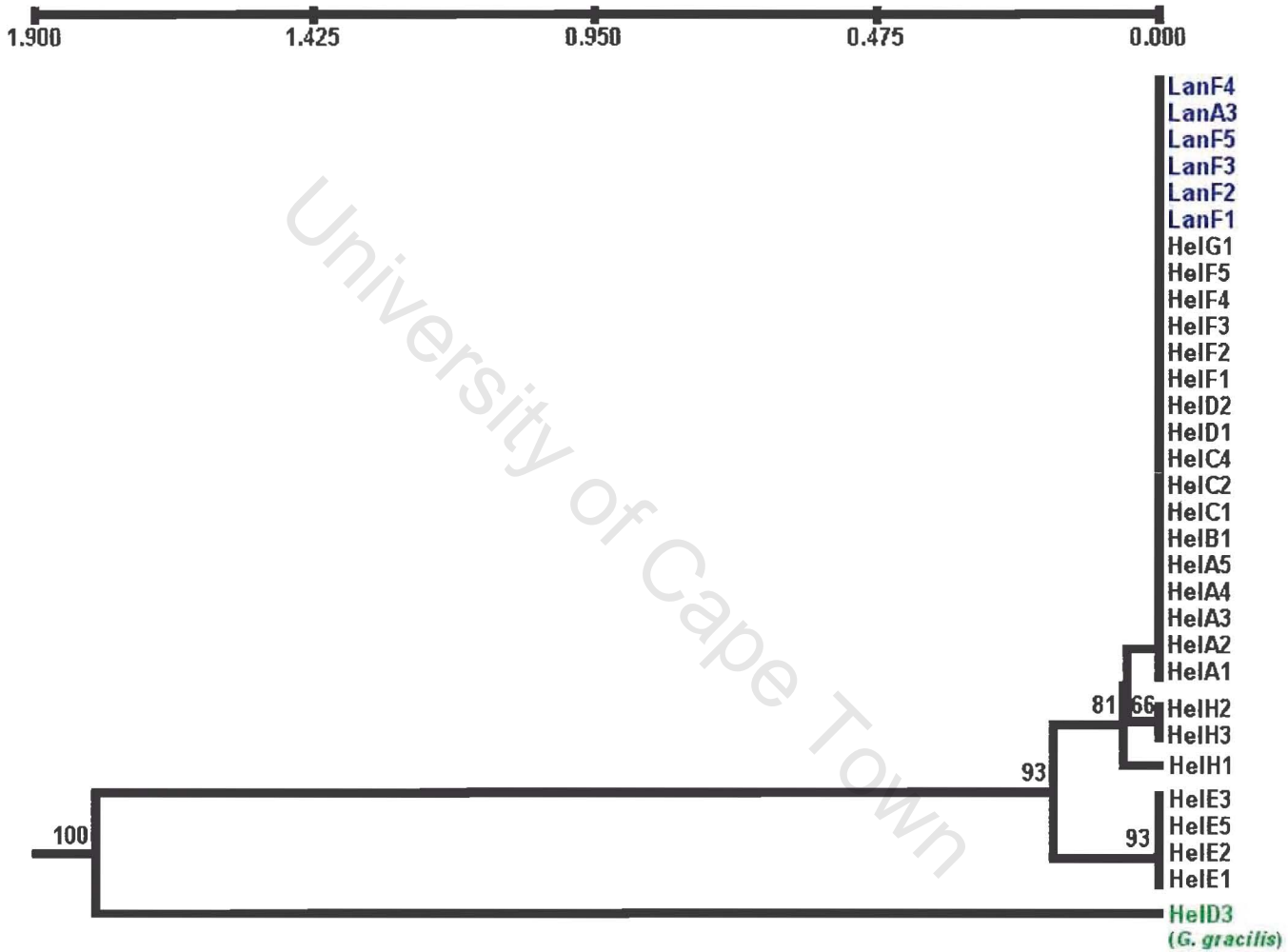

Fig. 4.6 Phenogram of St. Helena Bay (LanA3 and LanF1-F5 are also included). The tree is rooted with *G. gracilis* (HelD3) as the outgroup. Numbers above the branches indicate bootstrap values (percentage of 100 replicates; values below 50% are not included). Scale is based on Nei's unbiased (1978) distance measures. Refer to section 2.2.1 for nomenclature of isolates.

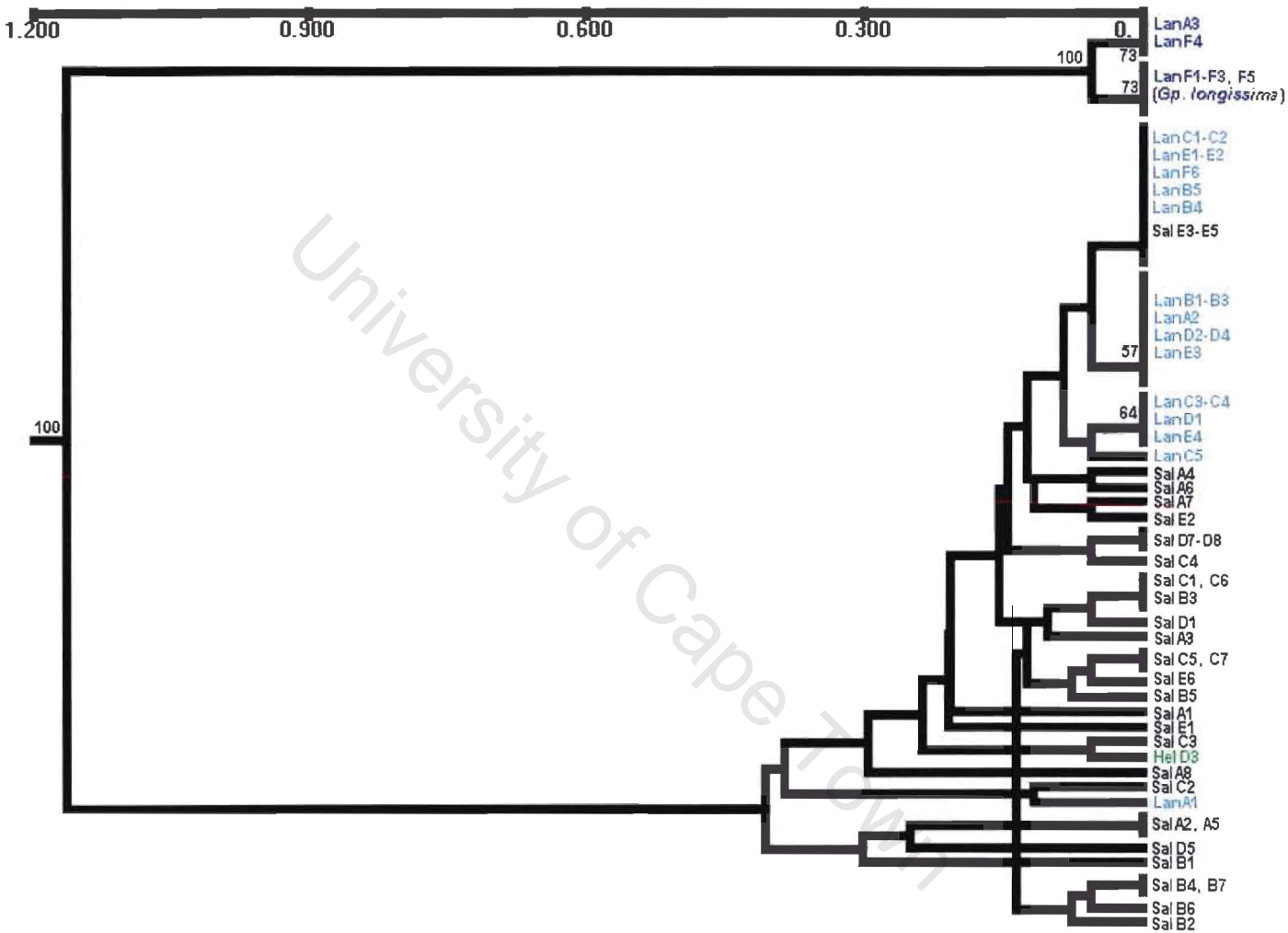

Fig. 4.7 Phenogram of Langebaan Lagoon and Saldanha Bay isolates (HelD3 also included). The tree is rooted with *Gp. longissima* (LanF1) as the outgroup. Numbers above the branches indicate bootstrap values (percentage of 100 replicates; values below 50% are not included). Scale is based on Nei's unbiased (1978) distance measures. Refer to section 2.2.1 for nomenclature of isolates.

4.4 DISCUSSION

AFLP has many of the characteristics of an ideal system for detecting genetic variation. For example, variability is assessed at a large number of independent loci, AFLP markers are 'neutral' (i.e. not subject to natural selection), variation is revealed in any part of the genome and data is obtained very quickly (Majer *et al.*, 1996). It is therefore a suitable tool for population genetic studies.

The choice of either restriction enzyme or primer can affect the number of AFLP polymorphisms detected. For example, more polymorphisms are detected in barley with the combination of restriction enzymes *Pst*I-*Mse*I than with *Eco*RI-*Mse*I (Ridout and Donini, 1999). Primer pairs that could produce too many bands should be avoided owing to concerns regarding reproducibility and the greater probability of homoplasy (different fragments of the same size). The inconsistent digestion results obtained in this study using the *Bcl*I-*Taq*I restriction set could be due to unstable activity of one of the enzymes. The *Mse*I-*Eco*RI restriction set was therefore suitable as it resulted in complete digestion of genomic DNA. This could be because the A+T-rich recognition sequences such as those of *Eco*RI (G/AATTC) or *Mse*I (T/TAA) are more frequent in G+C-poor genomes including the Gracilariaceae. The range of mol% G+C content of the nuclear genome is small throughout the Gracilariaceae (c.a. 41.1-49.7%; Dutcher *et al.*, 1990).

There are several concerns with regard to AFLP reproducibility: (i) some studies have found different banding patterns when samples are re-run (Krauss and Peakall, 1998; Winfield *et al.*, 1998); (ii) the scoring of the AFLP bands and their inclusion in a data matrix is not

explicit, which suggests that different individuals will score AFLP patterns differently; and (iii) the method of genomic DNA preparation may affect banding patterns, e.g. partial digestion due to either poor DNA quality or insufficient restriction enzymes (Lin and Kuo, 1995).

AFLPs result in very distinct bands but bands are not always reproducible. Winfield *et al.* (1998) in an analysis of genetic diversity of Black Poplar, ran duplicate samples for five trees. Three duplicates returned exactly the same banding patterns, while the other two were 98.9% and 97.6% similar. The number of different bands this equates to, and whether these differences were band gains or losses, was not documented. Krauss and Peakall (1998) encountered a "rare" disappearing fragment, in which an initially polymorphic band was scored but subsequent analysis with a new DNA extraction and repeated AFLP amplification failed to reproduce this band. They suggested that this could be due to partial digestion of the template genomic DNA, poor amplification of this fragment during PCR or DNA contamination.

The problems associated with AFLPs can be divided into three types: (i) practical; (ii) data; and (iii) analysis. Many of these problems are not unique to AFLP methodologies, but apply to most molecular marker systems. An ideal marker would have: (i) sufficient variation for the problem under study; (ii) be reliable; and (iii) be simple to generate and interpret. Unfortunately, an ideal marker does not exist for use in all studies; rather a technique or techniques will be suited to a range of investigations (Karp *et al.*, 1997; Harris, 1999).

In existing protocols, AFLP products are separated by denaturing polyacrylamide gel electrophoresis (PAGE) and detected either radioisotopically (Vos *et al.*, 1995) or by silver staining (Falcone *et al.*, 1995). In this study a non-radioisotopic modification of the AFLP method that uses a 5' Cy5 end-labelled selective primer was utilised. Cy5 is a fluorescent molecule with absorption and emission maxima at 649 and 670 nm, respectively (Roman *et al.*, 1999). Although they are more expensive to synthesise than unlabelled oligonucleotides, the modification is stable for years, and a small amount of oligonucleotide can be used for many rounds of AFLP, hence providing a low cost per reaction method.

Because AFLPs and RAPDs generate dominant markers, the information available to the researcher is incomplete because heterozygotes cannot be distinguished. For dominant markers, each band is assumed to correspond to a single locus. The presence of the band is assumed to represent the dominant genotype while the absence of a band represents the homozygous recessive genotype. This means that it is assumed that there are only 2 alleles at each locus. Genotype information is entered for each locus as either a "1", indicating the presence of a band or a "2" indicating the absence of a band (Tables 4.5 and 4.6). Values greater than 2 are unacceptable for input in these data sets (Miller, 1997).

Because of the incomplete information generated by dominant markers, allele frequencies must be estimated. TPFGA provides three methods for generating these estimates. In the case of AFLPs or RAPDs, each band is assumed to represent the dominant genotype at a locus while the lack of that same band in another organism represents the alternate homozygous recessive genotype (2 alleles per locus is assumed). In the case of 2 alleles when heterozygotes cannot be distinguished, a potential estimate of the frequency of the recessive

allele is simply the square root of the frequency of "absent bands" seen at a locus (Weir, 1990). An alternative and apparently less biased estimator based on a Taylor expansion was outlined by Lynch and Milligan (1994). Both methods require the assumption that genotype frequencies are in Hardy-Weinberg equilibrium. One potential drawback to both of the above mentioned approaches is that estimates of allele frequencies generated in this fashion often do not correspond to a possible genotype outcome. However it is not believed that this has an adverse effect on AFLP analysis (Miller, 1997).

There is a paradigm in conservation biology that genetic variation and population viability increase with population size, but decrease with population isolation (Ellstrand and Elam, 1993; Young *et al.*, 1996; Menges and Dolan, 1998). Many recent investigations support the hypothesis that small population size leads to low per capita reproductive rates (Allee effect; Lamont *et al.*, 1993; Agren, 1996; Groom, 1998; Oostermeijer *et al.*, 1998) and thus population size itself is the best predictor of population viability.

These hypotheses are partially supported in this study. Langebaan Lagoon and Saldanha Bay have the largest population size (based on observed density of material during sampling; Chapter 2) compared to St. Helena Bay which has the smallest population size (based on observed scarcity of material during sampling, Chapter 2). However, St. Helena Bay displays similar overall genetic diversity to Langebaan Lagoon, yet lower variation than observed in Saldanha Bay. It would seem that ecological conditions have a large impact on genetic diversity. This can be due to other factors including historical developments of population sizes (Ouborg and van Treuren, 1995) and the degree of isolation from other

populations. Processes of genetic erosion (genetic drift and inbreeding) tend to lower genetic diversity.

Schmidt and Jensen (2000) found that genetic variability was independent of actual population size, and suggested that historical population processes had to also be taken into account. They concluded that populations of the rare biennial plant, *Pedicularis palustris* (Scrophulariaceae) are genetically isolated and that reproductive success most likely is influenced by population size, genetic variability, and habitat quality.

Saldanha Bay isolates display some degree of genetic divergence (c.a. 55% similarity), more than isolates occurring in the other two localities. There are three possible reasons for the levels of genetic variation found in Saldanha Bay:

(i) The main problem with Saldanha Bay is the seasonal increase and decrease of nitrogen levels in the water. Previous research revealed that summer conditions of warm, nutrient depleted surface waters following prolonged southerly winds have a deleterious effect on the growth of *Gracilaria*. The influx of waste from fish factories has the effect of increasing epiphytic levels. Previous collapses in *G. gracilis* populations were quite pronounced due to this combination of factors. New populations are believed to be derived from a few individuals by fragmentation. In support of this argument, Anderson *et al.* (1989) found that there was almost no material left in Saldanha Bay. However, the biomass recovered fully over a period of just 2 months. It seems that the recovery was based on rapid growth of relatively little material. However, it may be possible that populations were generated from a large number of founder specimens.

(ii) Fertile specimens were previously observed on a regular basis by Isaac (1956) in Saldanha Bay. Only during the last few decades has there been a scarcity of fertile material. It is possible that ancestrally divergent material has survived through the recent collapses in populations. Also fertile specimens of *G. gracilis* have been observed in Saldanha Bay during the last ten years on a few occasions, albeit rarely (Anderson, pers. comm.). Sexual reproduction tends to increase genetic variation and could possibly explain the moderate divergence.

(iii) Saldanha Bay remains a favourable location for the growth of *G. gracilis*, especially during the winter months. Populations have been observed to often recover within a few months after a collapse. This high turnover of material may be the cause of the moderate variation.

Strain selection processes in seaweeds often have assumed that sterile clones could be maintained for long periods in a diversity of environments without major genetic changes (Meneses and Santelices, 1999). However, clonal species such as *Gracilaria chilensis* exhibit intra-clonal variation in performance, and ongoing studies suggest such changes may be due to rapid changes in DNA composition via mitotic recombinations during growth. Meneses and Santelices (1999) found that transfer to laboratory conditions reduced the frequency of low similarity values and increased the frequency of intermediate similarity values in DNA banding patterns, suggesting that the branchlets produced under controlled laboratory conditions have less genetic variability than plants that were recently collected in the field. Their results suggest that the dynamic of genetic changes in vegetative clones of *G. chilensis* is fast and strongly affected by the external environment. However, the genetic changes measured in vegetatively propagating clones of *G. chilensis* suggest two important

conclusions related to strain selection of clonal seaweeds: (i) In clonal thalli the dynamics of genetic change seems to be fast. It is not known if these changes are faster than in sexually reproducing populations. (ii) The importance of the external environment in determining these changes.

Langebaan Lagoon, being a very shallow and sheltered lagoon, seems to be suitable for vegetative growth of *Gracilaria* and some *Gracilariopsis* isolates. Interestingly, water flow in the lagoon is quite high due to the presence of tidal flow through channels. This would favour increased reproductive rates and hence greater genetic variability. However, the opposite is observed, in that vegetative growth occurs (c.a. 78% similarity). It would seem that other ecological factors or a combination of factors have a greater impact on the genetic composition of these isolates. Interestingly, it has been observed (Anderson, pers. comm.) that strong currents in Langebaan Lagoon led to the establishment of several isolated populations, and fragments from these populations are simply lost to the system. There is therefore almost no loose, drifting material such as is common in Saldanha Bay. In Saldanha Bay, material rolling around on the bottom can shed fragments or spores (although seldom fertile) over a wide area.

AFLP results suggest that specimens in the St. Helena Bay system are more similar to each other (c.a. 90% similarity). Being an open bay there should be much variation in the ecological conditions and therefore a strong need for these algae to adapt. The size of St. Helena Bay lends itself to being an ecologically diverse habitat. River mouths and salt marshes are examples of ecological niches that occur here. These could definitely affect genetic diversity due to selective pressures and adaptive responses. This could possibly

account for isolates of Patch E (HelE1, E2, E3, and E5) clustering together. These isolates are physically separated from the bay system and would have adapted to conditions prevalent within the salt marsh from which they were obtained. The apparent clonal growth within the bay itself is possibly due to occasional toxic H_2S events (black tides) which kill much of the biota (including seaweeds). These natural events are a result of the anoxic decay of phytoplankton blooms (Mathews and Pitcher, 1996). The resulting population would be of recent origin, and its low level of genetic diversity may be caused by a founder effect. Hence, it is quite possible that these occasional large reductions in biomass could cause a decrease in genetic variation.

Mariculture and research rafts within Small Bay have frequently been seeded with material from St. Helena Bay. Hence the presence of *Gp. longissima* populations would be expected in this system. However, this is not the case. It is possible that Saldanha Bay (Small Bay) is a homogenous environment which is favourable for the growth of *G. gracilis*, whereas certain ecological conditions are possibly adverse for growth of *Gp. longissima* explaining the absence of this species. The presence of *Gp. longissima* in Langebaan Lagoon could be attributed to contamination by either loose material from rafts or from material attached to or washed off boats. It is possible that Langebaan Lagoon is a more heterogenous environment as *G. gracilis* and *Gp. longissima* were found to co-exist within the same patch in two separate locales (Patch A and F).

This study was therefore beneficial in determining the degree of genetic variation in the three areas studied. This study was also capable of discriminating between specimens of *G. gracilis* and *Gp. longissima*. From this data it was determined that a small population of

Gp. longissima co-exists with *G. gracilis* populations in Langebaan Lagoon. It is now evident that most of the isolates found in Langebaan Lagoon and St. Helena Bay are clonal, as the majority of isolates from the respective systems clustered together indicating that they may belong to a single strain. Also it can be inferred that Saldanha Bay material displays relatively more variation. It can be concluded that AFLPs can be utilised effectively to determine genetic variation within populations and in distinguishing between *G. gracilis* and *Gp. longissima.*

Chapter 5

GENERAL DISCUSSION

Gracilarioid algae from the south western Cape propagate mainly by asexual reproduction. Fragmentation generally results in clonal populations. In corals this occurs by partial mortality of a group of intervening polyps in a colony or by breakage in branching species (Jackson, 1985). In land plants, propagation by fragments is widespread and among seaweeds, fragmentation is a frequent means of maintaining free-living populations (Lobban and Harrison, 1994). Vegetative propagation through thallus fragmentation is an advantageous aspect of commercial algal species. Simple thallus fragments are likely to grow and spread faster than spores or other types of microscopic propagules and the features selected in one particular strain are maintained by growing self-replicating fragments, which are probably identical genetic units. In *Gracilaria gracilis*, propagation by thallus fragmentation is the method most used to farm the species.

Gracilaria yields good quality and quantities of agar. Traditionally, economically important seaweeds are collected from beach-cast (e.g. *G. gracilis* from Saldanha Bay and, to a much smaller extent, *Gracilariopsis longissima* from St. Helena Bay) or harvested from natural populations. A worldwide increase in demand has led to overexploitation of some populations, resulting in shortages that are exacerbated by the inability of some populations, such as *Gracilaria* in Chile (Westermeier *et al.*, 1993), to recover sufficiently for sustainable utilisation. The demand for agar by many countries is increasing, providing South Africa with another important raw material for export. *Gracilaria* is also important to abalone farmers in South Africa in which case the *Gracilaria* is used as abalone feed. Marine Growers Limited,

needs to be determined. This is therefore a viable method to identify tetrasporophytic plants that could be manipulated under laboratory conditions to produce male and female gametophytes. In this study the cultivation of 1 cm excised tips of *Gracilaria* and *Gracilariopsis* was not successful in the long term. More detailed culture experiments are necessary to study variation within genotypes. Colour mutants, similar to the one found in this study, could provide information as to how these gracilarioids mutate.

Vegetative morphology lacks taxonomic significance due to the isomorphic nature of terete taxa. One cannot differentiate between *G. gracilis* and *Gp. longissima* by external morphology. Adding to this, it was found that under varying ecological conditions different morphologies occur. Although these specimens appear different externally, molecular studies show that they belong to the same species. Hence these specimens would therefore represent ecads. Cross sections of cystocarpic material from the Langebaan Lagoon-Saldanha Bay system and St. Helena Bay specimens display similarities to previously referenced *Gracilaria* and *Gracilariopsis* sp., respectively. This data correlates with 18S rDNA sequence data. Sectioning of vegetative thalli of *Gracilaria* has revealed prominent medullary cell walls, which are inconspicuous in *Gracilariopsis*. This is therefore a possible taxonomic character.

To confirm the identity of isolates in the studied location, molecular investigations were therefore necessary. DNA extraction from *Gracilaria* and *Gracilariopsis* is very difficult because of the high polysaccharide content (mainly due to agarose polymers). Therefore rapid and robust DNA techniques, such as the one utilised in this study, are essential for genetic studies. This method yielded DNA of sufficient quality and quantity for molecular studies.

This study to determine the nature of the red algal populations prevalent in the Langebaan Lagoon-Saldanha Bay system and in St. Helena Bay has provided insight into the existence of two taxa of the Gracilariaceae within these areas. *Gracilaria gracilis* dominates the Langebaan Lagoon-Saldanha Bay system and *Gp. longissima* predominates in St. Helena Bay, with a small isolated population in Langebaan Lagoon. Sequencing of a 299 bp variable region of the 18S rRNA gene readily distinguished between *G. gracilis* and *Gp. longissima* samples. This method has proved to be a simple and inexpensive method in that sequencing of a 299 bp variable region could differentiate between the two genera rather than sequencing the entire gene. *Gracilaria gracilis* and *Gp. longissima* specimens were well supported within the phylogenetic trees and resulted in monophyletic clades with referenced species.

Single stranded conformation polymorphisms (SSCP's) were unsuccessful in this study. Therefore there was a need to obtain an alternative method for detecting genetic variation within populations of *Gracilaria* and *Gracilariopsis* specimens. Amplified fragment length polymorphisms (AFLPs) employed in this study was successful. The general conclusion drawn was that Langebaan Lagoon and St. Helena Bay isolates are clonal, whereas Saldanha Bay isolates display a small degree of divergence. The method was also suitable for identifying *Gp. longissima* and *G. gracilis*. A single *G. gracilis* specimen was identified in St. Helena Bay and two populations of *Gp. longissima* were identified in Langebaan Lagoon. A significant difference observed in the clustering of isolates in St. Helena Bay was that all isolates from the salt marsh clustered together. These isolates are physically separated from the bay system and would have adapted to conditions prevalent within this habitat.

Apart from AFLPs, other molecular techniques have been successfully applied in population studies. (i) The mitochondrial control region has been used frequently and very successfully in systematic, phylogenetic and population studies, due to its high mutation rate (Avise, 1994; Zuccarello *et al.*, 1999a). (ii) Microsatellite data reported by Wattier *et al.* (1997) revealed a high level of intra-population polymorphism in *G. gracilis* and their results also suggest that the polymorphic locus Gv2CT may provide a valuable genetic marker within the different species of the Gracilariaceae. (iii) SSCP was used for the detection of plastid variation within populations of the red alga, *Caloglossa leprieurii* (Zuccarello *et al.*, 1999b). (iv) DNA sequence variation in the *rbc*L-*rbc*S intergenic spacer region has provided useful data for the reconstruction of inter- and intra-species phylogenies (Goff *et al.*, 1994; Stache-Craine *et al.*, 1997; Kamiya *et al.*, 1998). In these studies, the intergenic region has been used to analyse isolates from different biogeographic locations, but in only one case did sequence data reveal variation at the population level (Zuccarrello and West, 1997).

No single objection to the use of AFLPs for systematic studies exists, but the weight of circumstantial evidence cautions against their use. Firstly, the problems of non-homology and non-independence of the AFLP data have the potential to seriously mis-estimate similarity and distance, and these two problems cannot be overcome without extensive testing. Donaldson *et al.* (2000) subjected the AFLP procedure to reproducibility testing and three shortcomings were noted: (i) failure to reproduce band intensity between replicate runs for the same individual and primer pair; (ii) failure of some bands to replicate; (iii) lack of reproducibility for complete replicate runs for some individuals and primer pairs. In addition, the problems of scoring bias introduced by dominance, reproducibility problems, the effect of polyploids, as well as practical problems need to be addressed. There are other methods of

inferring phylogenies which are more rigorous, such as sequencing and plastid RFLPs, and which are no more expensive than AFLPs. For any problem under investigation it is the nature of the problem that should dictate the method of analysis, the most modern method being not always the best or most cost-effective way of addressing the problem. Robinson and Harris (1999) concluded that AFLPs should not be considered for phylogenetic analysis above the species level. AFLP is a valuable method for addressing population genetics and plant breeding issues, but when used for phylogeny reconstruction and taxonomy the technique is at best problematic and at worst misleading.

Some questions are raised in this study. (i) Do the two genera have different ecological requirements and are conditions in the two systems more suitable for the growth of the respective species that predominate? Hence could this have an impact on mariculture? (ii) How relevant is population size to genetic variability?

Due to the nature of this study and large sample number, material was only collected once during this study. Therefore future studies could be undertaken to trace the genetic variation of these samples over a period of time by collecting material from locations indicated in this study and performing further investigations. This would have the greatest significance if genetic variation was analysed soon after a population collapse. Three questions have been raised concerning population genetics during and after an adverse ecological event. (i) Is there a general collapse in population, with a few random genotypes surviving? Are these the genotypes which occur in the largest numbers? (ii) Does adaptation play a role in survival of specimens? If so, do these adverse ecological events drive the genetic evolution of the seaweed to a highly adapted "super-strain"? (iii) Is the genetic

makeup of the population similar to the present study, or is there a decrease in diversity indicating genetic erosion.

A further study involves the evaluation of 'strains' for their suitability for mariculture. For the export industry, samples with high agar content and increased growth rate would be ideal. For abalone farming, samples with high nutrient content and tolerance for growth in artificial ponds and tanks would be most suitable. For example, isolates of Patch E (salt marsh) in St. Helena Bay have been shown to be different from other *Gp. longissima* isolates. One would expect these seaweeds to be adapted to survival under extreme environmental conditions (high salt, water temperature and dehydration). Tests would have to be performed to determine the physiological characteristics of the different strains.

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Appendices

APPENDIX A: BUFFERS AND SOLUTIONS

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Water purified using a Milli-RO Plus (Millipore) water purification system, was used for making solutions and diluting buffers. Ultrapure water was obtained by further purification of the above water using a Milli-Q Plus (Millipore) water purification system.

A.1 BUFFERS

A.1.1 TE buffer (pH 8)

1 M Tris-HCl (pH 8)	10 μM
0.5 M EDTA (pH 8)	100 μM

A.1.2 Tris-Acetate-EDTA (TAE) buffer (50X stock)

Tris base (Boehringer Mannheim)	2 M
glacial acetic acid (Saarchem)	1 M
0.5 M EDTA (pH 8)	50 mM

A.1.3 Tris-Borate-EDTA (TBE) buffer (10X stock)

Tris base	890 mM
boric acid (Saarchem)	890 mM
0.5 M EDTA (pH 8)	10 mM

A.1.4 Lysis buffer

CTAB	1 %
PVP-40	5 %
NaCl	1.4 M
EDTA	20 mM
Tris.Cl	10 mM

A.1.5 DNA electrophoresis loading buffer

bromophenol blue	0.25 % (w/v)
sucrose	40 % (w/v)
0.5 M EDTA (pH 8)	20 mM

A.2 SOLUTIONS

A.2.1 ES-enriched seawater medium (Provasoli, 1963)

Fe-solution

Dissolve 351 mg of $Fe(NH_4)_2(SO_4)_2.6H_2O$ and 300 mg of Na_2EDTA in 500 ml of glass-distilled water. Autoclave.

PII metal solution	
Na_2EDTA	100 mg
H_3BO_3	114 mg
$FeCl_3.6H_2O$	4.9 mg
$MnSO_4.H_2O$	16.4 mg
$ZnSO_4.7H_2O$	2.2 mg
$CoSO_4.7H_2O$	0.48 mg

Dissolve all materials in 100 ml glass-distilled water. Adjust to pH 7.8 and autoclave. Store at 10°C.

ES-enrichment solution	
$NaNO_3$	350 mg
Na_2glycerophosphate.$5H_2O$	50 mg
Fe-solution	25 ml
PII metals	25 ml
vitamin B12	10 g
thiamine	0.5 mg
biotin	5 g
Tris buffer (Sigma)	500 mg

Dissolve all materials (except Fe-solution and PII metals) in 50 ml glass distilled water. Autoclave. Add 25 ml Fe solution and 25 ml PII metals and store at 10°C. Dissolve 6 ml ES-enriched solution in 1000 ml autoclaved, filtered seawater.

A.2.2 RNase A

Dissolve RNase A at a concentration of 10 mg/ml in 10 mM Tris.Cl (pH 7.5), 15 mM NaCl. Heat to 100°C for 15 min. Allow to cool slowly to room temperature. Dispense into aliquots and store at -20°C.

A.2.3 Denaturing solution

98 % formamide

10 mM NaOH

0.025% bromophenol blue

0.025% xylene cyanol

A.2.4 3% non-denaturing gel for SSCP

40% acrylogel (BDH)	7.5 ml
5X TBE	20 ml
water	71.5 ml

Prior to pouring the gel, the following was added:

10% (w/v) ammonium persulphate (Pharmacia)	1000 µl
N,N,N',N'-tetramethylethylene-diamine (TEMED; Sigma)	35 µl

The above solution was mixed well in a fumehood (without aerating too much) and rapidly syringed between two glass plates using a 60 cc syringe. The acrylamide was left to polymerise for at least 2 hrs.

A.2.5 Hamilton's freezing medium

gum arabic	1 g
sucrose	30 g
thymol	1 crystal
water to	100ml

APPENDIX B: DNA PRIMER SEQUENCES AND PCR CYCLE PROFILES

APPENDIX B: DNA PRIMER SEQUENCES AND PCR CYCLE PROFILES DNA

B.1 DNA primer sequences

All synthetic oligonucleotide sequences were supplied by the Oligonucleotide Synthesising Service of the Biochemistry Department, University of Cape Town, South Africa.

B.1.1 Primers for 18S rDNA amplification

K18SF	5'	CTC TGT AAG CTT ATA CCA AAG TTG TTG CAG	3'
K18SR	5'	CTA CGG ATC CCT TGT TAC GAC TTC TCC	3'

B.1.2 Nested primers for 18S rDNA sequencing and amplification SSCP products

sscpF	5'	GCC AGC AGC CGC GGT AAT TCC	3'
sscpR	5'	CTT GGC AGA CGC TTT CGC AGC	3'

B.1.3 Oligonucleotides and primers used in AFLP protocol

Oligo1	5'	CTC GTA GAC TGC GTA CC	3'
Oligo2	5'	TAG GTA CGC AGT CTA C	3'
Oligo3	5'	GAC GAT GAG TCC TGA C	3'
Oligo4	5'	AAT TGT CAG GAC TCA T	3'
Primer5	5'	CTC GTA GAC TGC GTA CCT AA	3'
Primer6	5'	GACGAT GAG TCC TGA CAA TTT C	3'
Primer7	5'	GTA GAC TGC GTA CCT AAN N	3'
Primer8	5'	ATG AGT CCT GAC AAT TCN N	3'

N = A, T, G or C

B.2 PCR cycle profiles

B.2.1 PCR cycle profile 1 (for 18S rDNA and SSCP amplification)

1 cycle:	denaturation	96°C	300 s
30 cycles:	denaturation	96°C	30 s
	annealing	57°C	60 s
	extension	72°C	120 s
1 cycle:	extension	72°C	600 s

B.2.2 PCR cycle profile 2 (Pre-selective PCR for AFLP protocol)

20 cycles:	denaturation	94°C	30 s
	annealing	56°C	60 s
	extension	72°C	60 s

B.2.3 PCR cycle profile 3 (Selective PCR for AFLP protocol)

1 cycle:	denaturation	94°C	30 s
	annealing	65°C	30 s
	extension	72°C	60 s

12 cycles:	denaturation	94°C	30 s
	annealing	x°C	30 s
	extension	72°C	60 s
	where x = :	64.3°C	for cycle 1
		63.6°C	for cycle 2
		62.9°C	for cycle 3
		62.2°C	for cycle 4
		61.5°C	for cycle 5
		60.8°C	for cycle 6
		60.1°C	for cycle 7
		59.4°C	for cycle 8
		58.7°C	for cycle 9
		58.0°C	for cycle 10
		57.3°C	for cycle 11
		56.6°C	for cycle 12
23 cycles of:	denaturation	94°C	30 s
	annealing	56°C	30 s
	extension	72°C	60 s

APPENDIX C: STANDARD METHODS

APPENDIX C: STANDARD METHODS

C.1 Agarose gel electrophoresis

Agarose gel electrophoresis of DNA was carried out according to Sambrook *et al.*, 1989. Agarose (D1 LE, Whitehead Scientific) was dissolved in TAE buffer (Appendix A) and 5 µl ethidium bromide (10 mg/ml) was added per 100 ml agarose solution. DNA electrophoresis loading buffer (Appendix A) was added to DNA samples (in a ratio of 1:6) prior to loading of DNA. DNA was visualised on a 264 nm transilluminator (UVP).

C.2 Annealing of complementary oligonucleotides to form adaptors

Ten microlitres of Oligo 1 (5 mM; Appendix B) was annealed to 10 µl primer 2 (5 mM; Appendix B) by incubating sequentially at 65°C, 37°C and 22°C for 10 min at each temperature and subsequently chilling on ice. Oligo 3 (50 mM; Appendix B) was similarly annealed to 50 mM oligo 4 (Appendix B). Oligo 1 annealed to oligo 2 generated *Mse*I sticky-ends and oligo 3 annealed to oligo 4 generated *Eco*RI sticky-ends. Annealed adaptors were aliquoted into 5 µl volumes and stored at -20°C.