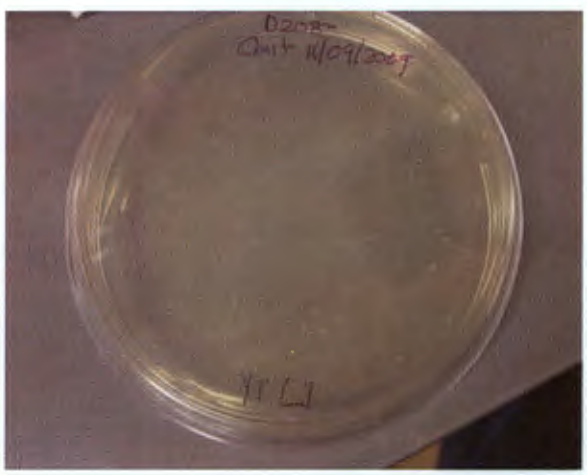
Taxonomy and Phylogeny of the South African *Plocamium* species (Rhodophyta, Plocamiaceae)

Plocamium suhrii

Plocamium beckeri

Plocamium rigidum

Plocamium beckeri



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Abstract

A number of taxonomic accounts have been produced for species of the red alga *Plocamium* in South Africa and the rest of the world; yet, the use of phylogenetic analyses to understand relationships within the genus has only been incorporated in the last decade in Japan and Europe. We used morphological and DNA sequence characters (*ITS1-5.8S-ITS2)* in both independent and combined Bayesian and parsimony analyses to investigate the relationships within South African *Plocamium* species and between South African *Plocamium* species and those from elsewhere. South African *Plocamium* forms a monophyletic group within the genus, and *Plocamium* is paraphyletic with respect to *Plocamiocolax,* the other genus in the family *Plocamiaceae*. The morphological and molecular phylogenies are incongruent; morphologically relationships resolve poorly toward the tips and several of the sister relationships resolved on the basis of the nuclear ITS marker are questionable. The results of this study revealed that phylogenetic relationships within *Plocamium* in South Africa are highly complex and particularly when evaluated in the context of the genus worldwide. The worldwide taxonomy of the genus is in need of full revision as this and several recent studies have highlighted incongruencies in the previous morphological taxonomic accounts of the genus.

Key words: Taxonomy, phylogeny, Rhodophyta, *Plocamium*

INTRODUCTION

The terrestrial flora of South Africa, in particular the Cape Floristic Region (CFR), has been the focus of many phylogenetic and taxonomic studies (e.g. Verboom et al 2009, Goldblatt & Manning 2000, 2002, Muasya and Simpson 2002, Muasya 2001). The marine flora of South Africa, in contrast, has been largely understudied in light of the advancement in molecular techniques (e.g. genomic DNA extraction methods designed for particular groups, polymerase chain reaction [PCR]) and expertise in the field over the last two decades. With close to 900 species (JJ Bolton, pers. comm.) many of which are endemic (Bolton and Anderson 1997), the South African seaweed flora is one of the richer marine floras globally, alongside southern Australia, Japan and the Philippines to name a few (Bolton and Stegenga 2002). Recent taxonomic studies (both morphological and molecular) on the *Gelidiaceae* (Tronchin *et al.* 2006), *Gracilariaceae* (Iyer *et al.* 2005), *Grateloupia* (De Clerck *et al.* 2005a) have made invaluable contributions to classification of a small number of South African red seaweed taxa. The first two studies identified more species growing in South Africa in their respective families than had previously been known, while De Clerck uncovered cryptic species within *Grateloupia filicina*, which was initially thought to be widespread.

Three divisions - Chlorophyta, Ochrophyta and Rhodophyta - are commonly referred to as the green, brown and red seaweeds respectively. These macroalgae are united superficially on the basis of their ecological similarity (Graham and Wilcox 2000). This is as a result of convergent evolution – the independent development of congruent features within taxa sharing a similar environment, habitat and/or function (De Clerck *et al* 2005b).

Phylogenetic studies into these phyla have shown that the "emergence of the red algae is the most ancient event detected so far in the evolution of the eukaryotes…" (Hori and Osawa 1987). Suggestion by authors such as Tappan (1980) and Hori & Osawa (1987) place this emergence between 1.3 and 2 billion years ago while Graham and Wilcox (2000) report the oldest known red algal fossil existed in the pre-Cambrian between 750 million and 1250 million years ago. Ragan *et al.* (1994) believe the Rhodophyta to represent one of the major radiations in the eukaryotes. Historically

the Rhodophyta were split into two subclasses (or classes depending on the taxonomist) Bangiophycidae and Florideophycidae (4 and 14-17 orders respectively) based on several characters such as sexual reproduction, thallus complexity and plastid location, none of which is taxonomically stable or a positive synapomorphy (Ragan *et al.* 1994). The Bangiophycidae are polyphyletic (i.e. the most recent common ancestor is assigned to a different group of taxa than the taxon itself (Quicke 1993)) while the Florideophycideae are strongly monophyletic i.e. all descendents share a common ancestor and together form a group of taxa (Quicke 1993) with nodal support at 100%.

Biochemical studies of several Florideophycideae revealed that a large proportion of the taxa contain compounds with potential medicinal use (e.g. the genus *Laurencia* has over 700 halogenated secondary metabolites [Jung *et al.* 2008]). The genus *Plocamium* Lamouroux contains a similar suite of metabolites, and several species such as *P. telfairae* and *P. corallorhiza* have been studied for the potential application of these chemical compounds in among others anticancer, antimicrobial and antitubercular activities (Knott *et al.* 2005; Kanagasabhapathy *et al.* 2008).

Distribution and form of *Plocamium* Lamouroux

Plocamium is a widespread genus and its global distribution spans tropical to cold-temperate seas (Yano *et al.* 2004). In South Africa specimens occur around the entire coastline, from cool temperate Atlantic Ocean waters on the west coast to warm tropical Indian Ocean waters on the east coast.

This genus is characterized by thalli consisting of a series of simple laterals and compound laterals that subtend a variable number of branchlets. These laterals are arranged in an alternate or zigzag fashion on the branches and subtend very distinctive pectinate (comb-like) branchlets. Typical plant sizes range from 40mm (e.g. in *P. beckeri*) to over 300mm (e.g. in *P. corallorhiza*). Thallus colour ranges from bright red to reddish brown (Stegenga *et al* 1997) and tooth-like structures are present on the margins (abaxially) of laterals in at least four (4) of the species in the genus.

Historical background on the classification of the genus *Plocamium*

J.V.F. Lamouroux first described the red algal genus *Plocamium* in 1813 (Lamouroux, 1813). Since that time various taxonomic treatments and revisions attempted to classify species and of these Simons (1964) and Stegenga *et al.* (1997) have become the standard references for South African species. Simons (1964) studied the genus along the coastline of southern Africa while Stegenga *et al.* (1997) focused primarily from the west coast to Cape Agulhas. Table 1 lists the entities recognized by these two authors as well as those listed in the Catalogue of Benthic Marine Algae of the Indian Ocean (Silva *et al.* 1996) and the Algaebase™ internet species database (Guiry and Guiry 2009).

General classification

Division: Rhodophyta

Class: Florideophyceae

Subclass: Florideophycidae

Order: Plocamiales or Gigartinales

Family: Plocamiaceae Kützing (1843b: 442 - 449)

Genus: Plocamium Lamouroux 1813

One of 14 to 17 orders recognised in the Floridiophyceae, Plocamiales, was previously recognised under the order Gigartinales as species exhibit the morphological features of the order i.e. generally erect & terete or folioceous plants with multiaxial structures (Silva *et al.* 1996 & Stegenga *et al* 1997). The family *Plocamiaceae* was raised to ordinal rank by Saunders & Kraft (1994) on the basis of the small-subunit ribosomal RNA gene as well as anatomical features both negating a close relationship to other members of the order (Silva *et al* 1996). Guiry and Guiry (2009) do not recognise the ordinal status Plocamiales and still rank the *Plocamiaceae* under the order Gigartinales.

The name *Plocamiaceae* was conserved against *Thamnophoraceae* - the earliest name for the family (Silva *et al* 1996). Two genera make up this family: *Plocamium* Lamouroux 1813 and *Plocamiocolax* Setchell 1923. The South African species *Plocamiocolax papenfussianus* is an unpigmented small adelphoparasite (i.e. it is related to the host plant) on *Plocamium corallorhiza* (Martin & Pocock 1953).

The holotype of the genus is *Plocamium vulgare* Lamouroux (Guiry and Guiry 2009), while the currently accepted type is *Plocamium cartilagineum* (Linnaeus) Dixon. *Plocamium vulgare* has been synonymised with *P. cartilagineum* (Guiry and Guiry 2009). The type locality for *P. cartilagineum* is Northern Europe but the species is reportedly cosmopolitan with distributions reaching as far as Western Australia (Silva *et al.* 1996). Silva *et al.* (1996) date the first description of a *Plocamium* as 1753 by Carl Linnaeus who described *Fucus cartilagineus* (= *P. cartilagineum).*

The number of species recognised in the genus worldwide varies – Saunders and Lehmkuhl (2005) state that there are more than 40 species in the genus. Wynne (2002) recognised 35 species while Guiry and Guiry (2009) recognise 39 currently accepted names of species in *Plocamium.*

In South Africa, there are 10 recognised species (from Silva *et al* 1996 and Stegenga *et al* 1997). The species *P. affine* was recorded by Silva *et al.* (1996), but neither Simons (1964) nor Stegenga *et al.* (1997) included it in their treatment of the genus. The latter is expected as the type species of *P. affine* was recorded from Durban (then Port Natal) and Stegenga *et al.* (1997) is a west coast guide. Simons (1964) makes no mention of the species at all in his account of *Plocamium* on the South African coast. *Plocamium telfairiae* was recently recorded in South Africa (De Clerck et al 2002) for the first time and is included in the summary of species for South Africa according to various authors (Table 1).

Alternative taxonomic treatment of the genus

Two significant contributions to the taxonomy of the genus in South Africa have been produced in the last 45 years – Simons (1964) and Stegenga *et al.* (1997). In the taxonomic treatments by Stegenga *et al.* 1997 and Silva *et al.* 1996 each author used vegetative features as well as reproductive structures (if possible) to delineate species in the genus.

Table 1: Recent listings of the genus in South Africa and species assigned by each author (+ indicates species is recognised; – indicates species is not recognised and * indicates species is a putative synonym)

Putative species	Simons (1964)	Silva *et al* (1996)	Stegenga *et al* (1997)	Guiry and Guiry (2009)
P. corallorhiza	+	+	+	+
*P. robertiae**	-	-	-	-
P. cornutum	+	+	+	+
P. beckeri	+	+	+	+
P. maxillosum	+	+	+	+
P. suhrii	+	+	+	+
P. rigidum	+	+	+	+
P. rigidum var tenuior	-	+	-	+
P. glomeratum	+	+	+	+
P. telfairiae	-	+	-	+
P. affine	-	+	-	+

Nine putative species and one putative synonym have been recorded in South Africa for *Plocamium*. Simons (1964) is the primary study on which classification within the genus is based and it was his opinion that *P. robertiae* was the same species as *P. corallorhiza*. Prior to this *P. robertiae,* described by Schmitz (1908), was recognised by workers such as Delf & Mitchell (1921), De Toni (1924) and Eyre & Stephenson (1938) (from Silva *et al.* 1996). Subsequent taxonomic treatments of the genus in southern Africa accepted the synonymy proposed by Simons (1964) (See Table 1). Simons (1964) does mention "more delicate forms" of *P. rigidum* that he suspected were specimens of *P. rigidum* var. *tenuior,* but attempts at finding a character to distinguish it from the type specimen proved unsuccessful.

Morphology based versus molecular based taxonomy

Morphological phylogenies have been produced for a number of macroalgal groups since taxonomic description of these organisms began, which molecular phylogenetic studies are on the increase (e.g. Yano *et al.* 2004, Yano *et al.* 2005, Oliveira *et al.*. 2004) with at least three local examples focused on taxa in the Rhodophyta (Tronchin *et al.* 2006: Iyer *et al* 2005; De Clerck *et al* 2005).

Molecular-based phylogenies were performed on the genus *Plocamium* from Europe (specifically *P. cartilagineum*: Saunders and Lehmkuhl 2005) and Japan (all described species in the region: Yano *et al.* 2004, 2005). The former study uncovered

four cryptic species within European material of the type species of *Plocamium*, *P. cartilagineum*. In the Japanese study a traditional morphological phylogeny was compared to a molecular phylogeny (based on nuclear ITS1, ITS 2 and the plastid Rubisco spacer) and the trees were found to be incongruent with one another. Several of the morphological characters used to identify the species were found to be homoplasious i.e. character states similar or the same as a result of parallel evolution rather than homology. The phylogenetic reconstruction revealed the Japanese *Plocamium* species to be para/polyphyletic rather than monophyletic as morphology had previously suggested.

The status quo of the genus in South Africa

Plocamium is not well studied in South Africa, and current taxonomy is based largely on gross morphological characters with brief inclusions of anatomy (e.g. distinct apical cell, parenchymatous thalli and a number of 'small, isodiametric cells surrounding a medulla of larger cells" in the cortex [Saunders and Lehmkuhl 2005]). The reproductive structures such as stichidia (specialised branchlets developed by tetrasporophytes housing cells that through meiosis develop into zonate tetrasporangia or spermatangia [Stegenga *et al.* 1997]) and their structural diversity (e.g. Simons 1964 & Stegenga *et al.* 1997) have also served as a taxonomically distinct character. The morphological traits used to identify species are not clear-cut (often based on dimensions, which are notoriously variable in seaweeds and which tend to have high levels of phenotypic plasticity) making *in situ* identification difficult at best. Considering the incongruence between morphological and molecular phylogenies found in the Japanese members of the genus, especially the recognition of homoplasy in morphological traits, is morphology a reliable tool to distinguish species? Of the ten recognised species of *Plocamium* in South Africa, one (*P. maxillosum*), is endemic to the country (Stegenga *et* al 1997), while several others are endemic to the southern African region – i.e. Angola, Namibia and South Africa. To date there is no phylogeny for this genus and this study is the first to produce both morphological and molecular phylogenies for South Africa. The major disagreement was in whether *P. corallorhiza* is in fact one or two species. On the basis of this long-standing taxonomic confusion, this study however used the information in Table 1 as a departure point and treated it as a hypothesis that was tested.

This study aimed to address the gap in the taxonomic and phylogenetic knowledge of *Plocamium* and contribute to the growing work on algal taxonomy and systematics in South Africa, by:

- Testing the monophyly of the genus *Plocamium,*
- Identifying diagnostic characters that can be used to validly define taxa,
- Investigating phylogenetic relationships within the South African members of the genus
- Comparing the phylogeny derived from vegetative morphological characters with that based on molecular character data.

MATERIALS AND METHODS

Molecular and morphological investigations

Taxa sampling

Molecular data

Eight taxa (*P. robertiae, P. suhrii, P. rigidum, P. cornutum, P. maxillosum, P. corallorhiza, P. beckeri* and *P. glomeratum*) were sampled for molecular analyses (Table 2). Molecular sequence data on species from outside South Africa (*P. cartilagineum, P. telfairiae, P. patagiatum, P. recurvatum, P. oregonum, P. violaceum*) was retrieved from Genbank and included in the analysis. For rooting purposed molecular data on two specimens from the other genus in the family, *Plocamiocolax,* were included: *Plocamiocolax pulvinata* and an unknown species of *Plocamiocolax* from Palmer Peninsula, Antarctica. My plan was to have at least duplicate samples for molecular analysis for each taxon extracted in this study from South African material. Unfortunately this was not possible due to sampling constraints.

Morphology

8 taxa were sampled from collections of Prof JJ Bolton and Assoc. Prof RJ Anderson, and the University of Cape Town's Bolus Herbarium (Table 2). Three to four samples were included (unless multiple specimens were not available as with *P. vulgare*) for each taxon to increase the likelihood of covering the range in variation within a species and also to sample specimens from different habitat conditions such as cold, Atlantic water specimens and warm termperate and tropical waters along the coast of South Africa.

Table 2: Taxa sampled for molecular and morphological investigations
(RSA = South Africa, CMF = taxa extracted and sequenced in this study; * indicates sequencing successful)

Species Name	Locality	Genbank Accession No.
Plocamium beckeri Schmitz ex Simons	Trailer Bay, Northern Cape, RSA	CMF*
	Romansbaai, Western Cape, RSA	CMF
Plocamium cartilagineum (Linnaeus) Dixon	Santa Cruz, California	U30355
Plocamium corallorhiza (Turner) J. Hooker & Harvey	Romansbaai, Western Cape, RSA	CMF
	Kenton On Sea, Western Cape, RSA	CMF

	Port Alfred, Eastern Cape, RSA	CMF
	Kommetjie, Western Cape, RSA	CMF
	De Hoop, Western Cape, RSA	CMF
	False Bay, Western Cape, RSA	CMF
Plocamium cornutum (Turner) Harvey	False Bay, Western Cape, RSA	CMF*
	Trailer Bay, Northern Cape, RSA	CMF
	Jacobsbaai, Western Cape, RSA	CMF
Plocamium glomeratum J. Agardh	De Hoop, Western Cape, RSA	CMF
Plocamium maxillosum (Poiret) Lamouroux	Tietiesbaai, Western Cape, RSA	CMF
	Knysna, Western Cape, RSA	CMF
Plocamium oregonum Doty	Oregon, USA	U30354
Plocamium patagiatum J. Agardh	Encounter Bay, South Australia	U30349
Plocamium recurvatum Okamura	Japan	AB205554
Plocamium rigidum Bory de Saint-Vincent	Kommetjie, Western Cape, RSA	CMF*
	Tsitsikamma, Eastern Cape, RSA	CMF*
	Tietiesbaai, Western Cape, RSA	CMF
Plocamium robertiae Schmitz ex Mazza*	Drift: Port Alfred, Eastern Cape, RSA	CMF*
Plocamium suhrii Kützing	Tsitsikamma, Eastern Cape, RSA	CMF*
Plocamium telfairiae (Hooker & Harvey) Hooker	Ibaraki, Ooarai, Japan	AB205555
Plocamium violaceum Farlow	Santa Cruz, California	U30350
Plocamium vulgare	Terrace Bay, Namibia	-
Plocamiocolax pulvinata Setchell	Japan	U30356
Plocamiocolax sp.	Palmer Peninsula, Antarctica	U30352

Molecular procedure

DNA extraction, PCR and Sequencing

Genomic DNA was isolated from 35 specimens (amplification with success indicated with an * in Table 2) from the collections of Prof JJ Bolton and Assoc. Prof. RJ Anderson (Department of Botany, UCT & Marine and Coastal Management

respectively) using the Red Seaweed (Rhodophyta) Combined DNA isolation protocol developed by Wattier *et al.* (2000). The following exceptions were made to the 2-day isolation method:

- In the working extraction buffer 25% SDS was used instead of the 20% SDS in the protocol.
- Total thalli tissue weight for all specimens was less than the 0.1g dry weight recommended and subsequently final DNA pellet was resuspended in 30µl of TE buffer (pH 8.0).

The final DNA suspension was quantified using the Nanodrop^{TM} ND-1000 spectrophotometer to determine the concentration of DNA present in the respective specimens.

The nuclear Internal Transcribed Spacer (ITS1- 5.8SrDNA-ITS2) and a plastid Rubisco spacer were sequenced through Polymerase Chain Reaction (PCR) (Saiki *et al.* 1988) in both forward and reverse direction. Our choice of molecular markers was guided by previous work on the genus *Plocamium*. The plastid spacer has proven successful in sequencing and revealing cryptic species especially within *Plocamium* species using Japanese and European material (Kamiya 2004). The nuclear ITS1-5.8S-ITS2 has been sequenced successfully in red algae, and it is a preferred region for inferring relationships between species (Iyer *et al* 2005). For the nuclear region, amplification was done in three parts: 18F & 5.8R, 18F & 28R and 5.8F & 28R whereas the chloroplast spacer was sequenced in just a single reaction using primers as listed in Table 3. Upon the direction of M. Kamiya (co-author of numerous Japanese *Plocamium* studies e.g. Yano *et al.* 2004 &2005), the PCRs were performed in 30µl volumes using KapaTaq DNA Polymerase kits (Kapa Biosystems). The reactions consisted of 18.6µl sterile distilled water, 3µl of 10x DNA polymerase buffer, 3µl of $MgCl_2$ (50mM), 1µl each of the forward and reverse primers (10µM), 1.2µl of dNTP (10mM), 0.2µl of *Taq* DNA polymerase and 2µl of template DNA with adjustment of water and DNA template as it suited the sample. All reactions were carried out on an applied Biosystems GeneAmp 2700 thermal cycler (Applied Biosystems). The PCR profile had an initial denaturation phase of 2 minutes at 94°C, followed by 35 cycles of 60 seconds at 94°C, 60 seconds at 48°C and 2 minutes at 72°C. For the Rubisco spacer the PCR profile was as follows: an initial denaturation

phase of 60 seconds at 94°C, followed by 35 cycles of 60 seconds at 94°C, 30 seconds at 48°C and 2 minutes at 72°C. The final extension phase (for both regions) of 7 minutes was done at 72°C and held at 94°C for infinity. The products of the PCR were run on a 1% agarose gel mixed with Goldview™ Nucleic Acid stainto evaluate the success of the PCR amplification and the size of PCR products determined by comparison to the 100 base pair DNA ladder (Biolabs).

Table 3: Synthetic oligonucleotide primers used to amplify and sequence DNA in this study

Complete Sequence Region: Nuclear **ITS1-5.8S-ITS2**		
Primer name	Primer sequence (5'– 3')	Reference
18F	gag gaa gga gaa gtc gta aca	Yano *et al* 2005
28R	ggg atc cat atg ctt aag ttc agc ggg t	Yano *et al* 2005
5.8F	aac tcg taa cgg tgg atg tct	Yano *et al* 2005
5.8R	aga cat cca ccg tta cga gtt	Yano *et al* 2005
Complete Sequence Region: Plastid **Rubisco spacer**		
Primer name	Primer sequence (5'– 3')	Reference
rbcL	tat act tct aca gac aca gct ga	Yano *et al* 2005
rbcS	ayr tca aaw aaw ggw arw ccc ca	Yano *et al* 2005

The first PCRs yielded no products and as such genomic DNA purification protocol using GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences) was performed on all specimens in an attempt to remove excess salts that may hinder the amplification process. In addition to purification, the genomic DNA was diluted to 5ng/µl and these resulted in amplification in both regions but at annealing temperatures different from those found in published literature (Rubisco spacer: 50°C as opposed to 48°C, ITS: 48°C as opposed to 52°C). Successfully amplified PCR products were sent to the DNA sequencing facility at Stellenbosch University, South Africa for purification, cycle sequencing and sequencing. The sequencing of some samples was problematic so a decision was taken to take them through the cloning procedure.

Cloning procedure

This is a technique for obtaining DNA sequence information and it involves cloning the desired insert (in this case the *ITS* and *Rubisco* spacer) into competent bacterial cells such as *Escerichia coli* cells. This procedure is guaranteed to have a high success rate (99.9%) and was performed on the samples with which we had had success in amplification using the protocol as outlined in the *E. cloni* & CloneJet™ cloning kit (Fermentas). The *E. cloni* cells containing the insert were grown overnight and these colonies were re-suspended in pH 8.0 TE buffer incubated at 95°C for 10 minutes before performing the colony PCR.

Cloning primers (which guarantee product specificity) included in the CloneJet™ cloning kit were used as opposed to gene specific primers, which had previously delivered ambiguities in PCR amplification. The profile as outlined in the CloneJet™ protocol was followed with one alteration; colonies grown overnight were not used in the PCR 'cocktail' directly but rather resuspended in a pH 8.0 TE buffer as mentioned earlier.

The colony PCR products where run on a standard 1% agarose gel as explained earlier and successfully amplified samples were cleaned using the protocol as outlined in the Biospin Plasmid DNA Extraction kit (BioFlux, Bioer). All samples were diluted to 10ng/µl and then sent for sequencing.

Morphological data

A total of twelve (12) morphological characters were established and character states were derived from these characters (Table 4). This study relied on dry specimens and as such only vegetative characters were considered. All specimens were first examined for possible character choice using a Leica L-1000 Dissecting Microscope, and a Vernier Calliper was used to measure characters such as thallus height and thallus axis width.

In addition descriptions of vegetative morphology were made and diagrams of each taxon studied prepared were drawn to illlustrate some of the characters used in this study (See Figure 1 for diagrams).

Table 4: Morphological characters and character states used in cladistic analysis of *Plocamium spp.*

Character	Character states and coding
1. Teeth on margin of lateral	Absent (0); Present (1)
2. Tooth pronouncement	None (0); Less pronounced (1); Well-developed (2)
3. Tooth spacing and shape	None (0); Spaced (1) Compact (2)
4. Tooth position	Throughout thallus (0); restricted to apical laterals (1)
5. Habit	Spread-out (0); Bushy (1)
6. Definition of Axis	Ill-defined (0); well-defined (1); very well-defined (2)
7. Main axis width (μm)	≤100 (0); 101-500 (1); 501-900 (2); 901-1300 (3); ≥ 1301
8. Curvature of simple lateral	Slightly incurved (0); incurved (1); Linear & pointed (2); Linear & triangular (3)
9. Curvature of compound lateral	Slightly incurved (0); incurved (1);
10. Overlap of simple and compound lateral with each other	Do not overlap (0); Overlap (1)
11. Plant height (mm)	≤50 (0); 51-100 (1); 101-150 (2); 151-200 (3); ≥201 (4)
12. Number of branchlets on compound lateral	2 (0); 2-3 (1); 2-4 (2); 3 (3); 3-4 (4); 4 (5); 4-5 (6)

Data analysis

DNA sequence editing, assembling and alignment

The generated nucleotide sequences were edited and assembled in the Staden Package (Staden *et al.*. 2003) and aligned in BioEdit (Hall 1999). The ITS1-5.8S-ITS2 region was sequenced but there were missing data for some taxa due to difficulty in sequencing the 5.8F spacer. Only two of the seven (7) species sequenced successfully for the Rubisco spacer and the region was excluded from analyses for this reason.

ITS1 is a non-coding region, which tends to have a high rate of substitution allowing comparisons between relatively recently diverged taxa (Provan *et al.* 2004).

Morphological characters and character polarity

Character states were scored and in performing the cladistic analysis, all characters with multiple states were treated as unordered because the direction of state changes are unknown and therefore each state was equally likely to be plesiomorphic and apomorphic (Fitch 1971, Quicke 1993).

Molecular approaches used to analyze DNA sequence data and morphological character data

The combined morphological and molecular matrix consisted of 922 characters: 910 molecular characters gained from the *ITS1-5.8S-ITS2* nuclear marker after alignment as well as twelve (12) morphological characters per specimen. Before alignment the DNA sequences were 721 base pairs long; gaps were coded as missing data in the DNA matrix as well as where no specimens were available for morphological character scoring. Base pair insertions and deletions were not coded separately from the rest of the sequence. Morphological and molecular datasets were analysed independently and in combination on the taxa in this study. Combining independent datasets into a single phylogenetic analysis does not receive equal support by phylogeneticists. According to Wiens (1998) & Farias *et al.* (2000), this can be done when the data partitions are less heterogeneous than expected, as is the case for the nuclear and morphological datasets in this study. The phylogenetic inferences used to analyze relationships between these taxa were done using the parsimony algorithm and Bayesian which were performed in PAUP* version 4.0b10 (Swofford 2002) and in MrBayes Version 3.12 (Huelsenbeck & Ronquist 2003), respectively. Under the Fitch criterion of unordered, equal weights (Fitch 1971) heuristic tree searches were performed with 1000 random replicates and tree bisection-reconnection (TBR) swapping holding only ten (10) trees at each replicate to reduce time spent searching on below-optimum trees. All trees generated were swapped to completion. Nodal support was evaluated through bootstrap analysis of 1000 replicates (Felsenstein, 1985) with characters sampled using equal weighting (Fitch 1971). Trees were constructed on the basis of simple taxon addition and TBR branch swapping; groups with frequencies greater than 50% in the final bootstrap consensus tree were retained. Following Muasya *et al.* (2001) the following

descriptions for categories of bootstrap support were used: weak, 50-74%; moderate, 75-84%; strong, 85-100%.

In Bayesian inference of phylogeny posterior probabilities (PP) were used as measures of support. The ITS data matrix was analysed under the GTR+I+Γ model of molecular evolution and default MrBayes priors. This model was chosen because the impact of overparameterization on the accuracy of the model search in the tree space is lower than that of underparameterization (Huelsenbeck & Rannala 2004). A MarkovChain MonteCarlo (MCMC) algorithm was employed in the analyses. Two simultaneous runs were done starting from random trees with four chains (three heated and one cold chain) set and the temperature was set at the default 0.2. The analysis ran for 1 million generations and the sampling frequency was set at 100 i.e. Markov chains were sampled every 100^{th} generation. Analyses were run until the average standard deviation of the split frequencies approached 0.10, which is indicative of convergence onto a stationary distribution. Calculation of posterior probability (PP) was done after discarding 10 000 trees sampled during this burn-in period.

RESULTS

Morphological descriptions and taxonomic key

Genus *Plocamium* JV Lamouroux 1813:

The genus is generally characterized by erect thalli that develop sympodially and is primarily complanate in appearance. Branching axes are all robust, often terete and generally well defined from the main axis. Branching structures are termed laterals; simple and compound laterals branch alternately throughout the thalli. The simple lateral forms the lowest branch of a couplet of laterals together with the compound lateral. The compound lateral bears 2 or more pectinate branchlets the number of which broadly characterises the species.

Terminology used in descriptions:

Complanate: Flattened in one plane

Pectinate: Comb-like in appearance

Terete: Circular in cross section

Thalli: the entire 'body' of the alga; plural of thallus

Species description based on morphological features

Plocamium corallorhiza (Turner) J.D. Hooker & Harvey 1845: 542

BASIONYM:

Fucus corallorhiza Turner 1808-1809: 70-71, pl. 96

SYNONYM(S):

Plocamium robertiae F. Schmitz ex Mazza* 1908

Fucus corallorhiza Turner 1808

Thamnophora corallorhiza (Turner) C. Agardh 1822

*According to Simons (1964)

HABITAT: Marine epilithic species in the sublittoral

DESCRIPTION:

Plants are large in size with the length generally around 16cm but can extend to 30cm; a complanate thallus and broad branching axes largely undefined from the main axis the latter is generally 7-10mm in width. Numerous alternating laterals each comprising a group or couplet which in turn is composed of one simple and one compound lateral. Compound laterals are slightly incurved and support two (2)

pectinate branchlets that are broad, while simple laterals are slightly incurved. Both laterals have pronounced compact teeth-like structures on the margins throughout the thallus.

DISTRIBUTION:

Southern Africa: Recorded from northern Namibia (Stegenda *et al.* 1997). In South Africa: Yzerfontein in the Western Cape to Salt Rock Beach in Kwa-Zulu Natal (Stegengna *et al.* 1997, Simons 1964), Madagascar, Amsterdam & St Paul Islands and Mozambique (Silva *et al.*1996).

P. corallorhiza is distinguished by its large size, flattened and broad thallus with wide axes, compactly arranged teeth on the margin of laterals throughout the thallus visible to the naked eye and the presences of two branchlets per compound lateral (see Figure 1).

Plocamium robertiae* F. Schmitz ex Mazza 1908

BASIONYM: *Plocamium robertiae* Schmitz ex Mazza 1908

SYNONYM (s): none

HABITAT: Marine species that is presumed to be subtidal

DESCRIPTION:

Plants generally around 20cm long and possess a complanate thallus with narrow axes between 9mm and 13mm in width. Numerous alternating laterals each comprising a group or couplet which in turn is composed of one simple and one compound lateral. Compound laterals are slightly incurved and support two (2) branchlets with the one branchlet much larger in size than the other; simple laterals are linear and triangularly shaped. Both laterals present spaced teeth-like structures that are very reduced and generally only present on the apical or terminal couplets (see Figure 1).

DISTRIBUTION:

Eastern Cape – Port Alfred

P. robertiae was considered a narrower form of *P. corallorhiza* by Simons (1964) but several morphological features such as the narrow main axes; linear, triangulate simple laterals, reduced and spaced tooth-like structures (found in terminal lateral

couplets only - see Figure 1) and an observed difference in size between the branchlets distinguish it from the latter species.

Plocamium maxillosum (Poiret) JV Lamouroux 1813: pp138

BASIONYM: *Fucus maxillosus* Poiret 1808

SYNONYM(S):

Fucus maxillosus Poiret 1808

Plocamium membranaceum Suhr 1840

HABITAT: Marine species assumed to be present in the sublittoral zone

DESCRIPTION:

Generally small plants, around 9cm long, but can reach up to 20cm; thallus complanate and axes exceeding 1.3 mm in width. Numerous alternating laterals each comprising a group or couplet which in turn is composed of one simple and one compound lateral. Compound laterals are slightly incurved and support 3-4 pectinate branchlets; simple laterals are incurved. Both laterals have structures on the margin that are bumps rather than the tooth-like structure found in *P. corallorhiza* and *P. robertiae*.

Distribution: From Hondeklipbaai to Cape Agulhas in the Western Cape, South Africa. The inclusion of Mozambique (Silva *et al.* 1997) in the distribution of this species seems unlikely.

P. maxillosum is a distinctive plant, appearing almost membranous and fan-shaped as the axes are flattened and spread out.

Plocamium suhrii Kützing 1849: 886

BASIONYM: *Plocamium suhrii* Kützing 1849: 886

SYNONYM(S):

Plocamium nobile J. Agardh 1851

Plocamium fullerae F. Schmitz ex Mazza 1908

Habitat: Marine species present in the sublittoral zone and in lower intertidal pools

DESCRIPTION:

Plants are generally small ca. 4cm long but can grow to 15cm; the single Western Cape specimen was 6cm larger than the 'typical' East and South coast specimens that

had an average length of 4cm. Plants display a 'spread and flattened' appearance similar to *P. corallorhiza* with numerous alternating laterals each comprising a group or couplet which in turn is composed of one simple and one compound lateral. Main axis width generally broader than 1.3mm with some reaching up to 2mm. Compound laterals support 2-3 pectinate branchlets and are slightly incurved; simple laterals are slightly incurved as well. Both types of laterals have structures on the margin that are bumps rather than tooth-like and the laterals overlap at the tips.

DISTRIBUTION: Southern Africa; occurring along the Southwest, South and East coasts of South Africa, northern Namibia and Angola as well as Madagascar, Amsterdam and St Paul Islands.

P. suhrii appears similar to *P. corallorhiza* in having broad branchlets and a complanate thallus, but it is much smaller. The distribution of *P. suhrii* in the Western Cape is questionable – Simons (1964) excluded taxa located west of Knysna but specimens have been collected in Muizenberg and Kalk Bay (including the type specimen) (Stegenga *et al* 1997). However, the west coast material itself is also questionable, as it does not differ greatly from *P. maxillosum* according to Stegenga *et al.* (1997).

Plocamium glomeratum J. Agardh 1851: 397

BASIONYM: *Plocamium glomeratum* J. Agardh 1851: 397

SYNONYM(S):

Plocamium subfastigiatum Kützing 1866

Plocamium membranaceum f. subfastigiatum (Kützing) De Toni 1900

HABITAT: Marine species present in both sand-affected and rocky habitats in the sublittoral fringe or in tidal pools.

DESCRIPTION:

Plants are generally small, around 5cm long, but can reach 10cm. Plant habit is spread and complanate with numerous alternating laterals; it is often difficult to distinguish the compound lateral from the simple lateral in the lateral couplet. Main axis width ranges between 0.1mm and 0.5mm, but can reach 1mm. Compound laterals support 4-5 pectinate branchlets and are incurved; simple laterals are generally slightly incurved but at times linear. Both laterals have smooth margins i.e. no tooth-like structures.

DISTRIBUTION: A southern African endemic with species recorded form Namibia to Still Bay, Western Cape, in South Africa (Stegenga *et al.* 1997) as well as from Madagascar and Mozambique.

P. glomeratum has a distinctive lateral couplet structure and the compound lateral is difficult to distinguish. In addition to this the compound lateral is characterised by the presence of at least 4 branchlets if not more. Simons (1964) and Stegenga *et al* (1997) disagree on what the compound lateral is in this species. Simons (1964) considers the subtending 'branchlets' to be multiple laterals or pinnae as he refers to them, but Stegenga *et al* (1997) treats them as branchlets on the compound lateral. The distribution of the species cited by each author overlaps.

Plocamium cornutum (Turner) Harvey 1849: (1847-1849): 123

BASIONYM: *Fucus cornutus* Turner 1819

SYNONYM(S):

Fucus cornutus Turner 1819

Thamnophora cornuta (Turner) Greville 1830: xlix

Thamnocarpus cornutus (Turner) Kützing 1843b: 450

HABITAT: Epilithic marine species in the lower to mid intertidal zone, generally on rather wave-exposed shores.

DESCRIPTION:

Plants are small generally around 6cm in length, but can grow to 20cm. They are bushy in habit with numerous alternating laterals each comprising a group or couplet which in turn is composed of one simple and one compound lateral. Main axes width ranges between 0.5mm and 0.9mm with larger specimens extending up to 1.5mm; branches terete. Compound laterals support 2-3 pectinate branchlets and are slightly incurved; simple laterals are linear in mature plants. Both laterals have smooth margins, i.e. bear no tooth-like structures, and generally overlap at the tip.

DISTRIBUTION: South Africa: abundant along the west coast but rarer along the south Coast (Eastern Cape). World: Indonesia, Madagascar, Mauritius and Reunion.

P. cornutum displays lateral crossing at the tips and simple laterals in juvenile plants are strongly recurving (See Figure 2).

Plocamium beckeri F. Schmitz ex Simons 1964a: 185, 187

BASIONYM: *Plocamium beckeri* F. Schmitz ex Simons 1964a: 185, 187

SYNONYM(S): None.

MISAPPLIED NAMES:

Plocamium glomeratum (Kylin 1938: 12 pl. 3)

Plocamium coccineum (Suhr 1834: 726)

HABITAT: Marine species collected from mid to low intertidal pools.

DESCRIPTION:

Plants are generally small (around 5cm in length), but can grow to 15cm. They are bushy in habit with numerous alternating laterals each comprising a group or couplet which in turn is composed of one simple and one compound lateral. Main axes width between 0.1mm to 0.5mm. Compound laterals support 3 branchlets and are slightly incurved; simple laterals are linear. Both laterals have smooth margins i.e. bear no teeth-like structures.

DISTRIBUTION: Angola, Mozambique and South Africa: Romaansbaai, Western Cape to Umfolosi, Kwa-Zulu Natal, also recorded from Angola and Mozambique.

Silva *et al.*. (1996) says that Simons (1964) excludes specimens west of Arniston in the distribution of *P. beckeri*. Simons (1964) believed there to be some confusion amongst collectors at the time between the northern Hemisphere *P. coccineum* (a synonym of *P. cartilagineum*) and *P. beckeri*. According to Simons (1964) *P. beckeri* differs from *P. coccineum* in that laterals almost always alternate in threes in the former.

Plocamium rigidum Bory de Saint-Vincent 1834: 164

BASIONYM: *Plocamium rigidum* Bory de Saint-Vincent 1834: 164

SYNONYM(S):

Plocamium condensatum Kützing 1866

Plocamium latiusculum Kützing 1866

Nereidea rigida (Bory de Saint-Vincent) Kuntze 1891

HABITAT: Marine species common to the intertidal

DESCRIPTION:

Plants are delicate; most are small with an average height of 8cm but they can reach to 20cm. Richly branched in habit giving a clumped appearance with numerous

alternating laterals each comprising a group or couplet which in turn is composed of one simple and one compound lateral. Main axes width between 0.1mm and 0.5mm. Compound laterals support 3 pectinate branchlets and are slightly incurved; simple laterals are linear and pointed at the tip. Both laterals present with smooth margins i.e. no teeth-like structures are visible.

DISTRIBUTION:

South Africa: Muizenberg, Western Cape to Palm Beach, Kwa-Zulu Natal. Silva *et al.* (1996) includes Amsterdam Island in the distribution of the species.

P. rigidum is somewhat similar in appearance to *P. beckeri* but the former is larger and branching axes are more delicate than those of *P. beckeri*. In addition the simple laterals are pointed in *P. rigidum* as opposed to rounded at the tip as in *P. beckeri.*

Taxonomic Key to the South African *Plocamium* material studied on the basis of vegetative characters

1. Teeth on margin of lateral
    a. Present
        i. Well-developed and visible to the naked eye
            1. Compact.....***P. corallorhiza***
            2. Spaced.....***P. robertiae***
        ii. Less pronounced and appear bump-like
            1. Habit: Branches bushy.....***P. maxillosum***
            2. Habit: Branches spread.....***P. suhrii***
    b. Absent
        i. Habit: Branches spread-out.....***P. glomeratum***
        ii. Habit: Clumped with numerous indistinguishable branches
            1. Apical laterals cross.....***P. cornutum***
            2. Apical laterals do not cross
                a. Main axes: robust.....***P. beckeri***
                b. Main axes: delicate.....***P. rigidum***

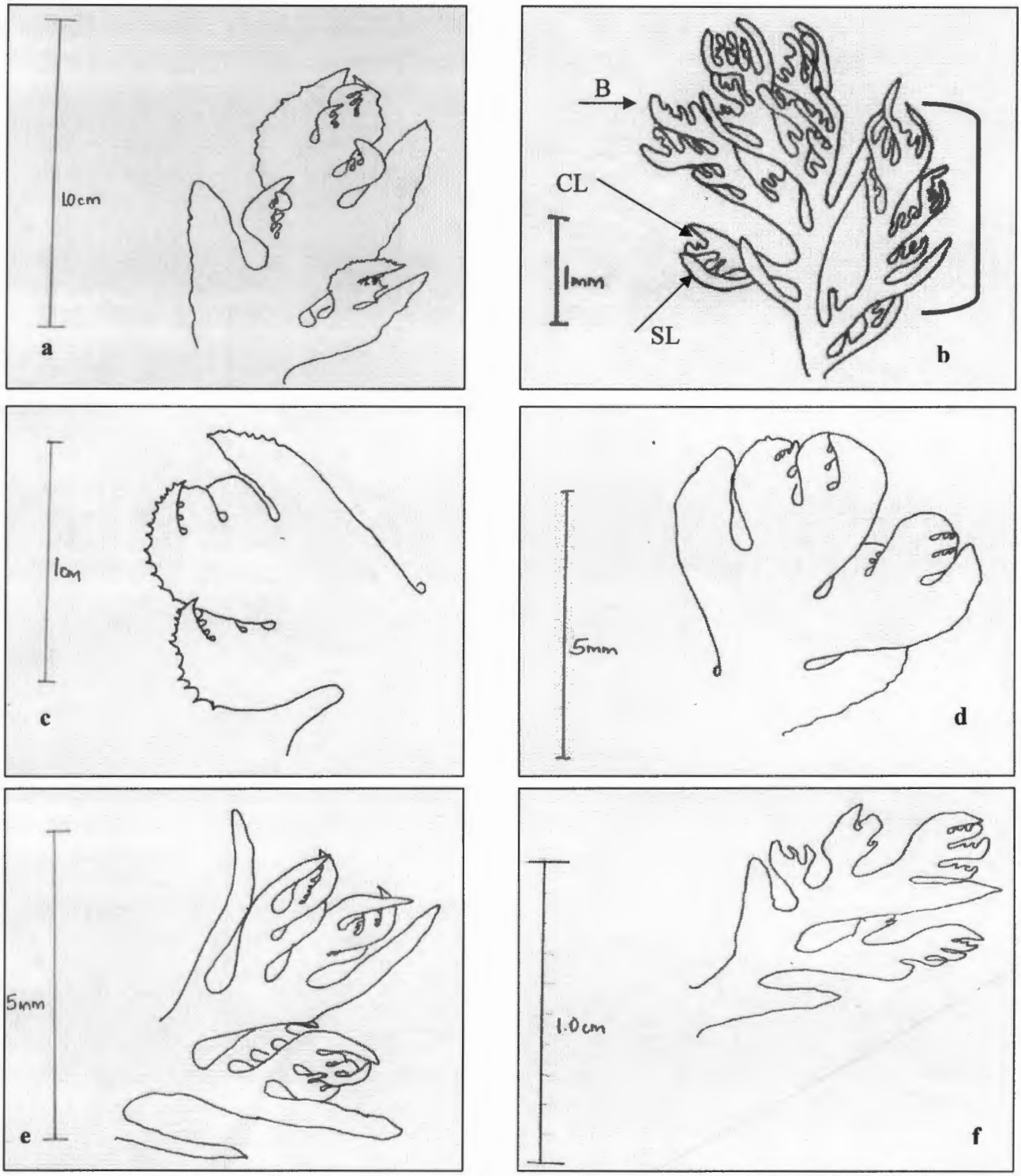

Figure 1 a, c-f: Diagrams of the apical lateral couplet of South African *Plocamium* species.
Figure 1b: Thallus of *P. beckeri*. (Bracket = lateral couplet) [Key: Species name (magnification)]

KEY: a. *Plocamium robertiae* (1.6X)
b. *Plocamium beckeri* – thallus (SL=Simple Lateral; CL=Compound Lateral; B=branchlet)
c. *Plocamium corallorhiza* (1X)
d. *Plocamium suhrii* (2.5X)
e. *Plocamium rigidum* (2.5X)
f. *Plocamium beckeri*(1.6X)

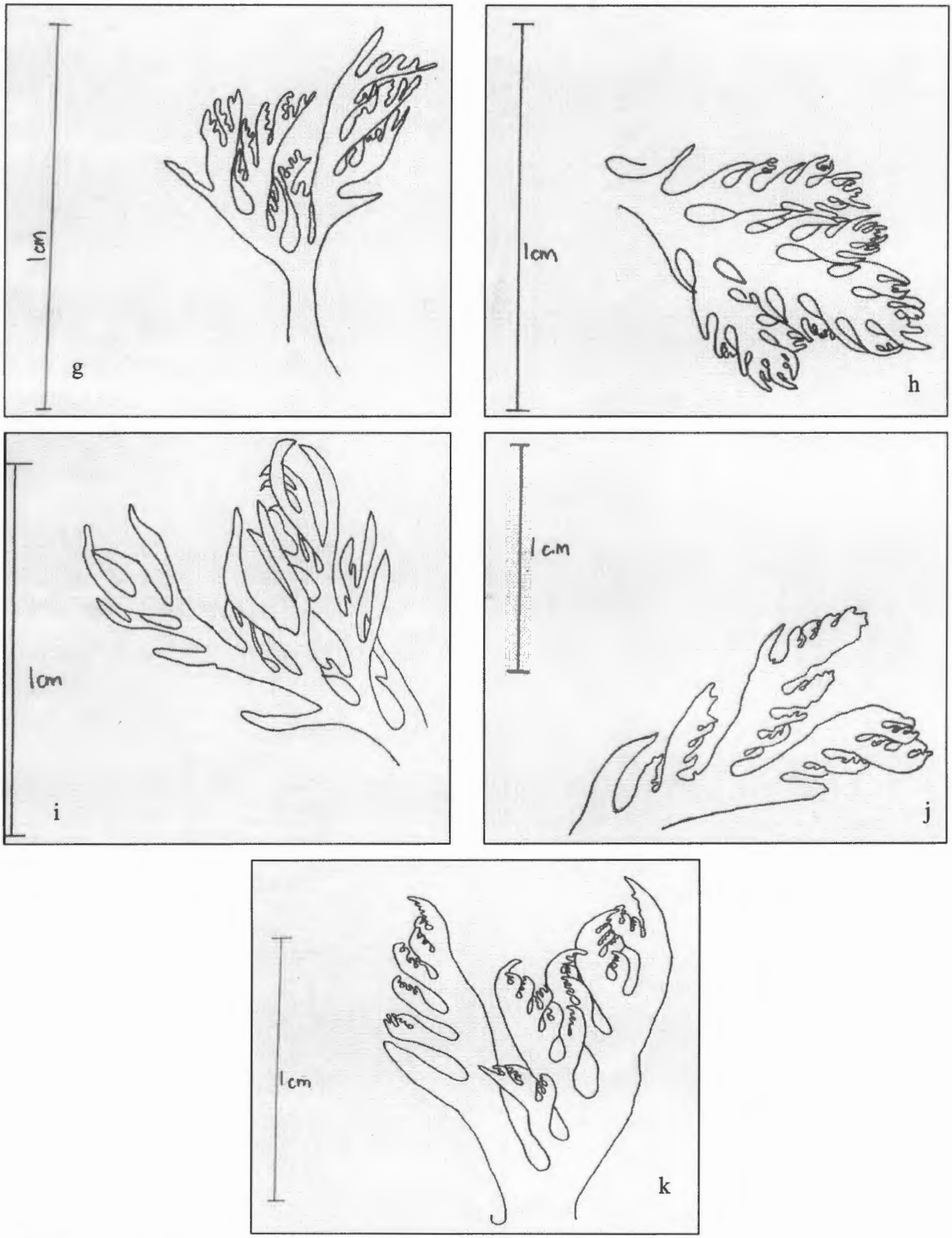

Figure 1 g-k: Diagrams of the apical lateral couplet of South African *Plocamium* species.
[Key: Species name (magnification)]

KEY: g. *Plocamium vulgare* (1.6X)
h. *Plocamium telfairiae* (1.6X)
i. *Plocamium cornutum* (1.6X)
j. *Plocamium maxillosum* (1X)
k. *Plocamium glomeratum* (1.6X)

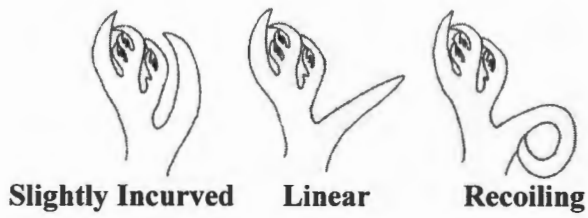

Figure 2: Simple lateral curvature in *Plocamium* (Adapted form Yano *et al.* 2004)

Phylogenetic analyses

Morphology

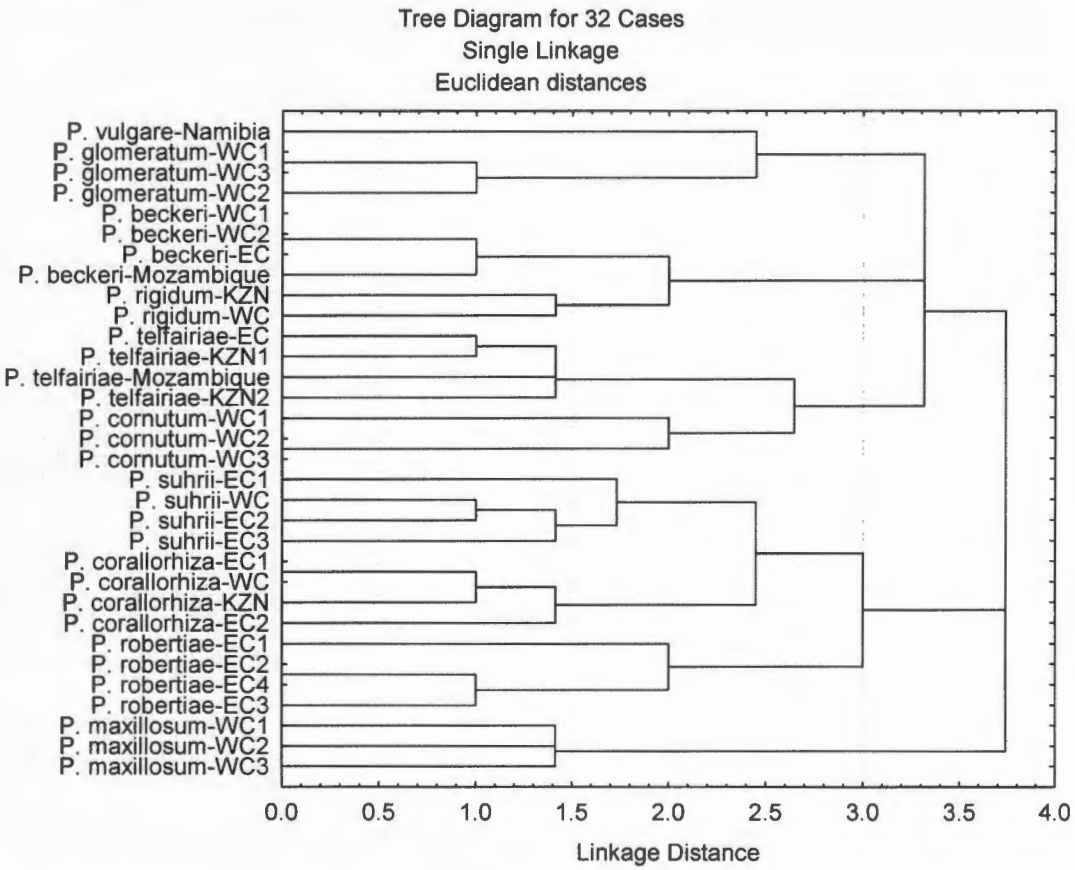

Figure 3: Cluster analysis of southern African *Plocamium* species based on morphology. (Single-Linkage algorithm using Euclidean distance measured between characters)

The genus *Plocamium* and its associated species were described on the basis of several morphological (both vegetative and reproductive) features in the earlier

publications. Figure 3 above represents the phenetic relationship between the taxa in the genus on the basis of overall similarity using the 12 vegetative characters scored in this study. Broad phenotypic similarities between sets of taxa are resolved e.g. *P. rigidum* and *P. beckeri* as well as *P. suhrii, P. corallorhiza* and *P. robertiae.* Despite the fact that *P. suhrii* and *P. corallorhiza* are each other's nearest neighbours, *P. robertiae* still emerges as *P. corallorhiza*'s phenetic nearest neighbour.** I do not know what this means? Suhri and Corallorhiza are nearest neighbours in the analysis (with robertiae the next nearest??)The splitting of taxa in Figure 3 *(P. suhrii-P. corallorhiza-P. robertiae-P. maxillosum* clade *versus P. vulgare-P. glomeratum-P. beckeri-P. rigidum-P. telfairiae-P. cornutum* clade) most likely corresponds to the broad morphological characters that define larger groups within the genus. This split could be attributed to either the presence or absence of teeth or bumpy teeth-like structures on margins of laterals or the bushy versus spread aspect of the species' habit.

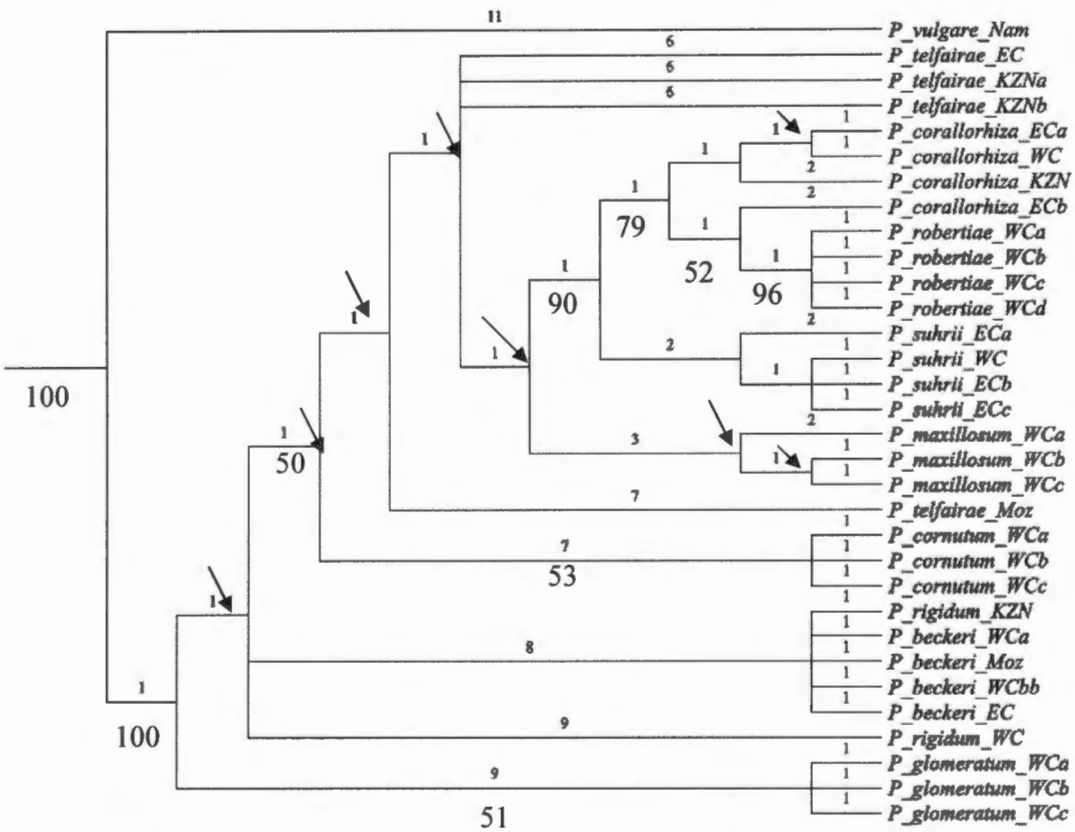

Figure 4: The most parsimonious tree (TL=50, CI=0.620, RI=0.878) based on 12 morphological features in southern African *Plocamium* species. The numbers above

the lines are branch lengths, which are proportional to the number of inferred substitutions. Branch support estimates for bootstrap values >50% are included below the relevant lines. Arrows indicate clades that collapse under strict consensus. (WC=Western Cape; EC=Eastern Cape, KZN=Kwa-Zulu Natal, Moz=Mozambique and Nam=Namibia)

For the morphological parsimony analysis all twelve characters analysed in the matrix were parsimony-informative. Gaps in the matrix were treated as missing data and the analysis returned 104 equally parsimonious trees of length (TL) of 50. The consistency index (CI) was 0.620 and the retention index (RI) was 0.878. Quicke (1993) states that a high CI value is indicative of a data set in which the characters are shared by descent (i.e. homologous) i.e. the topology is not overly influenced by homoplasious characters. A high RI value indicates a large number of synapomorphies are present in the dataset (Quicke 1993).

The phylogenetic relationships inferred from the twelve (12) morphological characters used in this study are presented in Figure 4. Several of the clades resolved in Figure 4 collapse under the strict parsimony consensus criterion to form polytomies with the exception of those clades with high bootstrap support (BP) for e.g. the node subtending the *P. corallorhiza, P. robertiae* and *P. suhrii* species have a bootstrap proportion of 90 and is retained in the strict consensus tree. *Plocamium maxillosum* is basal and belongs to the same clade with *P. corallorhiza, P. robertiae* and *P. suhrii* even though there is no support on the base of the clade. It is however paraphyletically associated with the three species *P. corallorhiza, P. robertiae* and *P. suhrii*. On the basis of morphology only there appears to be a split between *P. corallorhiza* and the putative species *P. robertiae* despite that *P. corallorhiza*_WCb only has the closest phylogenetic affinity with *P. robertiae* with BP of 51. The major split between *P. corallorhiza* and *P. robertiae* received strong BP support (79). The polytomy of *P. rigidium-P.beckeri* (excluding *P. rigidum_WC)* is the only one in the tree to contain two species. All other polytomies contain one species, although from different localities. *Plocamium glomeratum* is basal to all the species. While several polytomies are evident, generally the relationship amongst the South African *Plocamium spp.* is very strong (100 BP).

Molecular

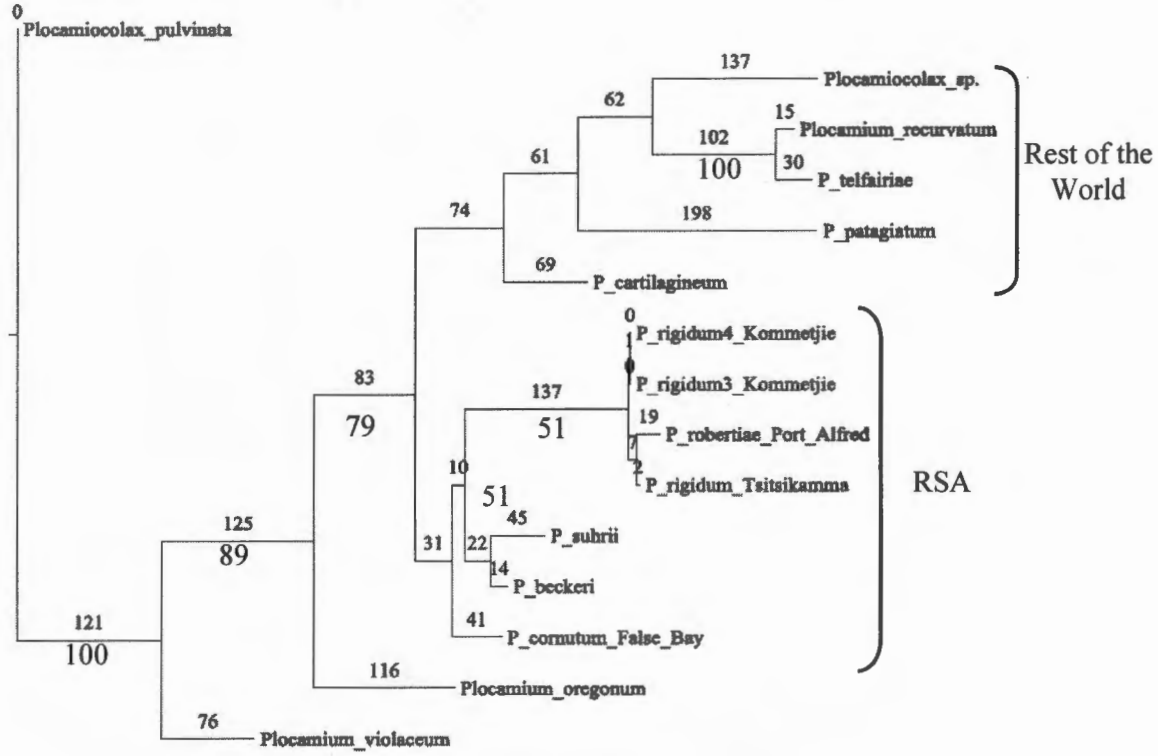

Figure 5: The most parsimonious tree (TL=1515, CI=0.530, RI=0.842) based on 910 molecular DNA characters present in the *ITS1-5.8S rDNA-ITS2* non-coding nuclear marker in *Plocamium* species from around the globe. The numbers above the lines are branch lengths, which are proportional to the number of inferred substitutions. Branch support estimates of bootstrap values >50% are included below the relevant lines. RSA = the South African contingent of the genus and Rest of the World = selected species in the genus from across the globe.

The molecular data matrix from which parsimony analysis was performed consisted of 910 characters when aligned, 202 characters were constant, 325 were parsimony-uninformative and 383 characters were parsimony-informative. Gaps in the matrix were treated as missing data and the analysis returned 91equally parsimonious trees of length (TL) 1515. The consistency index (CI) was 0.530 and the retention index (RI) was 0.842. Figure 5 depicts the relationship between certain South African *Plocamium* species as well as some Japanese and Western Northern Hemisphere species.

This analysis recovered two distinct clades – South Africa (RSA) and the Rest of the World (RW). The bootstrap support for the node supporting these clades is quite strong (BP=79). Numerous substitutions have occurred in both clades since the divergence from their common ancestor (RSA: 31 and RW: 74 substitutions each). *P. cornutum* appears basal to the South African taxa.

The sisterly relationship between *P. suhrii* and *P. beckeri* is questionable and it is very weakly supported by a bootstrap proportion much lower than 50 (BP=22). These two species are in turn sisters to the *P. rigidum-P. robertiae* group that has BP of 51. The *P.rigidum-P.robertiae* clade has weak nodal support (BP=51).

Combined morphological and molecular data

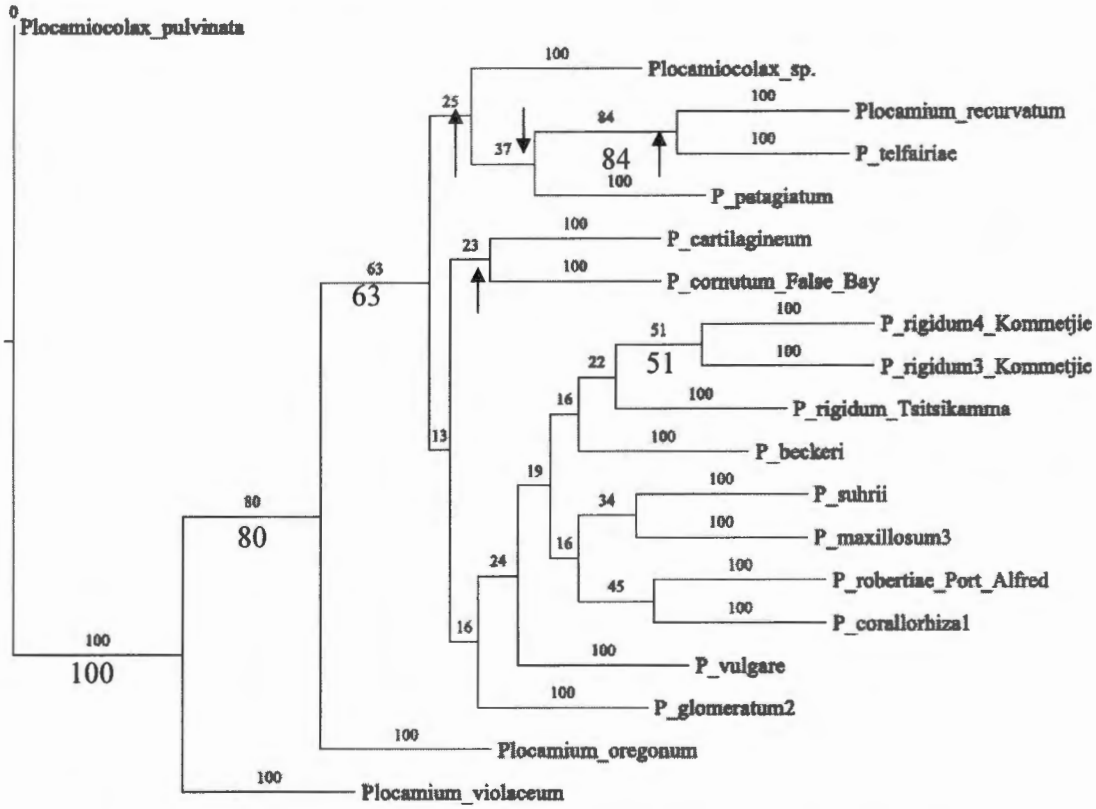

Figure 6: The single most parsimonious tree (TL=1557, CI=0.584 & RI=0.813) derived from combined DNA and/or morphological characters depicting relationships within *Plocamium*. The numbers above the lines are branch lengths, which are proportional to the number of inferred substitutions. Branch support estimates >50% are included below the relevant lines Arrows indicate the branches that collapse in the strict consensus tree.

The parsimony analysis of combined molecular sequence and morphological character matrix consisted of 922 characters, 202 were constant, 325 characters were parsimony-uninformative and 395 characters were parsimony-informative. Gaps in the matrix were treated as missing data and the analysis returned 21 equally parsimonious trees of length (TL) 1557. The consistency index (CI) was 0.584 and the retention index (RI) was 0.813.

The relationships within the combined tree are not as resolved as in Figure 4 and 5. *Plocamium beckeri* emerges as sister to the *P. rigidum* clade as opposed to the sister relationship with *P. suhrii* in Figure 5. Molecularly, the position of *P. corallorhiza* is questionable. *Plocamium vulgare* (synonym of *P. cartilagineum*) recorded from Namibia is embedded within the South African component of *Plocamium*. Several of the sister relationships resolved in the molecular tree are recovered in the combined morphology-molecular tree though in some cases the bootstrap support was much weaker. There is poor nodal support in this tree, but better placement of South African taxa.

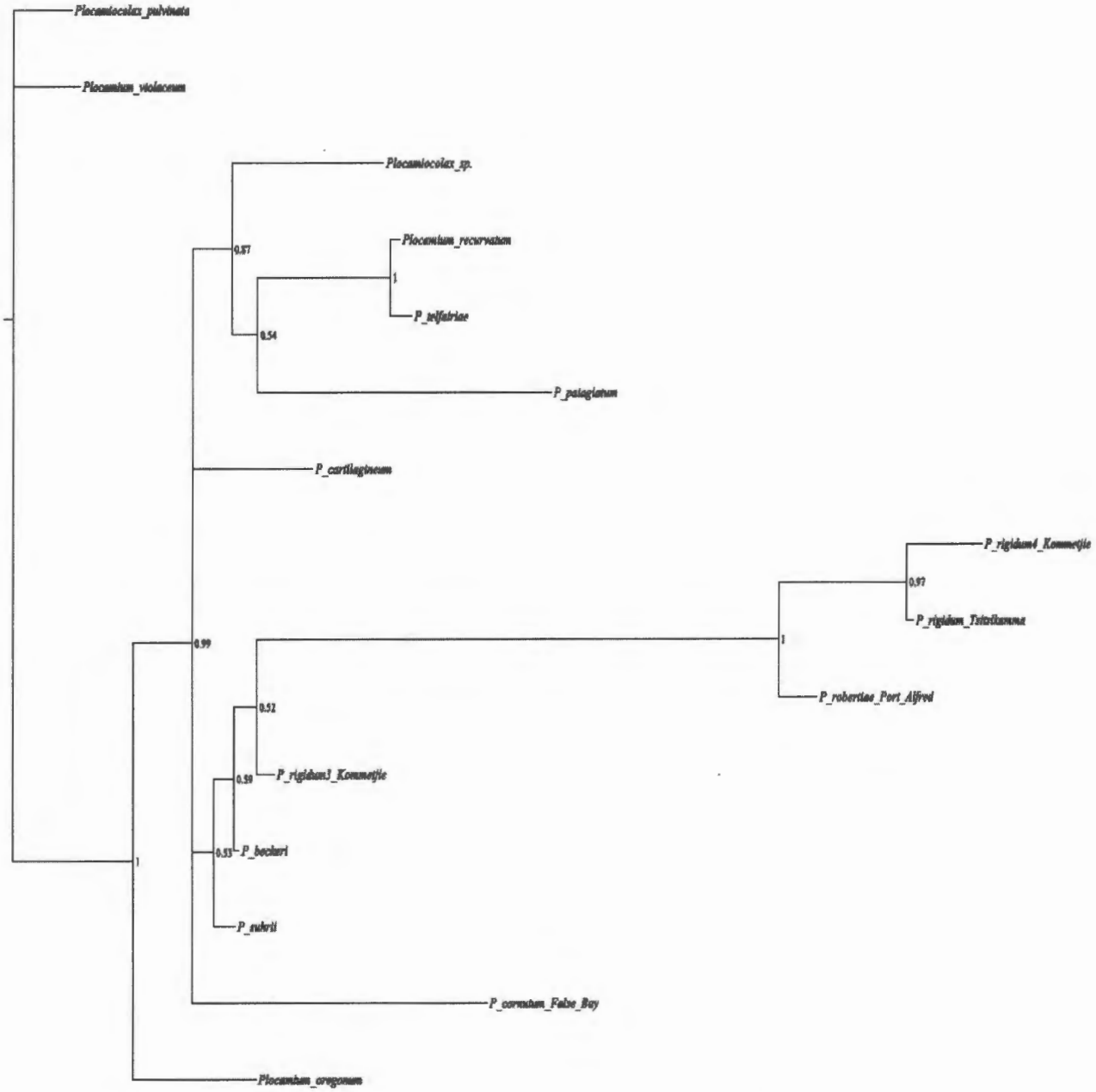

Figure 7: Phylogenetic relationships between species of the Plocamiaceae as inferred from the nuclear ITS region under the Bayesian inference of phylogeny using a GTR + I +G model of sequence evolution. Posterior probabilities (PP) adjacent to the relative node supported.

Figure 7 resolves a number of relationships similar to that of the molecular parsimony tree. However, the analysis couldn't resolve broad scale relationships producing a substantial polytomy dominating the tree (PP = 0.99). Towards the tips clades develop with many of these groups showing strong internal resolution (between PP = 0.52 and

PP=1 for different clades). *P. robertiae* is basal to two of the *P. rigidum* specimens (Kommetjie and Tsitsikamma) and the third *P. rigidum* (Kommetjie) is basal to that group. *Plocamium violaceum* is resolved as a basal taxon to the rest of the taxa alongside *Plocamiocolax pulvinata.*

Genetic Distances

The Kimura-2-Parameter pairwise distance calculation of the South African species exhibits low nucleotide sequence variation when compared to one another (*P. suhrii* and *P. rigidum* have a 0.01 pairwise distance between them). The two *P. rigidum* specimens from the Western Cape have no divergence in their nucleotides, but they do differ slightly from the same species in Tsitsikamma, Eastern Cape. The most divergent species is *P. cornutum* with divergences between this species and all other South African species ranging from 0.30 to 0.36 and 0.36 to 0.77 when comparing it to species from the rest of the world. *Plocamium cornutum* and *P. patagiatum* is the most divergent pair of species with a pairwise distance of 0.77. In general the South African species have divergence levels between 0.00 and 0.36, but when excluding *P. cornutum* the divergence levels drop dramatically to between 0.00 and 0.12. Unsurprisingly the species selected to broadly represent the members of the genus from around the world are more divergent among themselves and show no pattern of increase or decrease in divergence across the matrix.

Discussion

Phylogenetic Analyses

Does *Plocamium* form a monophyletic group?

Saunders and Lehmkuhl (2005) resolved a similar phylogeny in their study of the type species *P. cartilagineum* and state that *Plocamium* is paraphyletic when the adelphoparasite *Plocamiocolax* is considered a distinct genus. Our study used *Plocamiocolax pulvinata* as the root for both the molecular and combined dataset analyses (Figures 5 and 6 respectively). *Plocamiocolax pulvinata* was resolved as more closely related to *Plocamium violaceum* than its host *Plocamium pacificum* by Saunders and Lehmkuhl (2005). This close association between *Plocamiocolax pulvinata* and *Plocamium violaceum* is recovered again in Figure 7 with *Plocamium violaceum* resolved as basal to the rest of the taxa in the study alongside *Plocamiocolax pulvinata.*

The inclusion of *Plocamiocolax sp.* from the Palmer Peninsula in the Antarctic in the molecular (ITS1-5.8S-ITS2) and combined (ITS1-5.8S-ITS2 and morphology) analyses is controversial as there are a number of uncertainties linked to this species. These include the possibility of missing information in sequence and morphological data and of course that the taxon lacks a species name. The most pressing of these matters is that of the missing data, which may be exerting greater than expected influence on the positioning of the taxon in the tree. Wiens and Moen (2008) suggests that missing information does not have that drastic an effect on the topology. They do, however, qualify this statement by pointing out that this only holds if the dataset is sufficiently large to allow the taxon a number of characters to be placed accurately, if not precisely. The ITS1-5.8S-ITS2 data set consisted of 910 characters. The Antarctic *Plocamiocolax sp.* has a full complement of nucleotides in its sequence and so the possibility of missing sequence information influencing the structure of the tree - for this study - can safely be ruled out. In addition to there being no herbarium specimens to study for morphology many of the characters used in this study would not be present in the parasite.

Of course it is possible that the species was misidentified. The author associated with the sequence in Genbank is Lynda J. Goff (a world expert on rhodophyte parasites) and the paper attached to it is listed as unpublished. For the time being, with very little taxonomic information available regarding this species it would seem prudent to accept the identification of the species. The inferred relationship of this

adelphoparasite to species of the rest of the world (i.e. excluding South Africa) may well be real but the bootstrap support (BP) for this association is very weak (BP=25). Multiple sampling of taxa and sequencing additional and appropriate DNA regions may improve the resolution of the RW clade and the *Plocamiocolax sp.* associated with it (Figure 5). The resolution of the Antarctic *Plocamiocolax sp.* as sister to the Japanese members of the *Plocamium's* represented in Figure 5 and 6 (this result) supports the findings of Saunders and Lehmkuhl (2005) that *Plocamium* is paraphyletic.

What are the phylogenetic relationships amongst the South African *Plocamium* spp. & between South African species and the rest of the world?

A phylogeny of the South African *Plocamium* species, either molecular or morphological, has not been produced previously. The main features used to identify species are not discrete for e.g. the compound laterals in a single plant may support different numbers of pectinate branchlets and these numbers often overlap with those of another species. Figures 4, 5 and 6 depict relationships within the genus based on morphology, nuclear DNA and a combination of these datasets respectively.

Figure 4 depicts poor phylogenetic signal towards the tips of the tree and a number of polytomies are present in the tree. The clade consisting of *P. corallorhiza, P. robertiae* and *P. suhrii* is strongly supported (BP= 90). This close association on the basis of morphology is not unexpected, as at least two authors (Simons 1964; De Clerck *et al* 2005b) have noted the similarity between *P. suhrii* and *P. corallorhiza.* With the exception of teeth on the margins *P. suhrii* has the appearance of a small *P. corallorhiza.*

From Figure 4 the divergence between the '*P. corallorhiza'* and the '*P. robertiae-P. corallorhiza_ECb'* clades is well supported (BP=79) and the resolution of *P. corallorhiza_*ECb as basal to the *P. robertiae* polytomy (BP = 52) and this tentatively suggests that the putative species *P. robertiae* (supported by a BP of 96) may well be distinct from *P. corallorhiza.* Testing whether *P. robertiae* is divergent from *P. corallorhiza* on the molecular level is an essential component to the argument that the latter is a species. However, I failed to sequence *Plocamium corallorhiza* in this study and as such the molecular phylogeny (Figure 5) excludes this species instead resolving *P. robertiae* as sister to the *P. rigidium* from the same region – Eastern

Cape. This association highlights the need for a full species complement when resolving relationships in the genus on the basis of molecular characters, which themselves are susceptible to levels of homoplasy (Wiens 2004).

In addition, the divergence of *ITS* nucleotide sequences in the South African material (pairwise distance 0.00 - 0.36) is low in comparison to those of rest of the world (pairwise distance 0.36 – 0.77). The low level of divergence (pairwise distance 0.09) may well be one factor contributing to the resolution of *P. robertiae* as sister to *P. rigidum*. While speculative (and based on the obvious morphological association between *P. corallorhiza* and *P. robertiae*) the inclusion of DNA sequences of *P. corallorhiza* may alter the topology of the tree strongly. Indeed, under the combined morphology-molecular parsimony analysis (Figure 6) the *P. rigidum-P. robertiae* sister relationship is dissolved and each species is resolved in a separate clade.

In Figure 6, the combined dataset phylogeny, *P. vulgare* (a synonym of *P. cartilagineum*) is embedded in the clade of South African species resolved as monophyletic in relation to all other *Plocamium* spp. Silva *et al* (1996) noted that Simons (1964) did not recognise *P. cartilagineum* (then referred to as *P. coccineum* Lyngbye, *nom. illeg.*) as an entity within the South African flora as he believed it to have been confused with *P. beckeri* by previous collectors. Simons' study included the southern portion of the Namibian coastline (where the *P. vulgare* specimen was collected) and it is important to note that the *P. coccineum* west of Arniston in South Africa was not considered to be *P. beckeri* by Simons (1964). It is possible then that this specimen may be one of the cryptic species within *P. cartilagineum* identified by Saunders and Lehmkuhl (2005).

The studies by Iyer *et al.* (2005), Tronchin *et al.* (2006) and De Clerck *et al.* (2005a) tend to be of groups of related South African taxa (e.g. *Gelidium suhrii, G. micropterum, G. pristoides* in Tronchin *et al.* (2006); *Grateloupia capensis, G. longifolia* and *G. belangeri* in De Clerck *et al.* (2005a)). In comparison the molecular phylogeny in this study presents the South African material as a monophyletic group within the paraphyletic genus (see Figure 5), though the bootstrap support is very weak (less than 50). The divergence of the South African clade from the rest of the world (RW) is strongly supported (BP = 79). The 'RW' clade exhibit a number of

substitution events in the time since divergence from the South African clade (Branch length =74). While several relationships have low support the sister relationship between *P. recurvatum* and *P. telfairiae* is exceptionally well supported (BP = 100). This result was obtained in a study by Yano *et al.* (2005) who suggested that *P. recurvatum* is the same entity as *P. telfairiae* and proposed the former be synonymised with *P. telfairiae*. There is no pairwise nucleotide distance between these species on the basis of their nuclear ITS sequences and any difference in bootstrap support shown in the combined analysis (Figure 6) is a result of missing morphological data in the case of *P. recurvatum*.

Comparing molecular and morphological phylogenies

A comparison between the relationships resolved in the molecular and morphological phylogenetic analyses for the South African clade within *Plocamium* is hampered by the missing sequence data for *Plocamium corallorhiza, P. glomeratum* and *P. maxillosum*. Our ability to resolve the questionable relationship between *P. maxillosum* and *P. suhrii* of the west coast of South Africa as highlighted by Stegenga *et al* (1997) requires both morphological and sequence data to draw better informed conclusions. Morphological data resolve *P. maxillosum* as basal to the *P. corallorhiza-P.suhrii-P.robertiae* clade (Figure 4), but in the absence of sequences for either *P. corallorhiza* or *P. maxillosum* no such relationship is resolved (Figure 5). *Plocamium suhrii* is resolved as sister to *P. beckeri* (BP=51) and *P. robertiae* as sister to the *P. rigidum* of Tsitsikamma (Figure 5). Similarly, the existence of *P. robertiae* as an entity outside of *P. corallorhiza* requires sequence data to verify if the morphological divergence seen in the species is rooted in its genetic make-up. *Plocamium beckeri* and *P. rigidum* form the only polytomy containing two species in the morphological phylogeny (Figure 4), but despite having sequence data for both these species they are not resolved in the same clade in the molecular phylogeny. Instead they are within clades sister to one another with species with whom they share few to no physical similarities (Figure 5). It would seem that much like in the Japanese *Plocamium,* morphological and molecular data for South African *Plocamium* are incongruent with one another.

Taxonomy

The taxonomy of *Plocamium* worldwide and in South Africa requires extensive revision in the light of recent phylogenetic studies into the type species by (Saunders and Lehmkuhl 2005) and also the relationships resolved in the genus by studies focused in particular regions. The morphological, molecular and combined molecular and morphological data analyses of South African species in this study present a few taxonomic uncertainties that need to be addressed.

P. robertiae as a distinct species:

Simons (1964) proposed the synonymy of *Plocamium robertiae* with *P. corallorhiza* on the basis of the wide variation in the 'typical' features of the latter species, which he considered to encompass the traits of *P. robertiae*. In this study the morphological phylogeny showed that *P. robertiae* appears to be a distinct species with an exceptionally high bootstrap value supporting its divergence from the *P. corallorhiza*. Several physical features such as the narrower axis width, the pointed, triangular simple lateral and the appearance of teeth on the margin of the apical laterals support this tentative splitting between *P. corallorhiza* and *P. robertiae*. However, in the absence of molecular data on *P. corallorhiza* to serve as an additional source of evidence for the divergence of *P. robertiae*, the most I can say is that morphological data suggest the divergence of *P. robertiae* from *P. corallorhiza*.

P. glomeratum and its distribution in the world:

The key taxonomic treatments on the genus – Simons (1964) and Stegenga *et al* (1997) differ in their description of the physical features of *Plocamium glomeratum*. Simons (1964) does not recognise the lateral subtending the numerous branchlets as a 'true lateral' whereas Stegenga *et al* (1997) does consider it a compound lateral. The latter authors also extend the number of branchlets up to 10 (from a maximum of 6 by Simons) per compound lateral and considers the thallus of the alga to be fully complanate as opposed to alternating from terete at the base to complanate toward the apex as Simons (1964) stated. The specimens of *P. glomeratum* analysed in this study fit the description given by Stegenga *et al* (1997), with all specimens displaying the complanate thallus appearance and numerous branchlets.

Various authors place boundaries on the distribution of *P. glomeratum* that are questionable. Isaac (1956) cites Stephenson (1947) in recording the species only as far south as Port Elizabeth, while the author himself extends the range to inside the Western Cape near Mossel Bay. The implication is then that this is a warm water species and Silva *et al.* (1996) substantiate this finding in their listing of the species distribution in the Indian Ocean, which included Mozambique and Madagascar in the distributional range. Simons (1964) however, describes the range as one that is extensively west coast with Swakopmund, Namibia as the northernmost site and Still Bay the southernmost site. Simons (1964) stated that the specimens recorded by Kylin (1938) were misidentified and were in fact specimens of *Plocamium beckeri.* Specimens for this study were collected in the Western Cape only. In the absence of the specimen collected by Stephenson (1947) to verify that it is indeed *P. glomeratum,* there is no valid reason to exclude the warm water distribution of the species.

Plocamium affine:

Described by Kützing in 1849, *Plocamium affine* is supposedly a warm water species with type locality in Durban, South Africa (Silva *et al.* 1996). The most recent, published account of the species - J. Agardh, 1851: 405 - is 158 years old and Simons (1964) in his account of the genus in South Africa made no mention of the species either as an entity in its own right, a synonym or a misapplied name of any other species. Until a proper investigation into the collections of the species housed in the relevant herbaria can be performed, the existence of *P. affine* as an entity will remain uncertain.

Plocamium suhrii and taxonomic affinities:

Stegenga et al (1997) in their description of *P. suhrii* noted that the type of the species housed at Leiden Herbarium is "not very different from *P. maxillosum*" and an illustration of the species shows rows of three laterals as opposed to two as expected from the description. The discrepancies in the description, the type and the illustration of the species warrant closer inspection. In the current study all specimens shared the flattened, spread appearance of the thallus with both *P. maxillosum* and *P. corallorhiza. Plocamium suhrii* also exhibits the stout branchlets that *P. corallorhiza* has – and is almost like a miniature *P. corallorhiza* (De Clerck *et al* 2005b). Beyond

sharing a complanate thallus that is leathery in texture, *P. corallorhiza* and *P. maxillosum* are quite dissimilar. It has been suggested that *Plocamium suhrii* has a west and east coast form which may explain why the type specimen collected in Kalk Bay, in the Western Cape is similar to *P. maxillosum*, a predominantly west coast South African endemic. Furthermore, the specimens in this study and those in De Clerck *et al* (2005b) were collected along the south (this study) and east coast (De Clerck *et al* 2005b) where *P. corallorhiza* is abundant. *Plocamium suhrii* presents yet another species whose current taxonomic description does not adequately address the variation within the species. While the type species may resemble *P. maxillosum*, specimens in the field from warm water regions like KwaZulu-Natal resemble *P. corallorhiza* and the potential for misidentification of the species considering the dissimilarity between the two species is high.

Conclusions

Species within *Plocamium* in South Africa share a complex phylogenetic history, which is apparent in the difficulties several field ecologists experience when attempting to identify any species other than the large *P. corallorhiza* on the basis of key morphological characteristics. A phenetic analysis placed species into the relationships expected on the basis of morphology and appeared quite sensible though of no use in deducing the phylogenetic relationships between the species. Molecularly, several of the relationships are questionable which could be ascribed to the low levels of nucleotide sequence divergence seen in the nuclear ITS marker as well as to the absence of three species integral to the evaluation of phylogenetic relationships in the genus. The addition of *Rubisco* spacer for all species as well as the inclusion of the mitochondrial *cox 2-3* marker that has specifically been designed for red algae (see Provan *et al.* 2004) is necessary to resolve the relationships within the genus sensibly. In addition to this, the remaining species that failed to sequence in this study and at least two samples for each putative taxon are required for a phylogenetic analysis of the relationships within the genus to have a stronger footing to base inferences upon. The taxonomy of *Plocamium*, both in South Africa and worldwide, is in dire need of revision especially considering that several species boundaries are under question. Without an up-to-date and phylogenetically sound classification for *Plocamium*, further studies into the ecological or biochemical services provided by the genus will prove difficult at the least.

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