On the phylogenetic relationship of South African species of *Cladophora* (Chlorophyta) based on the 26S (LSU) gene region

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Submitted in partial fulfilment of the degree of BSc (Hons) in Botany at the University of Cape Town

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1. Abstract

In order to delineate the genus *Cladophora* in South Africa, several sites along the Cape peninsula were sampled and sequences for the partial 26S region of the rRNA were obtained and a phylogram was constructed. The aims of this study were to provide a robust phylogenetic tree describing the relationship between members of the genus *Cladophora* and related taxa and to determine the origins of South Africa's estuarine species of *Cladophora*. After DNA extraction and cycle sequencing of products, sequences were aligned and compared to several east coast species (obtained from GENBANK) and a phylogram was constructed. The results yielded several clades within *Cladophora*; *Clad. capensis* grouping with *Clad. isaacii, Clad sericea* and *Clad. dalmatica; Cheatomorpha* forming a polyphyletic group within *Cladophora*; and *Clad. vagabunda* and *Clad. laetevirens* grouping with the estuarine species. As no *Clad. vagabunda* or *Clad. laetevirens* are found along the coast of South Africa, the origins of its estuarine species still remain a mystery and needs further investigation.

2. Introduction

2.1. Background

In recent years, DNA analysis has become the basis upon which relationships between individuals and among species are based. It is fast replacing the older tradition of looking at morphological similarities to construct a cladogram because it provides a more robust tree - based on changes in DNA structure due to mutations, which eventually result in speciation - describing the relationships between the individuals being studied. This is accomplished by reconstructing ancestral states and determining the level of relatedness between individuals from the amount of changes in DNA structure.

These cladograms based on molecular characters – called phylogenetic trees – have led us to question many of our old groupings of individuals and also the morphological features we use as characters and how characters are defined (Pimentel & Riggins 1987). DNA analysis does not only provide a better criteria to base relationships between species on, it also shows us which morphological characters are based on common ancestry (as obtained from the molecular analysis) and which are as a result of homoplasy due to factors such as similar environmental conditions (for example, see van den Hoek & Chihara 2000).

Leliaert *et al.* (2003) published a paper in which they used both small sub-unit (SSU) and large sub-unit (LSU) rRNA to describe the phylogeny of the Cladophorophyceae. In their study, the objectives were to determine if the order Siphonocladales was a monophyletic group and to determine whether the morphological characters used to delineate the Cladophorophyceae were taxonomically significant. By adding their LSU sequences to the results published by Bakker *et al.* (1994) and Hanyuda *et al.* (2002) they hoped to resolve relationships between clades and species at all levels of the tree, which the SSU sequences alone could not do. The LSU region was found to be more variable and would therefore provide better clarity at higher levels of the tree where resolution was lacking, as shown by various others including Brosnan *et al.* (2003).

Several Cladophorales species from the East Coast of South Africa (Kwazulu-Natal) were included in the Cladophorophyceae study by Leliaert *et al* (2003a & b), the sequences of which will be incorporated into this study. These sequences (obtained from GENBANK) will be added to several West Coast and estuarine species to construct a tree representative of both West and East coast Cladophorales species.

2.2. The genus *Cladophora*

The genus *Cladophora* (type species *C. oligoclona* Kützing, Bakker *et al.* 1994) is characterised by species with simple thallus architecture and branched uniseriate filaments with multinucleate cells (van den Hoek & Chihara 2000). The genus has a worldwide distribution with an estimated 50 – 100 species and 11 recognisable architectural types.

Some other characteristics of the genus, many of which can also be used to identify members of the family Cladophorales, are: 1) cell walls composed of mainly crystalline cellulose; 2) irregular angular chloroplasts forming a parietal network in each cell (in both sporophytes and gametophytes); 3) fixed positioning of chloroplasts in the cell; 4) diplohaplontic and isomorphic life cycles for sexually reproducing species; and 5) cells grow almost exclusively by intercalary cell division (van den Hoek, 1963, van den Hoek & Chihara 2000).

Cladophora is believed to be the ancestral state of many taxa other genera, some of which have a reduced form, for example *Chaetomorpha*, while others have undergone elaborate changes in architectural structures, for example the blade-like *Microdictyon* (van den Hoek & Chihara 2000).

2.3. Aims

The main aim of this study will be to determine the level of relatedness between *Cladophora* species along the coast of South Africa.

Also, the theory that estuarine species originated from *Clad. vagabunda* will be tested. This will be done by determining whether the estuarine species has a marine origin (as a freshwater origin for the species is quite likely) by comparing various South African species to the estuarine species from the Bot River estuary.

Morphological studies suggest that estuarine species of *Cladophora* around the world most likely had a marine origin; possibly sharing a common ancestry with *Clad. vagabunda*, a metropolitan species with similar morphology to the estuarine species, which can grow in brackish water and tolerate slightly lower levels of salinity (van den Hoek 1963). Along the coast of South Africa however, no *Clad. vagabunda* has yet been described but another species, *Clad. dalmatica,* which looks similar to *Clad. vagabunda*, does occur along the South African coast and could possibly share a common ancestry with the estuarine species (J Bolton - pers. comm.).

When *Clad. vagabunda* enters an estuary individuals become smaller, resembling estuarine species. The estuarine species from the Bot River Estuary, however, more closely resemble *Clad. dalmatica*, with the only morphological difference being the tips of the estuarine species that are more curved, though it shares the common feature of second laterals (pers. Observation; compared to *Clad. dalmatica* as described by Stegenga *et al.* 1997).

Another possibility is that the estuarine species could share a common ancestry with *Clad. glomerata*, a freshwater species with a slight tolerance to increased levels of salinity, a species that is sometimes found in estuaries or areas with increased levels of salinity (van den Hoek 1963).

2.4. Hypothesis

It is hypothesised that the estuarine species has a marine origin. As no *Clad. vagabunda* has been described along the coasts of South Africa, the most likely sister taxon to the estuarine species is *Clad. dalmatica*, which has the most morphological similarities to the estuarine species. Alternatively, *Clad. glomerata*, a freshwater species which has been found to tolerate higher levels of salinity and has been seen inhabiting estuaries could be the sister taxon to the estuarine species. If the latter hypothesis were true, it would imply that the South African estuarine species of *Cladophora* has a freshwater rather than a marine origin.

This paper will also provide a phylogenetic tree describing the relationships between the sequenced Cladophorales species that are found along the East and West coasts of South Africa.

3. Materials and methods

3.1. Species identification

Specimens collected during this study are listed in appendix 1. The identification of these specimens are based on morphological characteristics as seen under a compound microscope at 4x – 10x magnification. The specimens were identified using the key from the Seaweeds of the South African West Coast (Stegenga *et al.* 1997).
Molecular analysis is based on the LSU rRNA region.

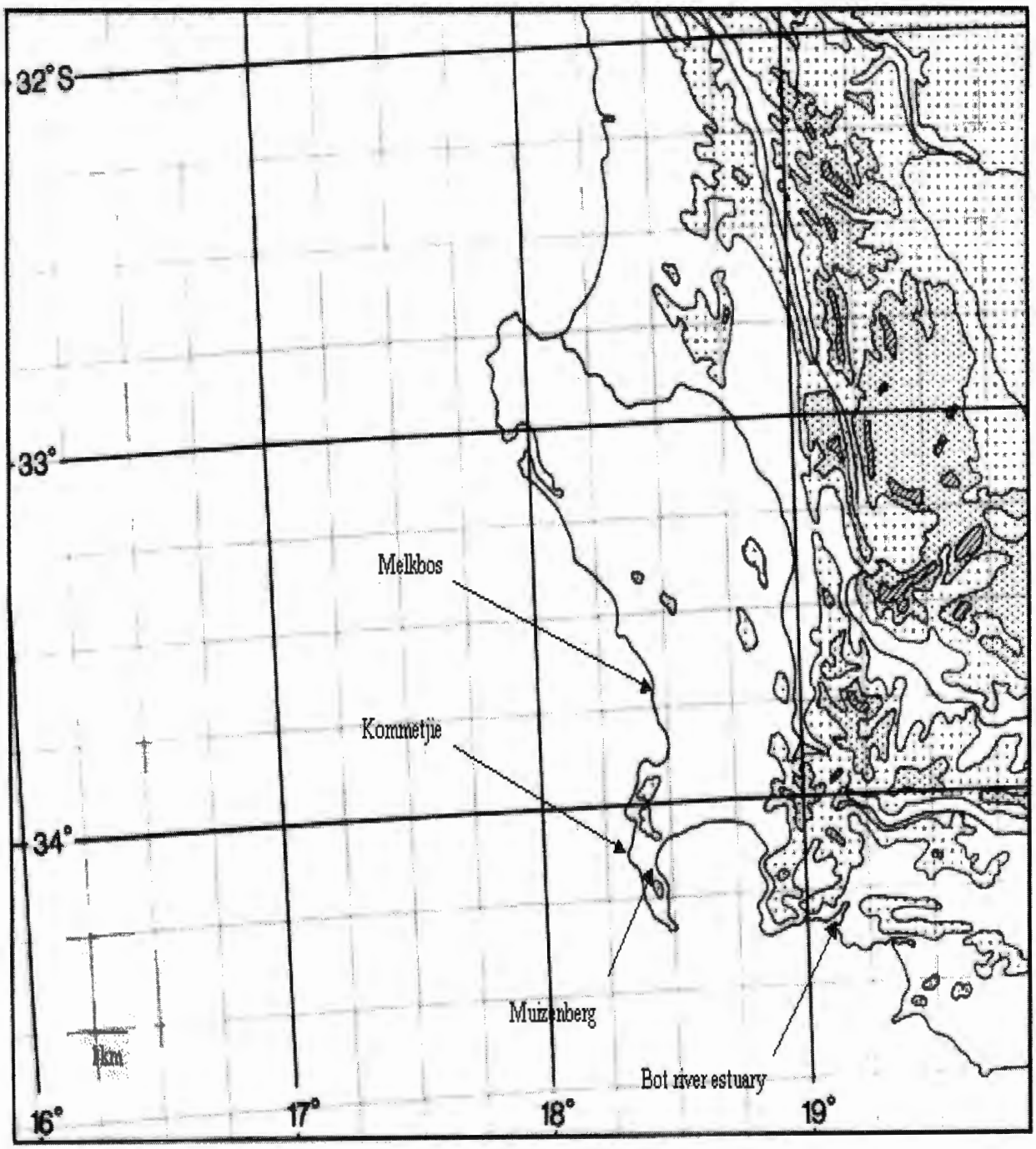

Figure 1: Map of the South-western Cape showing areas where marine and estuarine samples were collected. Sites of samples not collected by the author is not shown

3.2. Study area

Several areas in the Western Cape were sampled to obtain a record of the diversity of the Cladophora species around the Cape Peninsula (Fig. 1). These were added to sequences of Southern African *Cladophora* species obtained by Leliaert *et al.* (2003) and will be used to provide a broad overview of the relationships between the West and East coast Cladophora species of South Africa. Several estuarine specimens were also added to the study to aid in providing a more holistic view of Cladophora species in South Africa.

3.3. Sampling technique

Whole individuals (holdfast and stipe) were targeted (but not always obtained) to help with morphological identification to species level. Marine specimens were sampled at spring low tide in the shallow subtidal zone along rocky shores. The first sets of samples were collected in Kommetjie while further sampling occurred at Muizenberg and Melkbos beaches and at the Bot River estuary where, at the latter site, estuarine species were collected.

The samples were separated and parts of each individual were stored in dry silica gel (blue or yellow) where it was dried for sequencing while the rest of the individual (this included the holdfast, if the alga was removed with the holdfast intact) was placed in jars with 5% formalin seawater or estuarine water - depending on where the individual was collected - for reference and further identification based on morphological characters.

Samples were cleaned before being placed in silica gel by removing algal growth and sand particles, although not all epiphytes could be removed because of their small size and large number.

Fourteen samples coded ca001 – ca014 were collected at Kommetjie, 11 samples collected at Muizenberg were coded ca015 – ca025, the 13 samples collected at Melkbos beach were coded ca026 – ca38, and the four samples collected at the Bot River estuary were coded ca039 – ca042 (see Appendix 1). Not all samples could successfully be extracted and were thus discarded.

East coast species' sequences were obtained from GENBANK and after DNA extraction and PCR amplification of DNA of the collected samples; GENBANK sequences were added to the sequences obtained from the collected samples.

3.4. DNA extraction, amplification and sequencing

DNA was extracted following the CTAB-based method for extracting DNA from fresh leaf material described by Doyle & Doyle (1987), with the following modifications made to the protocol for scaling down of reagents for approximately 20mg of plant material. Samples were ground in a pestle and mortar with 700µl of extraction buffer and a pinch of PVP – 40 (polyvinylpyrrolidone) added to each. After incubation of samples, 600µl of chloroform:isoamyl alcohol (24:1 v/v) was added before being mixed by inversion for 5 min and spinning samples in a centrifuge.

The primers C 1 and D 2 (Leliaert *et al.* (2003) were used to amplify the 5 end of the 26S region of the nuclear encoded rDNA, thus making data generated in this study comparable to that of Leliaert *et al.* (2003).

PCR amplifications were performed using 0.75 units of BIOTAQ™ DNA polymerase (Bioline), 0.1mM of each dNTP, 0.3µM of each primer and 3µl of unquantified DNA template. Thermo-cycling was carried out on a Genamp® PCR system 2700 (Applied Biosystems) with the following thermal profile (modified from Leliaert *el al.* 2003): an initial denaturation step of 94°C for 3 min followed by 35 cycles of 30s at 94 C, 30s at 53 C, and 30s at 72 C, followed by the final extension step of 3 min at 72 C.

Amplified products were cleaned using the GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences), as per manufacturers instructions before being cycle sequenced in a Genamp® PCR system 2700 (Applied Biosystems). Amplification primers were also used for sequencing.

Both strands of the PCR product were cycle sequenced using the ABI PRISM® Big Dye™ Terminator v3.1 cycle sequencing Ready Reaction Kit (Applied Biosystems), as per manufacturers instructions. Cycle sequencing products were resolved using an ABI PRISM® 3001 Genetic Analyser (Applied Biosystems).

Sequences were assembled by eye and checked for inaccurate base calling using SeqMan II (LaserGene System Software, DNAStar, Inc.) to check for inaccurate base calling before being aligned manually using MegAlign (LaserGene System Software, DNAStar, Inc.).

Sequences of the east coast South African Cladophoraceae from the Leliaert *et al.* (2003) study as well as sequence of the freshwater *Clad. glomerata* - obtained from GENBANK – were added to the sequences of the west coast and estuarine species

obtained from this study. The GENBANK sequences were aligned preliminarily using MegAlign (LaserGene System Software, DNAStar, Inc.). Sequence extremities were trimmed for all species analysed to exclude regions for which many taxa had missing data.

Parsimony analysis were conducted using PAUP*4.0b10 (Swofford, 1998) to perform a full heuristic search of 10 000 replicates. Due to its basal position in the strict consensus phylogram obtained by Leliaert *et al.* (2003), *Cladophora horii* was chosen as the outgroup to which the rest of the species were rooted. Parsimony analysis was conducted using a TBR branch-swapping algorithm with two trees being held at each step; of which a consensus tree was created. A Jacknife was performed with 1 000 replicates with 33.67% deletion at each step to obtain nodal support. Gaps were treated as missing characters.

4. Results

4.1. Phylogeny of *Cladophora*

The aligned partial rRNA sequence yielded 485 characters of which 152 characters were parsimony-informative. Parsimony analysis, using a TBR (Tree-Bisection-Reconstruction) branch-swapping algorithm yielded 82 most parsimonious trees, of which the strict consensus tree was saved as a phylogram (Fig. 2). A Jacknife estimate of nodal support was performed and added to a strict consensus tree of *Cladophora* (Fig. 3, only nodal support above 60 shown).

Within the sampled taxa, *Cladophora coelothrix, Clad. sibogae, Cladophoropsis philippensis, Chameadoris delphinii* and *Cham. auriculata* were placed as sister to the rest of the taxa.

Cladophora capensis, along with *Clad. isaacii, Clad. sericea* and *Clad. dalmatica* form a distinct paraphyletic group, but is divided into two clades, one of which shares a common ancestry with *Chaetomorpha aerea*. This grouping is supported by high Jacknife value (93, Fig. 3) and there are few changes between the clades (< 10, Fig. 2).

The estuarine *Cladophora* species form a well-supported monophyletic group, with some divergence between individuals. The estuarine species also form a monophyletic group with *Clad. vagabunda* and *Clad. laetevirens* (Jacknife = 86), suggesting common ancestry between the estuarine species and *Clad. vagabunda*.

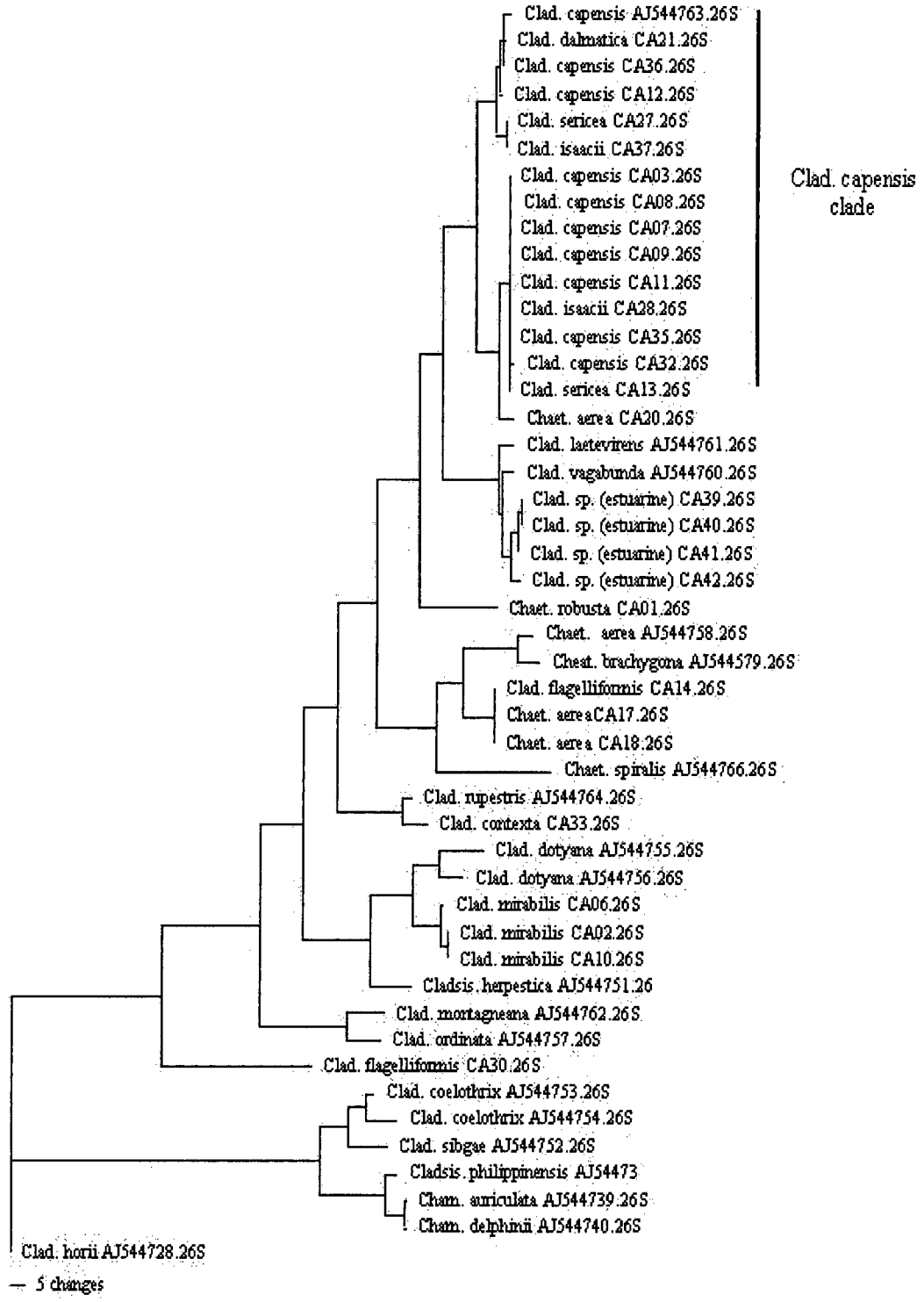

Figure 2: Strict consensus phylogram of the 82 most parsimonious trees as inferred from partial rRNA sequence data.

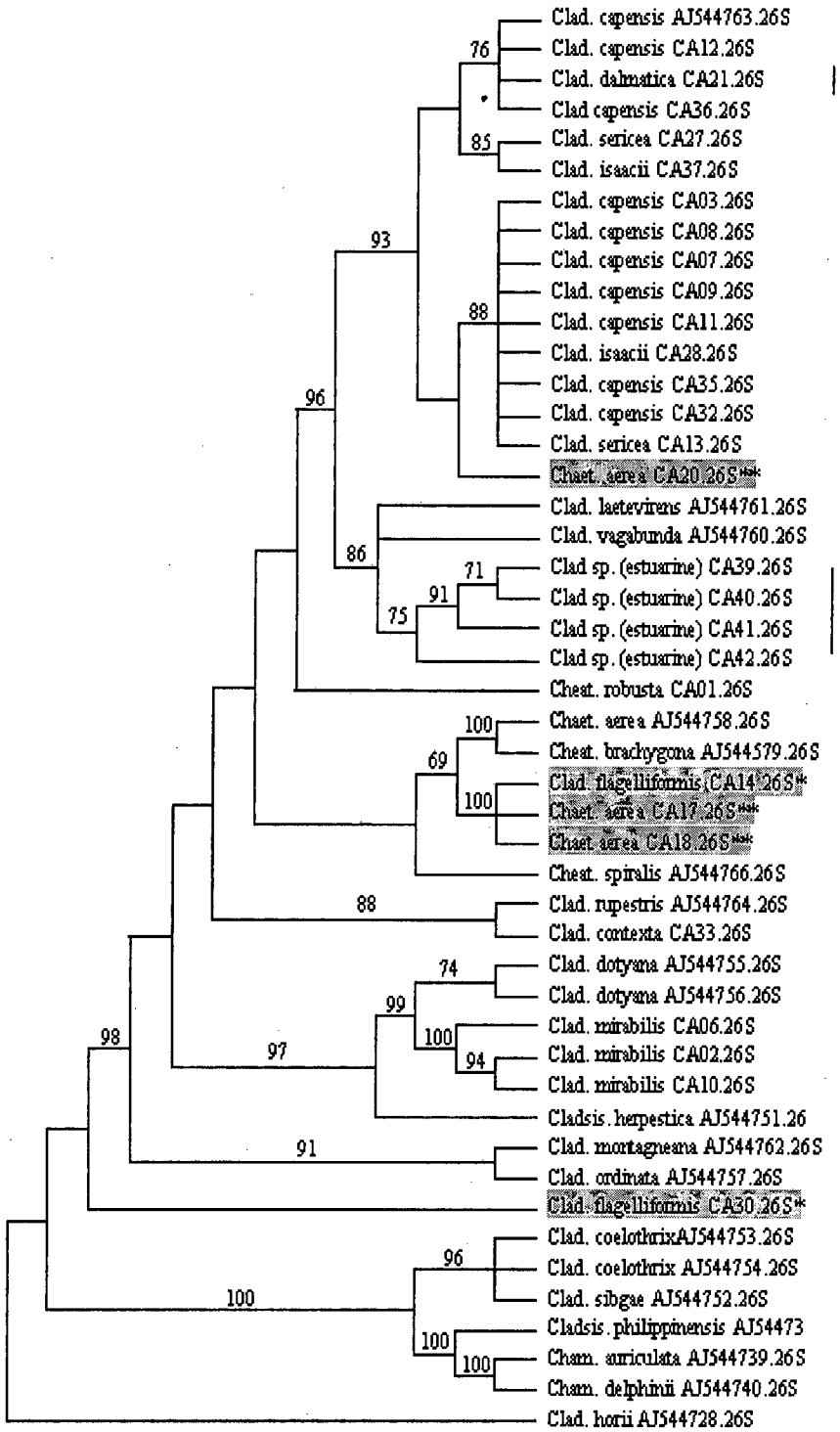

Figure 3: Strict consensus tree showing bootstrap values above branches

Chaetomorpha robusta groups sister to the *Clad. capensis* clade / *Clad.vagabunda* (and estuarine species) complex, with no other closely related taxa.

Chaetomorpha aerea forms a polyphyletic group, grouping with the *Clad. capensis* clade (Fig. 2) as well as with the rest of the *Chaetomorpha* species (excluding *Chaet. robusta*) sampled (Jacknife = 69, Fig. 3).

Clad. flagelliformis forms a polyphyletic group with a representative grouping with *Chaet. aerea* (Jacknife = 100) whereas the other forms a lone group between the basal group and the rest of the *Cladophora* species.

5. Discussion

5.1. Phylogeny of the genus *Cladophora*

5.1.1. The *Cladophora capensis* clade

Cladophora capensis forms a polyphyletic clade that includes *Clad. isaacii, Clad. dalmatica, Clad. sericea* and *Chaetomorpha aerea*. This is a well-supported clade with a high Jacknife value (93). The clade is divided into two subclades, each further divided into two (Fig. 3). The first clade at the lower level groups *Clad. capensis* with *Clad. dalmatica* (Jacknife = 76), the second groups *Clad. sericea* with *Clad. isaacii* (Jacknife = 85), the third groups *Clad. capensis* with *Clad. isaacii* and *Clad. sericea* (Jacknife = 88) and the fourth clade consist of one sample, *Chaet. aerea* (no Jacknife support).

This suggests that *Clad. capensis, Clad. isaacii* and *Clad. sericea* belong to the same clade (as seen from the third subclade) with some divergence between subclades. The grouping of *Clad. capensis* and *Clad. isaacii* is almost intuitive from a morphological point of view, as the only noticeable difference between the species is the shape of the apical cells, which are pointed in *Clad. capensis* and rounded in *Clad. isaacii* (Stegenga *et al. 1997). Clad. sericea* also show many similarities to *Clad. capensis*, with the only major difference being the higher number of branches and rows of *Clad. sericea* (van den Hoek1963). *Clad. dalmatica*, however, has several differences to the rest of the members of this *Clad. capensis* clade and more closely resemble *Clad. vagabunda* and the estuarine species than it does *Clad. capensis*, though some similarities do exist such as similarities between *Clad. dalmatica* and *Clad. albida*

(van den Hoek 1963), another species quite closely related to *Clad. sericea* (Bakker *et al.* 1994, Bakker *et al.* 1995).

5.1.2 Discrepancies in the phylogeny

There were only two discrepancies in the data set that are of note. The first was the inclusion of *Cheat. aerea* in the *Clad. capensis* clade. Even though it was hypothesized that *Cheatomorpha* species were derived from *Cladophora* species and that *Chaetomorpha* may have arisen several times (van den Hoek 1963) it is unlikely that these *Chaetomorpha* could be as different from each other as the phylogram proposed (the nearest common ancestor was about 20-50 changes), thus very little confidence can be placed in the grouping of any of the *Chaetomorpha aerea* and further investigation is needed before any conclusions can be made. The second discrepancy that existed in the data set was the positioning of the two samples of *Clad. flagelliformis*. The one sample is sister to all the *Cladophora* sampled except the basal clade that consisted of *Cladophora coelothrix, Clad. sibgae, Cladophoropsis philippensis, Chameadoris delphinii* and *Cham. auriculata*. The second sample formed a monophyletic group with two of the *Chaet. aerea* with a Jacknife support of 100. Very little confidence can thus be placed in this outcome of the positioning of *Clad. flagelliformis* within the genus *Cladophora* and its related taxa and further investigation is required.

5.2. Origins of the estuarine species

It is generally accepted that estuarine Cladophorales species from around the world had a marine origin (marine species have been known to inhabit areas with lowered salinities, as noted by van den Hoek 1963). Phylogenetic and morphological studies have shown that the most likely taxon to have shared a common ancestry to the estuarine species is *Cladophora vagabunda*; a marine alga that can survive slightly reduced levels of salinity, which is often found in estuaries (van den Hoek 1963).

Clad. vagabunda was found to tolerate a wide range of salinities ranging from as high as 50‰ - 200‰ in Lake Pomorie and its adjacent saltpans to an average of 16‰ – 18‰ in the black sea (Elenov et. al. 1996) to salinities as low as 6.3‰ in Mediterranean lagoons (van den Hoek 1963).

Clad. vagabunda has also been found to inhabit certain estuaries; with a reduction in size of individuals a noted adaptation. In South Africa however, no *Clad. vagabunda* has yet been found along the coast, yet the estuarine *Cladophor*a species is found in several of its estuaries.

A study of the morphological characters of the estuarine species shows that the estuarine species shares features with another marine species, *Clad. dalmatica,* which is found along the coast of South Africa. This analysis of the morphology of the species is based on the key from Stegenga *et al.* (1997) and Van Den Hoek (1963) and shows that the estuarine species is almost identical to *Clad. dalmatica*, with the only difference being the tips of the estuarine species, which are more curved than that of *Clad. dalmatica*. Both species have the following characteristics: 1) branches occur in long second rows; 2) apical cells are 200-250 μm long and 40-60 μm in diameter (pers. observation; also see Stegenga *et al.* 1997).

6. Conclusions

The phylogeny of *Cladophora* species of South Africa still remain unresolved, though many of the characters used to define species morphologically are supported by the phylogeny, though in some cases this was not the case, for example, *Clad. dalmatica* and the estuarine *Cladophora*. Though there are many groupings that seem intuitive, like grouping *Clad. isaacii* with *Clad. capensis*, discrepancies still exist in the results and further sampling needs to be performed . For example, two samples of *Chaetomorpha aerea* form a monophyletic group with *Clad. flagelliformis* showing a Jacknife value of 100, whereas the other sample of *Chaet. aerea*, obtained from the same location, groups with the *Clad. capensis* clade. Further investigation is needed to better resolve the phylogeny of the *Cladophora* and related species of South Africa.

Contrary to what was expected, the estuarine species of *Cladophora* are not closely related to *Clad. dalmatica*, even though morphology suggests otherwise. As no *Clad. vagabunda* is found along the coast of South Africa, the only plausible ancestor to the estuarine species is *Clad. glomerata,* a theory that should be supported or rejected by the addition of 26S data to the present data set.

7. Acknowledgements

I would like to thank my supervisors Prof. John Bolton for his help with collecting and species identification and Tracey Nowell for her help with DNA extraction, sequencing and analysis. I would also like to thank the University of Cape Town, the Harry Crossley foundation and the NRF for financial support.

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9. Appendix 1: List of species and species codes

Code	Date collected	Area sampled	Species
ca 001	08/05/2004	Kommetjie	Chaetomorpha robusta
ca 002	08/05/2004	Kommetjie	Cladophora mirabilis
ca 003	08/05/2004	Kommetjie	Cladophora capensis
ca 006	08/05/2004	Kommetjie	Cladophora mirabilis
ca 007	08/05/2004	Kommetjie	Cladophora capensis
ca 008	08/05/2004	Kommetjie	Cladophora capensis
ca 009	08/05/2004	Kommetjie	Cladophora capensis
ca 010	08/05/2004	Kommetjie	Cladophora mirabilis
ca 011	08/05/2004	Kommetjie	Cladophora capensis
ca 012	08/05/2004	Kommetjie	Cladophora capensis
ca 013	08/05/2004	Kommetjie	Cladophora sericea
ca 014	08/05/2004	Kommetjie	Cladophora flagelliformis
ca 017	20/07/2004	Muizenberg	Chaetomorpha aerea
ca 018	20/07/2004	Muizenberg	Chaetomorpha aerea
ca 020	20/07/2004	Muizenberg	Chaetomorpha aerea
ca 021	20/07/2004	Muizenberg	Cladophora dalmatica
ca 027	21/07/2004	Melkbos	Cladophora sericea
ca 028	21/07/2004	Melkbos	Cladophora isaacii
ca 030	21/07/2004	Melkbos	Cladophora flagelliformis
ca 032	21/07/2004	Melkbos	Cladophora capensis
ca 033	21/07/2004	Melkbos	Cladophora contexta
ca 035	21/07/2004	Melkbos	Cladophora capensis
ca 036	21/07/2004	Melkbos	Cladophora capensis
ca 037	21/07/2004	Melkbos	Cladophora isaacii
ca039	12/08/2004	Bot River estuary	Cladophora sp. (estuarine)
ca040	12/08/2004	Bot River estuary	Cladophora sp. (estuarine)
ca041	12/08/2004	Bot River estuary	Cladophora sp. (estuarine)
ca042	12/08/2004	Bot River estuary	Cladophora sp. (estuarine)