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Phylogenetic diversity in South African *Porphyra* (Bangiales, Rhodophyta) determined by nuclear SSU sequence analyses

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Specimens of *Porphyra* were collected at 10 sites along the western, southern and eastern Cape coasts of South Africa during late January to early February 2001. Sequence data from the variable V9 region of the nuclear small subunit rDNA (nSSU) locus were determined for 45 specimens. These data identified 11 distinct entities each with unique V9 sequences. Phylogenetic analyses incorporating sequences of *Porphyra* and *Bangia* from throughout the world revealed that eight of the entities examined in this study form a monophyletic group, apparently endemic to the southern African region. The other three entities exhibited no close phylogenetic relationship either with each other or with the other South African taxa. This survey indicates that the southern African coast is a repository of greater taxonomic diversity for this genus than has been previously reported.

Key words: 18S rDNA, Bangiales, Bangiophycidae, nuclear SSU, phylogenetics, *Porphyra*, small-subunit rDNA, South African biodiversity, species identification

Introduction

Intertidal red algae belonging to the genus *Porphyra* C. Agardh occur on rocky shores from polar to tropical seas (Bold & Wynne, 1978). Although the genus is widespread along most temperate oceanic coastlines, the recorded distribution of individual species tends to be more limited and species are generally considered to be confined to clearly demarcated geographical regions (Yoshida, 1997; Guiry & Nic Dhonncha, 2003).

More than 130 species of *Porphyra* have been described worldwide (Yoshida, 1997). However, species identification within the genus has been hampered by the simple morphology exhibited by *Porphyra* and a paucity of distinguishing features. Recently, both biochemical and molecular techniques have been employed in attempts to overcome some of the limitations associated with the classical morphological approach commonly used for identification of species within the genus (Stiller & Waaland, 1993; Oliveira *et al*., 1995; Kunimoto *et al*., 1999; Broom *et al*., 1999). Data obtained from isozyme electrophoresis have been used to aid species resolution within *Porphyra* (Lindstrom & Cole, 1993; Griffin *et al*., 1999*a*,*b*). More recently, DNA sequence data from the highly conserved nuclear SSU gene of *Porphyra* have emerged as a useful and reliable tool for species differentiation (Broom *et al*., 1999; Nelson *et al*., 2001). Nuclear SSU (nSSU) sequencing has been applied as a systematics tool to the *Porphyra* flora of New Zealand and surrounding regions, to assess diversity and establish species boundaries. This has revealed a much higher level of phylogenetic diversity than had been apparent based on morphological criteria alone (Broom *et al*., 1999).

South Africa has an extensive coastline covering a wide latitudinal spread, with water temperatures varying from subtropical to cold temperate. *Porphyra* species have been recorded around the majority of the rocky coastline of the western, southern and eastern Cape (Graves, 1969; Stegenga *et al*., 1997). Abundant populations are restricted to the temperate waters of the Atlantic Ocean on the western Cape shores (Griffin *et al*., 1999*a*,*b*). *Porphyra* has been recorded as occurring from Port St Johns on the east coast to Cape Frio, Namibia, on the west coast (Seagrief, 1984; Lluch & Garreta, 2002).

Kützing (1843) described the first two *Porphyra* species known from southern Africa, *P. capensis* Kützing and *P. augustinae nom. illeg*. Agardh

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(1883) considered that *P. augustinae* was conspecific with *P. capensis*, representing a developmental stage. Delf & Michell (1921) recorded *Porphyra vulgaris nom. illeg.* and *P. laciniata* (Lightfoot) C. Agardh from South Africa, but the material they examined has also been considered by later authors to be consistent with *P. capensis* (Seagrief, 1984; Silva *et al.*, 1996). Further reviews of the taxonomy of *Porphyra* in South Africa were undertaken by Isaac (1957) and Graves (1969). Both authors recognized a single species but noted that *P. capensis* exhibited considerable morphological plasticity. They identified three morphological variants but did not consider these to represent separate species. Chamberlain (1965) reported that *Porphyra tristanensis* Baardseth from Tristan da Cunha and Gough Island was indistinguishable from South African specimens.

More recently Stegenga *et al.* (1997) recorded three species of *Porphyra* from the South African coast: *P. capensis, P. carolinensis* Coll *et* Cox (recently reduced to synonymy with *P. suborbiculata* Kjellman; Broom *et al.*, 2002) and *P. gardneri* (G. M. Smith *et* Hollenberg) M. W. Hawkes (Hawkes, 1977). Stegenga *et al.* (1997) also described a new species, *P. saldanhae* Stegenga, J. J. Bolton *et* R. J. Anderson, and recorded an unnamed species epiphytic on the stipes of *Ecklonia maxima* (Osbeck) Papenfuss and *Laminaria pallida* Greville. Griffin *et al.* (1999*a*,*b*) described a new epiphytic species, *P. aeodis* Griffin, J. J. Bolton *et* R. J. Anderson. To date, therefore, five species and one unidentified species of *Porphyra* have been recorded from South African coasts.

This study reports the results of a survey of *Porphyra* sampled from sites along the west, south and east coasts of South Africa during late January and early February 2001. Nuclear SSU sequence data from specimens collected along the coast were used to assess phylogenetic diversity within the genus *Porphyra* in the Cape region.

Materials and methods

Seventeen sites extending from St Helena Bay on the west coast to the east coast beyond Port Alfred were surveyed for the presence of *Porphyra*. Fifty-eight specimens of *Porphyra* were collected at 10 of these sites between St Helena Bay and Port Alfred (Fig. 1). Fresh samples were collected at each site and morphological and ecological details recorded. Voucher specimens were prepared, and have been lodged in WELT (Herbarium, Museum of New Zealand Te Papa Tongarewa). A portion of each specimen was desiccated in silica gel for DNA analysis.

Of the 58 samples collected, 45 were selected for further investigation by DNA sequence analysis. Where samples collected from the same location exhibited similar morphology, only one individual was selected for sequencing.

Molecular analysis

DNA was extracted from desiccated samples using the Chelex extraction method of Goff & Moon (1993). PCR amplification of the nSSU rDNA region was performed by amplification of two overlapping fragments. Fragment R, ~1170 bp in size, was amplified by primers 18e (Hillis & Dixon, 1991) and NS4 (White *et al.*, 1990) and fragment Qs, ~850 bp in size, by primers G04 (Saunders & Kraft, 1994) and J04 (Broom *et al.*, 1999) as shown in Fig. 2. PCR amplifications were performed in a Stratagene Robocyler (Stratagene Corporation, La Jolla, CA, USA) according to Broom *et al.* (1999). Sizes and yields of PCR products were assessed by electrophoresis through a 1% agarose gel. Reaction products were purified by PEG precipitation and sequenced on an ABI 377 automatic sequencer (Perkin Elmer Applied Biosystems, Foster City, CA, USA) utilizing standard methods. Where possible, after initial amplification of the Qs product, the 3′ region of the nSSU rDNA was sequenced using primer G06 (Saunders & Kraft, 1994). This sequence, which spans approximately 450 bp and includes the V9 variable region of the gene (Neefs *et al.*, 1993), has been found to be a useful proxy for overall variation in the whole nSSU in *Porphyra* (Broom *et al.*, 1999). Specimens from which a particular V9 sequence was obtained were considered to represent that 'V9 entity' and assigned a three letter, three digit identifier beginning with the letter 'Z' to indicate the South African origin of the specimens. The complete nSSU locus was amplified and sequenced from one specimen of each V9 entity and from specimens of each V9 entity that differed in morphology. Internal primers G02 (Saunders & Kraft, 1994) and J05 (Broom *et al.*, 1999) were used to complete the R and Qs regions respectively (Fig. 2). It should be noted that this method for selecting samples for complete sequencing using their unique V9 region sequences will almost certainly miss some of the variation present; however, we have found it to be an effective compromise between rigour and economy.

Sequence identification and alignment

Sequences were compared with previously determined nSSU sequences using the GCG software package (Genetics Computer Group, 1994) and with existing *Porphyra* sequences in GenBank using BLAST (Altschul *et al.*, 1990).

Sequences were aligned interactively using Se-Al v2.0 (Rambaut, 2001; http://evolve.zoo.ox.ac.uk/software/Se-Al/main.html) after initial comparison with alignments obtained from the European small subunit ribosomal RNA database (Wuyts *et al.*, 2002). Nuclear SSU exon sequence data from 51 bangialean species obtained from GenBank were included in the alignment. The dataset contains *Porphyra* and *Bangia* sequences from both the northern and southern hemispheres, and from all major oceans. Nuclear SSU sequences of three erythropeltidalean taxa were included as outgroup taxa. Regions of the dataset that could not be unambiguously aligned, including intron sequences, were excluded from

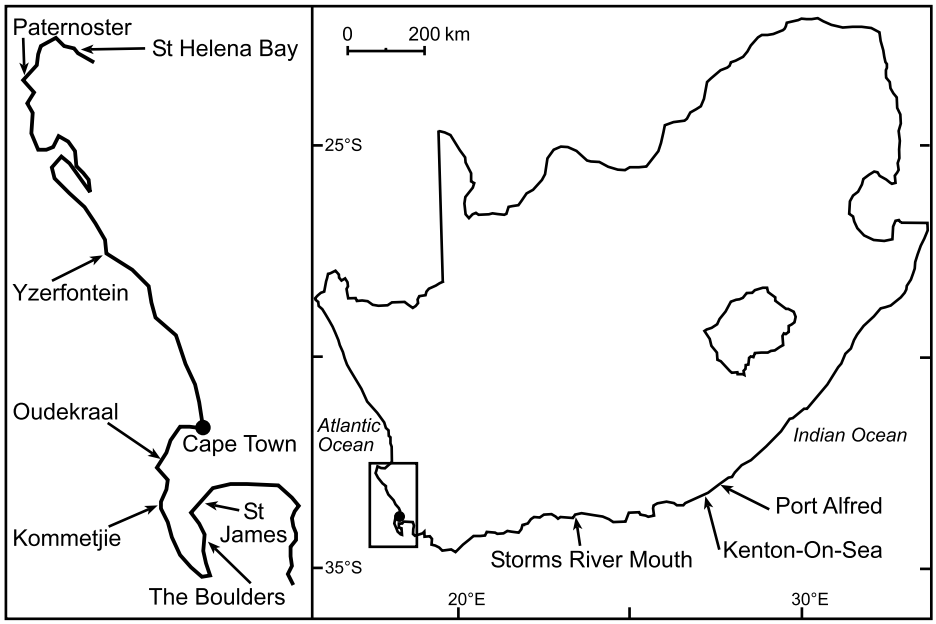

Fig. 1. Sites of *Porphyra* collections made in January and February 2001.

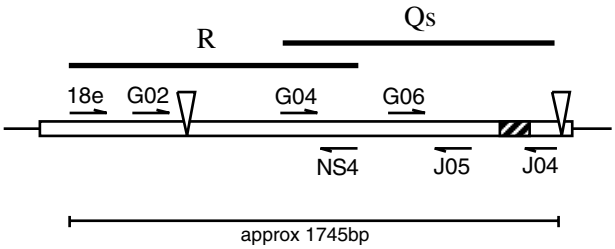

Fig. 2. Relative positions of primers used in this study (not to scale). R and Qs are the two overlapping pieces of the nSSU amplified by PCR. Triangles mark the insertion positions of two Group I introns commonly present in *Porphyra* spp., relative to these primers – only one of these is amplified with our primer set. Crosshatching indicates the variable V9 region used as an initial screen to assign samples to entities.

further analyses. Pairwise similarity scores over the whole alignment were calculated using PAUP*4.0b10 (Swofford, 2002) considering gaps as missing data. Two pairs of South African nSSU entities (ZIR901/ZIR970 and ZDR980/ZDR966) differed from one another by only a single nucleotide substitution. In both cases the sequence difference in the pair occurred in a highly variable region that was not retained in the final phylogenetic matrix, and each of these pairs is represented in the phylogenetic matrix by a single sequence representative (ZIR970 and ZDR980).

Phylogenetic analyses

Analyses were performed under maximum parsimony (MP), minimum evolution (ME) and maximum likelihood (ML) optimality criteria, and using the neighbour joining (NJ) algorithm in PAUP*4.0b10. MP trees were found by an initial heuristic search with 25 replicates of random order sequence addition followed by tree bisection-reconnection (TBR) branch swapping. This was followed by a further search with 100 replicates of random order sequence addition followed by TBR, saving no more than 10 000 trees of score greater than or equal to four steps greater than the best score found in the initial search (805) in each replicate. Consistency indices and rescaled consistency indices were calculated using PAUP*4.0b10; decay indices (CI, RI, Farris, 1989) were calculated using TreeRot version 2 (Sorenson, M.D. 1999; Boston University, Boston, MA, USA) and PAUP*4.0b10.

The ML tree was found by seven replicates of random order sequence addition followed by TBR branch swapping. The model of sequence evolution used for ML analyses and distance analyses was identified using the Akaike information criterion (Akaike, 1974) in MODELTEST v3.06 (Posada & Crandall, 1998). The model selected was the General Time Reversible (GTR) model allowing for invariant sites and with rate heterogeneity modelled by four gamma-distributed rate classes. Parameters were as follows: R(a) [A-C] = 1.2227, R(b) [A-G] = 3.6936, R(c) [A-T] = 1.8990, R(d) [C-G] = 1.0354, R(e) [C-T] = 8.8176, R(f) [G-T] = 1.0000, proportion of invariable sites = 0.5665, gamma distribution shape parameter = 0.5508. ME trees were found by TBR branch swapping on a NJ tree and were bootstrapped (1000 replicates) to assess support for nodes.

A full bootstrap analysis was not practicable for the MP and ML analyses due to the size of the dataset, however 1000 bootstrap replicates under the 'fast' bootstrap option of PAUP*4.0b10 were used to assess support for the tree under MP. This method finds trees for each replicate by stepwise addition without branch swapping, and has been criticized as likely to result in lowered bootstrap support values since some trees saved are certainly sub-optimal (Mort *et al.*, 2000). Mort *et al.* (2000) considered this method useful although they confirmed it gave lower support values than methods that included branch-swapping, while Salamin *et al.* (2003) concluded that such fast algorithms could give misleading support value estimates and should not be used. The MP bootstrap values in this analysis are therefore

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Table 1. GenBank accession numbers and references for samples used in this study. Samples are in the order they appear in Fig. 14

Species or entity	GenBank Acc. No.	Location	Reference
Erythrocladia sp.	L26188.1	–	Ragan *et al.* (1994)
Erythrotrichia carnea	L26189.1	–	Ragan *et al.* (1994)
Smithora naiadum	AF087126	Moss Beach, CA, USA	Rintoul *et al.* (1999)
Bangia sp. NC	AF043363	North Carolina, USA	Müller *et al.* (1998)
Bangia sp. MA	AF043362	Massachusetts, USA	Müller *et al.* (1998)
Bangia fuscopurpurea	AF342745	Strain SAG B 59.8	Müller *et al.* (2001b)
Porphyra umbilicalis	AB013179	Nahant, Mass., USA	Kunimoto *et al.* (1999)
P. lucasii	AY139685	Trigg Beach, WA, Australia	Farr *et al.* (2003)
Porphyra sp. LGW151	AY299973	Whahariki Beach, South Island, NZ	This study
P. purpurea	L26201	Avonport, Kings County, NS, Canada	Ragan *et al.* (1994)
P. coleana	AF136423	Leigh, Northland, NZ	Broom *et al.* (1999) (as PAP032)
Porphyra sp. LGD030	AF136422	Lyall Bay, Wellington, NZ	Broom *et al.* (1999)
Porphyra sp. WLR260	AY292644/ AY292645	Brighton, Otago, NZ	This study
Porphyra sp. GRB488	AY184350	Chatham Is, NZ	Broom *et al.*, in press
Porphyra sp. GRB368	AY292639	Kaka Point, Otago, NZ	This study
Porphyra sp. GRB145	AY184349	Te Henga, NZ	Broom *et al.*, in press
Porphyra sp. ZCE965	AY292627	The Boulders, SA	This study
Porphyra sp. ZDR980	AY292629	Kommetjie, SA	This study
Porphyra sp. ZDR966	AY292628	The Boulders, SA	This study
Porphyra sp. ZPP956	AY292636	Port Alfred Breakwater, SA	This study
Porphyra sp. ZGR903	AY292631	St Helena Bay, SA	This study
Porphyra sp. ZBS900	AY292626	St Helena Bay, SA	This study
Porphyra sp. ZIR970	AY292633	Kommetjie, SA	This study
Porphyra sp. ZIR901	AY292632	St Helena Bay, SA	This study
Bangia sp. Virgin Islands	AF043364	Virgin Islands	Müller *et al.* (1998)
Bangia sp. CA	AF043356	CA, USA	Müller *et al.* (1998)
Bangia sp. Victoria BC	AF043359	Victoria, BC, Canada	Müller *et al.* (1998)
Bangia sp. Japan (asexual)	AB053489	Tokyo, Haneda, Japan	Niwa *et al.* (2003)
Bangia sp. Japan (dioecious)	AB053488	Kanagawa, Enoshima, Japan	Niwa *et al.* (2003)
Bangia sp. OR	AF043358	Oregon, USA	Müller *et al.* (1998)
Bangia sp. Newfoundland	AF043357	Newfoundland, Canada	Müller *et al.* (1998)
Bangia atropurpurea. OM1	D88387	–	Shimimura *et al.* published only in database
Bangia sp. Nth BC	AF043360	Northern British Columbia, Canada	Müller *et al.* (1998)
Bangia sp. TX	AF043361	Texas, USA	Müller *et al.* (1998)
P. suborbiculata	AF136424	Wellington, NZ	Broom *et al.* (1999) (as *P. lilliputiana*)
B. gloiopeltidicola	AB053490	Hokkaido, Hakodate, Shinori, Japan	Niwa *et al.* (2003)
P. yezoensis	AB013177	Hakodate, Hokkaido, Japan	Kunimoto *et al.* (1999)
P. tenera	AB013175	Shinwa, Kumamoto, Japan	Kunimoto *et al.* (1999)
P. onoi	AB015794	–	Yamazaki *et al.* (1998) published only in database
P. tenuipedalis	AB015797	–	Yamazaki *et al.* (1998) published only in database
P. katadae	AB013184	Kawatana, Yamaguchi, Japan	Kunimoto *et al.* (1999)
P. leucosticta	L26199	Digby County, NS, Canada	Ragan *et al.* (1994)
P. rakiura	AF136425	Ocean View, Kaikoura, NZ	Broom *et al.* (1999) (as RAK049)
P. pseudolinearis	AB015793	–	Yamazaki *et al.*, (1998) published only in database
Porphyra sp. Japan	AB013182	Shimonoseki, Yamaguchi, Japan	Kunimoto *et al.* (1999)
Porphyra sp. PTK206	AY292641/AY292640	South East Bay, Three Kings Is, NZ	This study
Porphyra sp. ZEK881	AY292630	Paternoster, SA	This study
Porphyra sp. SSR053	AF136427	Ocean View, Kaikoura, NZ	Broom *et al.* (1999)
P. amplissima	L36048	Sandy Cove, Digby County, NS, Canada	Oliveira *et al.* (1995)
Bangia sp. AK	AF043355	Alaska, Greenland and Nunavut, Canada	Müller *et al.* (1998)
B. atropurpurea	L36066	Sandy Cove, Halifax County, NS, Canada	Oliveira *et al.* (1995)
Bangia sp. RI	AF043354	Rhode Island, USA	Müller *et al.* (1998)
P. dentata	AB013183	Koga Fukuoka, Japan	Kunimoto *et al.* (1999)
P. haitanensis	AB013181	Yuge Ehime, Japan	Kunimoto *et al.* (1999)
Porphyra sp. ZLI1045	AY292634/AY292635	Paternoster, SA	This study
P. acanthophora	L26197	Ubatuba, São Paulo, Brazil	Ragan *et al.* (1994)
P. spiralis	L26177	Ilha do Cardoso, São Paulo, Brazil	Ragan *et al.* (1994)
Porphyra sp. SSR091	AF136428	Brighton, Otago, NZ	Broom *et al.* (1999)
Porphyra sp. FIA1085	AY292637	Falkland Islands	This study

(*continued*)

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Table 1. (*continued*)

Species or entity	GenBank Acc. No.	Location	Reference
Porphyra sp. ZAE953	AY292624/ AY292625	Paternoster, SA	This study
Porphyra sp. FIC1084	AY292638	Falkland Islands	This study
Porphyra sp. ROS054	AF136426	Ocean View, Kaikoura, NZ	Broom *et al.* (1999)
P. virididentata	AF136421	Lyall Bay, Wellington, NZ	Broom *et al.* (1999) (as LGD018)
Porphyra sp. ROS204	AY292643/ AY292642	Campbell Island, NZ	This study
P. cinnamomea	AF136420	Bruce's Rock, Otago, NZ	Broom *et al.* (1999) (as BRU107)

Table 2. Absolute sequence differences (below diagonal) and % sequence divergence (above diagonal) of nSSU exon sequences from South African *Porphyra* entities identified in this study. Sequence length is slightly variable between entities due to the presence of indels, but is approximately 1730 bp.

	ZCE965	ZDR980	ZDR966	ZPP956	ZGR903	ZBS900	ZIR970	ZIR901	ZEK881	ZLI1045	ZAE953
ZCE965		1.87	1.79	0.81	0.92	1.21	1.27	1.22	8.06	8.57	8.46
ZDR980	32		0.06	1.58	1.69	1.52	1.63	1.57	7.96	8.5	8.31
ZDR966	31	1		1.50	1.62	1.44	1.56	1.50	7.93	8.46	8.34
ZPP956	14	27	26		0.69	0.75	0.87	0.75	8.10	8.51	8.39
ZGR903	16	29	28	12		0.29	0.35	0.41	8.11	8.63	8.45
ZBS900	21	26	25	13	5		0.17	0.23	8.05	8.57	8.39
ZIR970	22	28	27	15	6	3		0.06	8.05	8.63	8.45
ZIR901	21	27	26	13	7	4	1		7.93	8.51	8.36
ZEK881	138	136	136	139	139	138	138	136		3.17	3.69
ZLI1045	147	145	145	146	148	147	148	146	55		5.42
ZAE953	146	142	144	145	146	145	146	144	64	94	

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likely to be conservative, and should be interpreted with caution.

Results

Habitats of *Porphyra*

Porphyra was collected from 10 sites (Fig. 1), five of which (St Helena, Paternoster, Yzerfontein, Oudekraal and Kommetjie) are located on the Atlantic coast, with the latter two on the western shore of the Cape Peninsula. These sites supported abundant growth of *Porphyra*. The substrata at these five sites consist of granite, dolerite or sandstone and the shores have either north-facing aspects or broad, wave-cut platforms that provide some protection from the full impact of heavy surf.

Two of the collection sites (The Boulders and St James) are within False Bay near Cape Town. These sites consist of granite or sandstone substrata and are protected from heavy wave action. The water temperature on the False Bay side of the Cape Peninsula is normally several degrees higher than on the western side (Stegenga *et al.*, 1997). At these sites *Porphyra* was less abundant than at the Atlantic coast sites and occurred predominantly in the mid to upper intertidal zone.

Three sites located along the southern Indian Ocean coast, at Storms River Mouth, Kenton-on-Sea and Port Alfred, supported only one *Porphyra* entity, which occurred in isolated patches in the upper intertidal zone on sandstone and recently consolidated beach rock. No *Porphyra* was found along the southern coast at Mossel Bay, Knysna, Plettenberg Bay, Jeffrey's Bay, and on the sub-tropical Indian Ocean coast of Natal at Port Edward, Ballito Bay and Sheffield Beach.

Sequence data

Sequence data from the nSSU V9 region identified 10 unique sequences, designated V9 entities, and complete nSSU sequencing allowed us to identify a further entity (ZCE965), which is identical in the V9 region to entity ZPP956, but varies elsewhere in the nSSU. One sample initially failed to amplify using 'Qs' primers (Fig. 2) and was identified as belonging to entity ZGR903 based on sequence of the 'R' PCR product (5′ nSSU, Fig. 2), which also encompasses regions of significant sequence variation.

All sequences, except that of entity ZEK881, included a group I intron, inserted at the nSSU

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Table 3. Locations and gross morphology of South African entities found in this study.

Entity	Phylogenetic subgroup	n[a]	Coastline & sample site	Position on shore	Attachment	Thallus colour and form	Reproductive regions	Voucher specimens[b]
ZCE965	Cape cluster	1	**False Bay** – The Boulders	High shore	Epilithic on sandstone	Grey green sheets	Golden male areas, dark pink/red female areas; marginal and on separate regions of the blade edge	A23090
ZDR980	Cape cluster	3	**Atlantic** – Kommetjie, Oudekraal	Mid to low shore	Epilithic on sandstone	Grey green ribbons, divided near the base	Golden male areas, strongly pink female areas; milky/indistinct boundaries between male and female areas; both along upper margins of the blades; some blades appear solely female or solely male	A23099, A23100
ZDR966	Cape cluster	4	**False Bay** – St James, The Boulders	Mid shore	Epilithic on sandstone	Grey green deeply folded rosettes.	Golden yellow male areas, deep pink/magenta female areas; in separate regions of the margin around the rim of each rosette	A23095, A23096, A23098
ZPP956	Cape cluster	5	**Indian Ocean** – Storms River Mouth, Kenton-on-Sea, Port Alfred	High to mid shore	Epilithic on sandstone or beach rock	Deeply divided golden green blades with tightly rolled/folded ribbon-like fingers; also eroded pompoms	Pale cream to gold male areas; bright pink to brown female areas; both as marginal strips on separate fingers	A23091, A23093, A23094,
ZGR903	Cape cluster	2	**Atlantic** – St Helena, Kommetjie	High to mid shore	Epilithic on granite & sandstone	Golden green to green twisted ribbons also sheets with a rosette growth form	Pale milky male areas apical and marginal; brown female areas adjacent to male	A23079, A23106,
ZBS900	Cape cluster	4	**Atlantic** – St Helena, Paternoster	High to mid shore	Epilithic on granite; epizoic on mussels	Dark greenish brown; epilithic samples ribbons, epizoic sample broad blade	Milky golden male area marginal near apex; female areas deep red brown with indistinct boundary	A23080, A23081
ZIR970	Cape cluster	13	**Atlantic** – St Helena, Paternoster, Yzerfontein, Kommetjie	Mid to low shore	Epilithic & epizoic on limpets and mussels	Dark brown ribbons (mid shore) to large dark greenish brown/purplish black sheets (lower shore)	Male areas pale, milky golden pink; female areas deep magenta; monoecious, although some specimens have the appearance of being dioecious; some sectoring evident.	A23075, A23084, A23085, A23109, A23110
ZIR901	Cape cluster	4	**Atlantic** – St Helena, Paternoster, Yzerfontein. **False Bay** – The Boulders	High to mid shore	Epilithic on granite & dolerite; epizoic on mussels	Broad pink/grey/brown ruffled ribbons to sheets	Male areas golden and marginal; female areas pink brown to magenta	A23074, A23078, A23092, A23112,
ZEK881		3	**Atlantic** – Paternoster, Yzerfontein, Kommetjie	Subtidal to low shore	Epizoic on mussels; epiphytic on *Ecklonia maxima*	Pink brown, finely textured small blades to rosettes with deeply ruffled margins	Pale male areas as islands among dark pink/purple female areas giving a mottled appearance at the blade edge; sometimes occurring in sectors	A23103, A23104, A23113

(*continued*)

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Table 3. (*continued*)

Entity	Phylogenetic subgroup	n[a]	Coastline & sample site	Position on shore	Attachment	Thallus colour and form	Reproductive regions	Voucher specimens[b]
ZLI1045		1	**Atlantic** – Paternoster	Subtidal	Epizoic on *Patella compressa*	Dark purple/pink delicate oval blades	Pale male areas as marginal strip in lower blades and as pale islands in mid-upper blade; dark pink purple female areas	A23073
ZAE953		5	**Atlantic** – Paternoster, Yzerfontein	Low shore to subtidal	Epilithic on dolerite; epiphytic on *Aeodes orbitosa*; epizoic on mussels	Magenta brown, ribbons to large undivided deeply ruffled sheets	Pale male areas developing as marginal edge forming streaks or patches within dark magenta female areas; blades becoming mottled and deeply pigmented	A23101, A23102, A23107

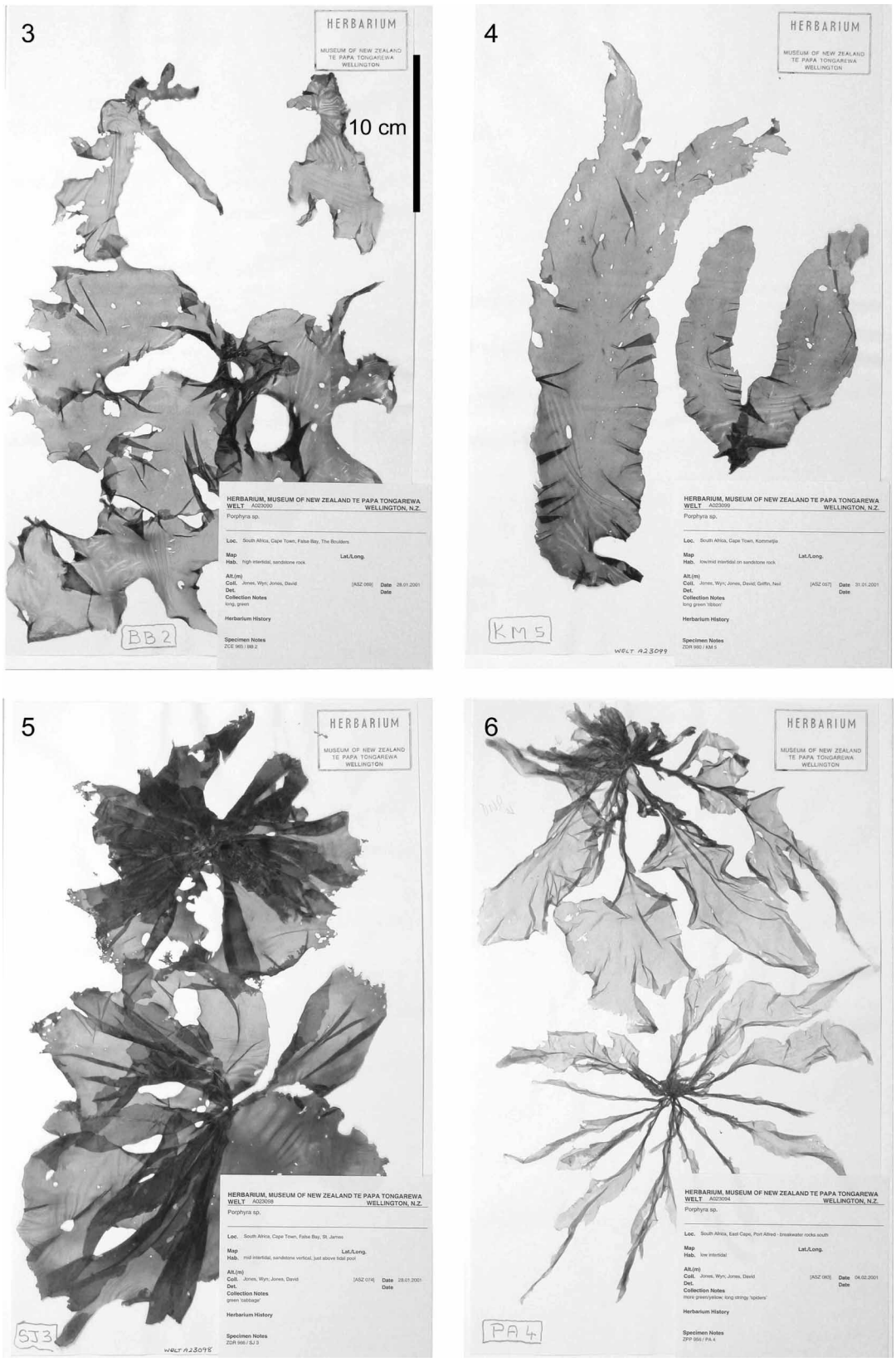
[a]n = number of specimens sequenced.
[b]Vouchers held at WELT.

position equivalent to base 516 in *Escherichia coli*, as is common in *Porphyra* and *Bangia* (Oliveira & Ragan, 1994; Oliveira *et al*., 1995; Müller *et al*., 1998, 2001*a*; Broom *et al*., 1999). Complete sequences of introns were obtained for at least one specimen from each V9 entity, except for ZAE953 and ZLI1045, for which sequences were only partial. Intron sizes varied from 550 bp (entity ZPP956) to 668 bp (entity ZDR966). The region amplified by the nSSU primers used in this study did not include the insertion position of a second group I intron commonly present in *Porphyra* and *Bangia* nSSU sequences (1506 in *E. coli* numbering). Sequence data spanning this insertion point were obtained for entity ZEK881, however, and no 1506 intron was present.

GenBank accession numbers for the nSSU sequences for each entity identified in this study along with their collection sites are listed in Table 1. The base pair differences and % divergence over the total nSSU exon sequences obtained for the 11 South African entities are given in Table 2. Information relating to the distribution, habitat, and morphology of the 11 entities are presented in Table 3. Voucher images of representative specimens of each entity are presented in Figs 3–13.

The final data matrix consisted of 1524 bp from 63 species and entities of possible species status. All three phylogenetic analyses – MP, ME and ML – generated trees with similar structure (Figs 14 and 15). In all trees *Porphyra* and *Bangia* are paraphyletic with respect to one another, as well established by Oliveira *et al*. (1995), Müller *et al*. (1998), and Broom *et al*. (1999). A basal subdivision within the Bangiales is well supported in all trees, as has been observed in previous analyses of nSSU sequences from the order (Broom *et al*., 1999, 2004; Müller *et al*., 2001*a*). South African entities are placed on both sides of this divide.

Eight of the 11 South African entities form a well-supported monophyletic group with no taxa from any other region. These entities differ from one another by 3 to 32 bp (Table 2) over approximately 1730 bp of nSSU sequence data. This group is designated the Cape cluster and is nested in a well-supported *Porphyra* clade which includes *P. coleana* W. A. Nelson from New Zealand, *P. lucasii* Levring from Western Australia and *P. umbilicalis* (L.) Kützing and *P. purpurea* Roth (C. Agardh) from the North Atlantic as well as a number of undescribed New Zealand entities (Broom & Nelson, unpublished data). The monophyly of the Cape cluster is well supported in all three analyses, but little other internal structure is resolved in the larger *Porphyra* clade. The parsimony grouping of *P. coleana* and *P. purpurea* has no bootstrap support and is not retained under either ML or ME. Both



Figs 3–6. Photographs of representative specimens of four of the 11 nSSU entities identified in this study. Entities are presented in the order in which they appear in Fig. 14. Fig. 3, ZIR970; Fig. 4, ZIR901; Fig. 5, ZBS900; Fig. 6, ZGR903. All figures are to the scale indicated in Fig. 3.

of these taxa are on long branches, which may promote artifactual grouping under parsimony (cf. Figs 14 and 15).

Within the Cape cluster, four entities (ZIR970/ ZIR901, ZBS900 and ZGR903) represented by 23 samples are consistently resolved as a monophyletic

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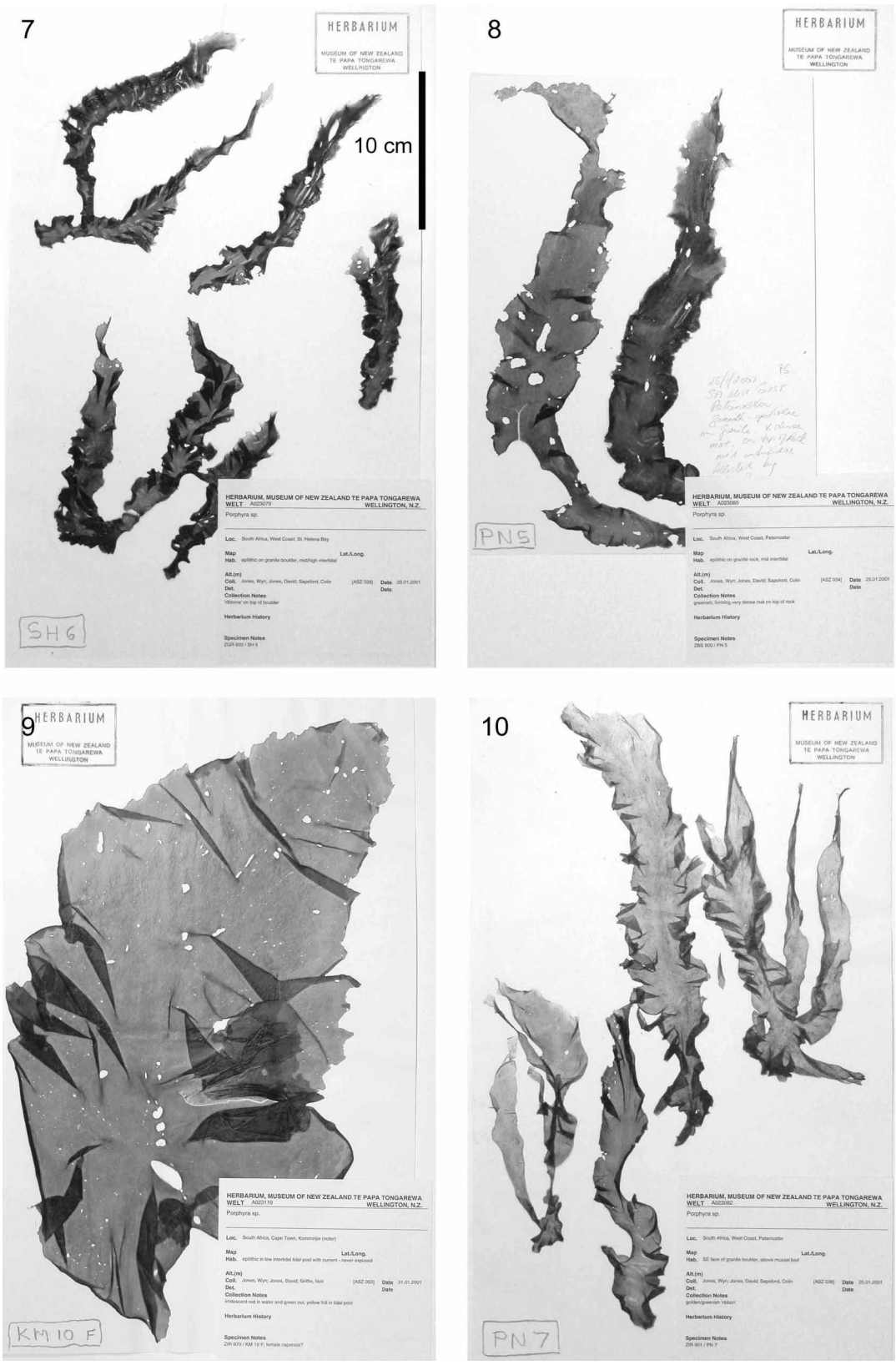

Figs 7 – 10. Photographs of representative specimens of four of the 11 nSSU entities identified in this study. Entities are presented in the order in which they appear in Fig. 14. Fig. 7, ZPP956; Fig. 8, ZCE965; Fig. 9, ZDR966; Fig. 10, ZDR980. All figures are to the scale indicated in Fig. 7.

group with nSSU sequences differing from one another by a maximum of only 7 bp (0.41%). With one exception all samples within this subgroup were collected on the Atlantic coast. Specimens of these four entities exhibit differences in colour, morphology and habitat, but differentiation based

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Figs 11–13. Photographs of representative specimens of three of the 11 nSSU entities identified in this study. Entities are presented in the order in which they appear in Fig. 14. Fig. 11, ZAE953; Fig. 12, ZEK881; Fig. 13, ZLI1045. All figures are to the scale indicated in Fig. 11.

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on these criteria alone is difficult and unreliable. The majority of samples belonging to ZIR970 occurred as large mature plants collected from gullies, channels or mussel beds in the lower intertidal region. Samples of the ZIR901, ZBS900 and ZGR903 entities occurred predominantly in the mid to high intertidal zone.

The other four entities belonging to the Cape cluster exhibit more extensive phylogenetic diversity. A relationship between entities ZCE965 and ZPP956 was supported under ME with moderate to low bootstrap support (65%, data not shown) but this association was not recovered in either the ML or MP analyses. MP placed these two entities on a polytomy with entity ZDR980 (also representing entity ZDR966), while the ML analysis placed ZDR980 basal to the Cape cluster and ZCE965 as a sister group to the Atlantic subgroup (Figs 14 and 15).

All five samples belonging to the ZPP956 entity were collected from the Eastern Cape coast of the Indian Ocean and exhibited a distinctive morphology and colouring (Fig. 7). The single sample belonging to the ZCE965 entity collected from the high intertidal zone in False Bay also exhibited a distinctive morphology (Fig. 8).

The remaining two entities, ZDR966 and ZDR980, represent a second closely related subgroup within the Cape cluster that differ from each other by only a single base difference in their nSSU sequences, but exhibit quite distinctive morphologies (Figs 9 and 10). All seven samples of these two entities were collected from the Cape Peninsula. The three samples of the ZDR980 entity were all collected on the colder western Atlantic side of the Peninsula and the four of the ZDR966 entity were collected on the warmer eastern False Bay coast.

The other three South African entities, ZAE953, ZEK881 and ZLI1045, are placed in a second well-supported and geographically widespread bangialean clade.

Entity ZAE953, collected on the Atlantic Ocean coast from the low intertidal and subtidal zones, occurred as an epiphyte on *Aeodes orbitosa* (Suhr) Schmitz, epilithically on dolerite, and epizoically on mussels [*Aulacomya atra* (Molina)]. Identical nSSU sequences were obtained from one epilithic and two epiphytic samples. Entity ZAE953 is placed in a *Porphyra* clade that is supported in all three analyses, with high bootstrap support under ME (94%), and with a decay index of five under MP. This clade consists of seven *Porphyra* entities from the Southern Ocean. In all three analyses ZAE953 is placed in a clade with nSSU sequences of two *Porphyra* specimens from the Falkland Islands, FIA1085 and FIC1084. This association is supported by a bootstrap value of 83% under ME and by a decay index of one under MP. The five other members of this well-supported clade are New Zealand entities, including *P. cinnamomea* W. A. Nelson and *P. viridididentata* W. A. Nelson.

The ZEK881 entity is represented by three samples collected from the subtidal or low intertidal zone on the Atlantic coast. These small

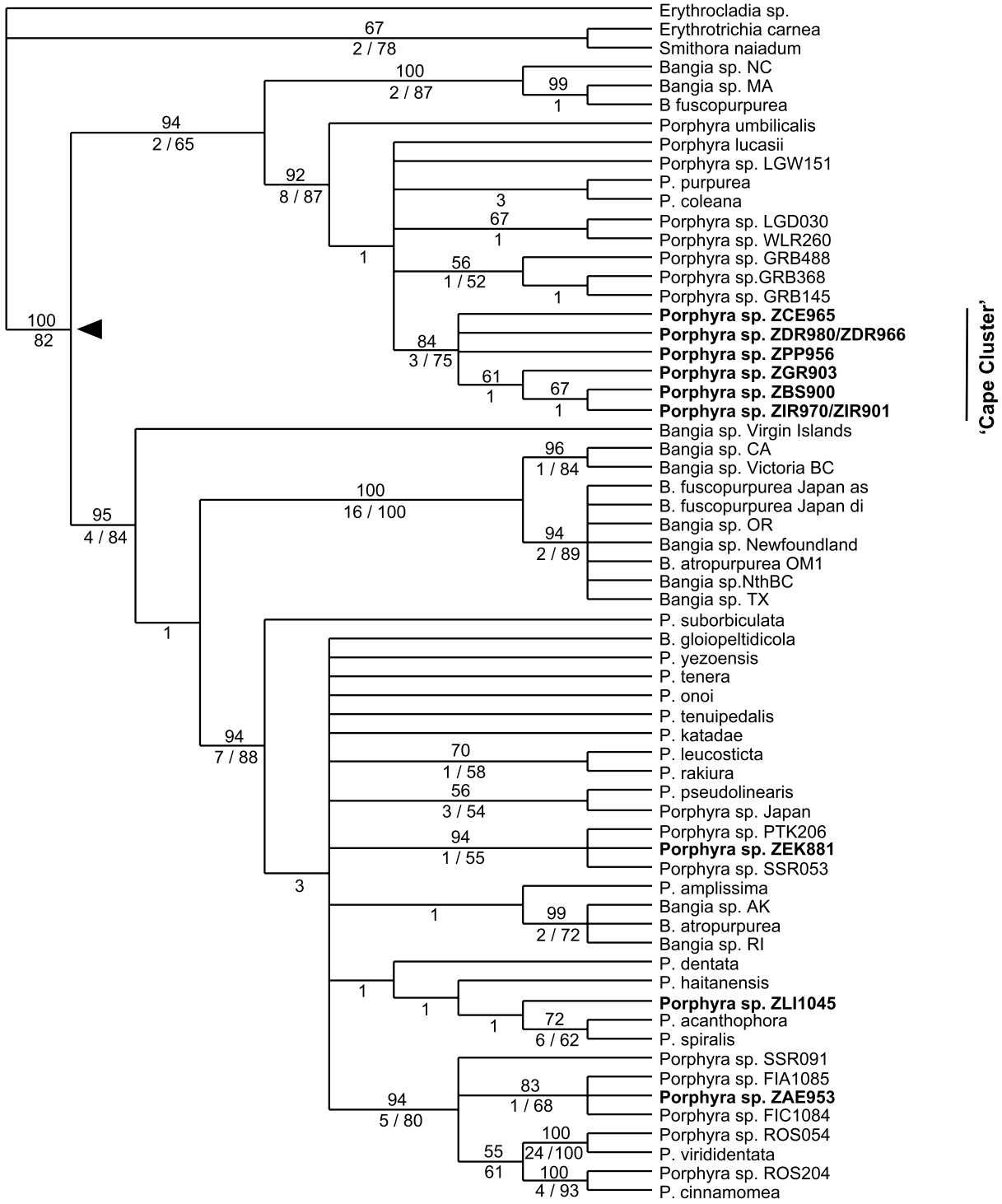

Fig. 14. The strict consensus of 20 193 trees (L = 805, CI = 0.573, RI = 0.842) found by maximum parsimony search of 63 Bangiales nSSU sequences. ME bootstrap values greater than 50% (1000 replicates) are shown above, decay indices and 'fast' MP bootstrap values greater than 50% (1000 replicates) below, branches leading to each node. South African entities are shown in bold type. The arrowhead marks the basal split in the *Porphyra*/*Bangia* clade.

epilithic, epizoic and epiphytic specimens exhibit a distinctive morphology (Fig. 12). Entity ZEK881 forms a monophyletic group with undescribed New Zealand entities SSR053 and PTK206. The nSSU exon sequences of ZEK881 and SSR053 are strikingly similar – only a single nucleotide substitution separates these two sequences.

The ZLI1045 entity (Fig. 13) is represented by a single sample collected from the sub tidal zone of the Atlantic coast, epizoic on the limpet *Patella compressa* which is itself restricted to *Ecklonia maxima*. Entity ZLI1045 is resolved on all three analyses as a member of a *Porphyra* clade that also includes *P. dentata* Kjellman and *P. haita-*

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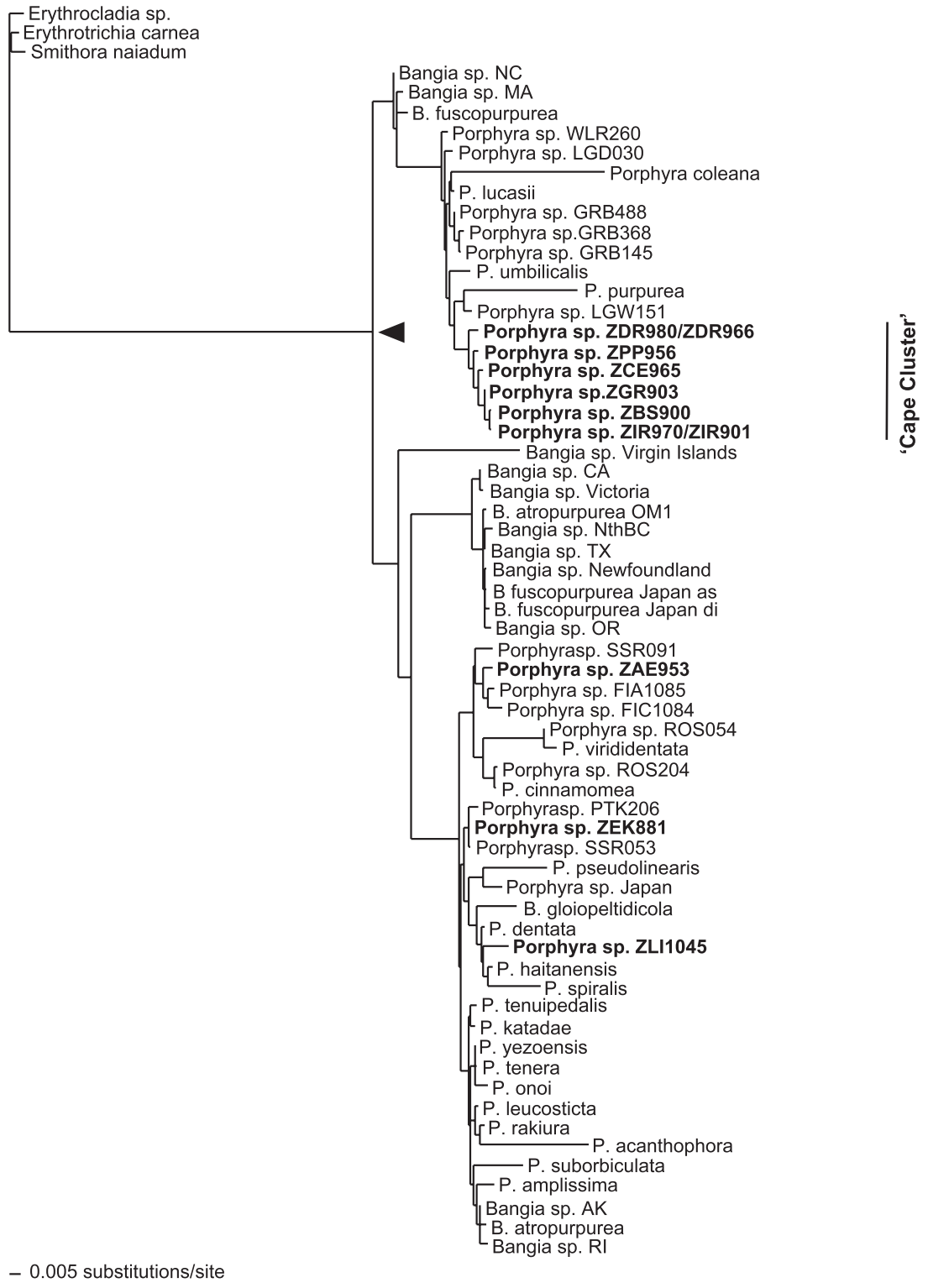

Fig. 15. Maximum likelihood phylogram found by heuristic search of the Bangiales dataset (see text for details). South African entities are shown in bold type; the arrowhead marks the basal split in the *Porphyra*/*Bangia* clade.

nensis Chang *et* Zheng. ME and ML analyses also include *P. acanthophora* Oliveira Filho *et* Coll *et P. spiralis* Oliveira Filho and Coll in this clade, although with only moderate bootstrap support under ME (63%). These latter two taxa are grouped together but placed on a basal polytomy under MP.

Discussion

This study reveals much greater diversity than has previously been recognized within the genus *Porphyra* in South Africa. Eleven entities with unique nSSU sequences were identified. Eight of these cluster together to form a closely related

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monophyletic group, separated from one another by a maximum of 32 bp (1.87%) differences in their nSSU sequences. This is suggestive of an independent radiation of species endemic to the southern tip of the African continent. The morphological characteristics of the eight entities belonging to the Cape cluster equate well with the current concept of *P. capensis* (Stegenga *et al*., 1997). Previous publications have commented on the high level of morphological variability within *P. capensis* and alluded to the existence of distinct subgroups within that species (Isaac, 1957; Graves, 1969; Stegenga *et al*., 1997). From the limited data generated from this survey it appears that the eight entities belonging to the Cape cluster exhibit distinctive regional distributions and habitats. Given the significant variation in nSSU sequences among members of the Cape cluster, re-examination of the application of the name *P. capensis* is called for. It is likely that sequencing of further collections would reveal additional entities within this Cape group. More extensive collections encompassing seasonal, ecological and geographic variation will be required to address this issue.

The morphology and habitat of specimens of the ZAE953 entity are similar to those reported for *P. saldanhae* (Stegenga *et al*., 1997) and *P. aeodis* (Griffin *et al*., 1999*a,b*). *P. saldanhae* is described as an epilithic species occurring as a winter annual in the lower intertidal and subtidal fringe of the west coast, and considered to be endemic to South Africa. *P. aeodis* is described as a summer annual, occurring as an epiphyte on *A. orbitosa*, that is morphologically similar to *P. saldanhae*. The two species are most readily distinguished using a combination of gametophyte morphology, seasonality and substratum choice (Griffin *et al*., 1999*a,b*). Recently Lluch & Garreta (2002) reported the occurrence of *P. saldanhae* from the coast of Namibia, collected from hard substrata and growing epiphytically on *A. orbitosa*. Although their specimens were collected in summer the anatomical features agree more closely with those of *P. saldanhae* than of *P. aeodis*. In the absence of isozyme data it is not possible to unambiguously identify specimens in this study. Well-defined *Porphyra* species sharing identical nSSU sequences have not previously been observed. However, the nSSU is a very conservative gene that accumulates mutations only slowly, and recently diverged species with identical nSSU sequences have been observed for some species of *Gelidium* (Bailey & Freshwater, 1997). It is therefore possible that these samples are in fact a mixed collection of both *P. aeodis* and *P. saldanhae*. Alternatively, seasonality combined with substratum choice may not be reliable for distinguishing between these two species. More variable genetic markers must be examined in order to determine whether the epilithic and epiphytic members of this nSSU entity form mutually exclusive monophyletic groups suggestive of mutual reproductive isolation and speciation.

Stegenga *et al*. (1997) record two species of small, subtidal *Porphyra, P. gardneri* and *Porphyra* sp. indet., that occur epiphytically on the large brown algae *E. maxima, Anthrophycus longifolius* (Turner) Kützing and *Laminaria pallida*. In the current study, entities ZEK881 and ZLI1045 were collected from deeper water on the Atlantic coast. The single sample of the ZLI1045 entity differed in its nSSU sequence from all other South African entities by at least 55 nucleotide substitutions (3.17% sequence difference). The relationship between ZEK881 and ZLI1045 entities and the deep water species discussed by Stegenga *et al*. (1997) requires further elucidation.

In entity ZIR970 a number of thalli appear to be solely female or solely male, suggesting that it may be dioecious, while others with the same overall morphology are clearly monoecious, with both male and female regions present on a single individual. This phenomenon was also observed in entities ZIR901 and ZDR980. It seems likely that this apparent dioecy is in fact due to sequential appearance of male and female fertility, rather than a commitment of any individual thallus to exclusively male or exclusively female development. Caution is therefore called for in describing *Porphyra* species as dioecious in the absence of extensive collections or life history information that reliably point to true separation of male and female thalli.

Porphyra in South Africa has been considered to occur predominantly as a winter annual, then being the dominant alga in the littorinid and balanoid zone (Branch & Branch, 1981; Lubke & de Moor, 1988). The luxuriant growth and the large size of many specimens collected from the Atlantic Ocean in mid-summer show that *Porphyra* is present throughout the year (Griffin *et al*., 1999*a,b*). It is clear from this study that the diversity of *Porphyra* in southern Africa has been substantially underestimated, and developing an adequate understanding of the seasonal distribution and annual growth of *Porphyra* in this region will require a broad-ranging study including extensive seasonal collections. Future studies would be facilitated by the determination of molecular taxonomic characteristics in addition to the morphological features that have, until recently, been employed in descriptive accounts of the South African *Porphyra*.

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