Eur. J. Phycol., (2012), **47(4)**: 355–365

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Phylogenetic analysis of *Codium* species from Brazil, with the description of the new species *C. pernambucensis* (Bryopsidales, Chlorophyta)

MARIA DE FÁTIMA DE OLIVEIRA-CARVALHO[1], MARIANA C. OLIVEIRA[2], SONIA MARIA BARRETO PEREIRA[1,3] AND HEROEN VERBRUGGEN[4,5]

[1]*Programa de Pós-Graduação em Botânica, Universidade Federal Rural de Pernambuco (UFRPE), Avenida Dom Manoel de Medeiros, s/n Dois Irmãos, CEP: 52171-900, Recife, PE, Brazil*
[2]*Instituto de Biociências, Universidade de São Paulo, Rua do Matão 277, CEP: 05508-900, São Paulo, SP, Brazil*
[3]*Departamento de Biologia da Universidade Federal Rural de PernambucoRecife, PE, Brazil*
[4]*Phycology Research Group, Ghent University, Krijgslaan 281 (WE11), 9000 Ghent, Belgium*
[5]*School of Botany, University of Melbourne, Victoria 3010, Australia*

(Received 21 November 2011; revised 20 April 2012; accepted 9 May 2012)

The genus *Codium* comprises *c*. 125 species widely distributed in marine coastal environments throughout the world. Due to morphological plasticity, the taxonomic delimitation of *Codium* species can be difficult. Sequences of the first exon of the large subunit of RUBISCO (*rbc*L) have been used in the molecular delimitation of species and for phylogenetic purposes. In the present study, we complement previous morphological work on Brazilian *Codium* species with molecular systematics. Based on the partial *rbc*L sequences, seven species are recognized along the Brazilian coast: *C. decorticatum*, *C. intertextum*, *C. isthmocladum*, *C. profundum*, *C. spongiosum*, *C. taylorii* and the new species *Codium pernambucensis*. Ten unique sequences were obtained among the samples examined, which we used in combination with previously published sequences to infer molecular phylogenies using various methods. The resulting trees showed three principal monophyletic groupings: Clade A with species having a prostrate habit, not branched, and mostly with small, grouped utricles; Clade B primarily consisting of upright species with cylindrical branches and large individual utricles; and Clade C composed of upright species with cylindrical branches that are slightly flattened, and have intermediate-sized individual utricles. The Brazilian species grouped with morphologically similar taxa from other geographic localities, and are present in all three main clades. A new sprawling species, *Codium pernambucensis* is described based on morphology and molecular analyses.

Key words: Brazil, Bryopsidales, Chlorophyta, *Codium pernambucensis sp. nov.*, molecular phylogenetics, *rbc*L, taxonomy

Introduction

Codium is a cosmopolitan genus widely distributed in marine environments throughout the world, with the exception of the polar regions, and currently comprises *c*. 125 species that are mainly found in temperate and subtropical zones (Goff *et al.*, 1992; Pedroche *et al.*, 2002; Verbruggen *et al.*, 2007). The thallus of this alga has a spongy nature due to its dense meshwork of coenocytic filaments, called siphons. Anatomically, the medullar region is composed of cylindrical, colourless siphons, which give rise to the enlarged, inflated utricles that form the cortical layer (van den Hoek *et al.*, 1995; Pedroche, 2001). The marked morphological plasticity within and between populations has been reported repeatedly (Silva, 1951; Pedroche *et al.*, 2002), and makes the systematics of the genus quite complex (Shimada *et al.*, 2004). In recent years, molecular markers have added to the toolbox of methods used to improve the understanding of the taxonomy and phylogeny of the genus.

While there exists a vast body of work on the biology of the invasive species *Codium fragile* (e.g. Francis *et al.*, 1987; Manhart *et al.*, 1989; Goff *et al.*, 1992), relatively few studies have addressed the phylogeny of the genus. Pedroche (2001) analysed fragments of 716 base pairs of the large subunit ribosomal gene (LSU rDNA) of the mitochondrial genome of ten Pacific Mexican *Codium* taxa. Subsequent phylogenetic studies have mostly been based on exon 1 of the *rbc*L gene that codes for the large subunit of the ribulose-1,5-bisphosphate carboxylase–oxygenase enzyme (Rubisco). Shimada *et al.* (2004) spearheaded the use of this

Correspondence to: Mariana C. Oliveira. E-mail: mcdolive@usp.br

ISSN 0967-0262 print/ISSN 1469-4433 online/12/00000355–365 © 2012 British Phycological Society
http://dx.doi.org/10.1080/09670262.2012.718363

gene in *Codium* for phylogenetic inference of 18 Japanese species. Verbruggen *et al.* (2007) broadened the taxonomic and geographic coverage to 74 *Codium* taxa from across the globe and proposed more detailed phylogenetic and biogeographical hypotheses for the genus.

Seven *Codium* species have been reported from Brazil: *C. decorticatum* (Woodward) M. Howe, *C. intertextum* Collins & Hervey, *C. isthmocladum* Vickers, *C. profundum* P.C. Silva & Chacana, *C. repens* P. Crouan & H. Crouan, *C. spongiosum* Harvey, and *C. taylorii* P.C. Silva. Nonetheless, our knowledge of the genus in Brazil is mostly limited to publications that have registered the presence of these taxa as part of floristic surveys (Joly, 1957, 1965; Ugadim & Pereira, 1978; Pereira *et al.*, 1981; Teixeira *et al.*, 1985, Pereira & Accioly, 1998; Pereira *et al.*, 2002; Oliveira-Carvalho *et al.*, 2003; Yoneshigue-Valentin *et al.*, 2006; Pereira *et al.*, 2007; Silva & Chacana, 2010). While taxonomic hypotheses have been proposed for Brazilian *Codium* taxa based on morphology (Silva, 1960; Oliveira-Carvalho *et al.*, 2010), they have not yet been studied using molecular systematic methods such as DNA barcoding and molecular phylogenetics.

The goal of the present study was to characterize the molecular diversity of the Brazilian *Codium* flora. Given the broad reference framework that exists for *rbc*L exon 1, we generated DNA barcodes of this marker for 32 Brazilian *Codium* specimens. We infer a phylogeny of the genus with newly generated plus already available sequence data, discuss the molecular systematics of the genus, and describe a new species based on morphological and molecular analyses.

Materials and methods

Codium *specimens and morphological observations*

Samples of *Codium* were collected in diverse localities along the Brazilian coast for molecular analysis (Table 1). Young portions of the thallus were collected in the field and cleaned using a toothbrush with flexible hairs in order to avoid including epiphytes. After cleaning, stem sections were dried using paper towels and divided into small pieces that were immediately placed in plastic jars containing silica gel or 100% ethanol. External features of the thallus were recorded and the anatomy was examined as described in Oliveira-Carvalho *et al.* (2010). Species identification was based on gross morphology (habit, branching pattern and the dimensions of the branches), anatomy (diameter of the medullary filaments, shape and dimensions of the utricles, and the placement of the hairs and scars), and reproductive characters (shape, dimensions and insertion of the gametangia), according to Silva (1951, 1959), Silva & Womersley (1956), Chacana *et al.* (1988), Van den heede & Coppejans (1996) and Pedroche *et al.* (2002). Whenever possible, 20 measurements for each structure were made with a Zeiss ocular micrometer (Axioskop 50, Jena, Germany). Digital photos of specimens were made by a Canon Power Shot 4.0 digital camera (Tokyo, Japan). The material was stained for anatomical examination using an aqueous solution of 1% Aniline Blue (Inlab, São Paulo, Brazil) and the images were made using a Leica DM 1000 stereomicroscope (Wetzlar, Germany) coupled to a digital photographic camera. Herbarium specimens of field material were deposited in the PEUFR herbarium. Species identification was based on Joly (1965), Taylor (1960), Silva (1952, 1960), Littler & Littler (2000) and Chacana *et al.* (2003).

DNA extraction

The samples of *Codium* were subjected to a manual DNA extraction protocol using CTAB (cetyl trimethyl ammonium bromide) extraction buffer (2% CTAB, 5% NaCl, 0.5 M EDTA, 1% polyvinylpyrrolidone (PVP), and 1 M Tris–HCl, pH 8). The *Codium* samples were macerated to a fine powder in liquid nitrogen, added to 700 µl of the CTAB buffer with 7 µl of protease K (20 mg ml^{-1}), and incubated at 60°C for 30–40 min. Subsequently, 250 µl of 3 M potassium acetate was added, and the sample was then maintained at −20°C for 30 min before being centrifuged at 24 511 × *g* (Mikro 220R, Hettich, Germany) at 4°C for 30 min. The aqueous phase of the sample was extracted twice with one volume of chloroform:isoamyl alcohol (24:1), and the sample was then centrifuged again for 10 min. DNA was precipitated with 0.8 volumes of isopropanol (100%) for 30 min at −20°C. After centrifuging (24 511 × *g*) for 20 min, the supernatant was discarded and the DNA contained in the tube was dried in a centrifuge under vacuum for 30 min. The DNA sample was then diluted in 50 µl of 0.1 × TE buffer (10 mM Tris, pH 8.0 and 1 mM EDTA) and stored at −20°C.

PCR amplification, purification and sequencing

Exon 1 of the *rbc*L gene was amplified with primers 12-34F and 799-778 R (Verbruggen *et al.*, 2007). The PCR reactions were performed in mini-tubes containing: 39.25 µl of milliQ water, 5 µl of 10 × buffer, 1.5 µl of 1.5 mM $MgCl_2$, 1 µl of 0.2 mM dNTP; 1 µl of 0.2 µM each primer; 1 µl of total DNA (~2 ng); and 0.25 µl of Taq DNA polymerase (1.25 U; Promega, Madison, WI) for a final volume of 50 µl. The reactions were performed in a Minicycler™ thermocycler (MJ Research) using the following cycling conditions: 94°C for 4 min; 35 cycles of 30 s at 94°C, 1 min at 45°C and 2 min at 72°C; followed by a final extension of 7 min at 72°C.

In order to minimize possible errors during PCR, three independent PCR reactions were performed for each DNA sample and pooled before purification (Baldwin *et al.* 1995). The products were purified in MicroSpin™ columns (Amersham Pharmacia Biotech, Buckinghamshire, UK), according to the manufacturer's instructions. The PCR products were analysed using 0.7% agar gel electrophoresis and verified by comparison

Table 1. Samples of *Codium* from Brazil used in sequencing exon 1 of *rbc*L, with information about their respective collections. Abbreviations: PEUFR: Professor Vasconcelos Sobrinho Herbarium at the Federal Rural University of Pernambuco, Brazil; GENT: Ghent University Herbarium, Ghent, Belgium.

Species	Collection site	Collector	Date	Sample	Voucher no.	GenBank accession no.
C. decorticatum	Florianópolis (Armação Beach), Santa Catarina (SC)	S.M.P.B. Guimarães	27 Nov 2005	Cdec5SC	PEUFR 48525	JQ950516
C. decorticatum	Florianópolis (Ponta das Canas Beach), SC	M.F. Oliveira-Carvalho & P. Horta.	21 Sep 2006	Cdec23SC	PEUFR 48529	JQ950515
C. decorticatum	Cabo Frio (Peró Beach), Rio de Janeiro (RJ)	V. Cassano	18 Jul 2006	Cdec16RJ	PEUFR 48533	JQ950517
C. decorticatum	Rio de Janeiro (Cavaleiros Beach), RJ	L.M. Gestinari	11 May 2006	Cdec24RJ	PEUFR 48532	JQ950518
C. decorticatum	Buzios (Forno Beach), RJ	H. Verbruggen	16 Sep 2010	HV2742	GENT HV2742	JQ950543
C. decorticatum	Buzios (Forno Beach), RJ	H. Verbruggen	16 Sep 2010	HV2757	GENT HV2757	JQ950541
C. decorticatum	Buzios (Forno Beach), RJ	H. Verbruggen	16 Sep 2010	HV2762	GENT HV2762	JQ950540
C. intertextum	Vila Velha (Baleia Beach), Espirito Santo (ES)	D. Barata	25 May 2005	Cint6ES	PEUFR 48557	JQ950521
C. intertextum	Vila Velha (Baleia Beach), ES	D. Barata	01 Jun 2006	Cint4ES	PEUFR 48558	JQ950520
C. intertextum	Canal de São Sebastião, São Paulo (SP)	M.F. Oliveira-Carvalho & F. Berchez	14 Jul 2006	Cint10SP	PEUFR 48560	JQ950519
C. intertextum	Cabo Frio (Peró Beach), RJ	V. Cassano	28 Jul 2006	Cint17RJ	PEUFR 48563	JQ950523
C. intertextum	Salvador (Stela Mares Beach), Bahia (BA)	D. Barata	15 Sep 2006	Cint18BA	PEUFR 48547	JQ950522
C. intertextum	Arquip. Fernando de Noronha (Boldró Beach), Pernambuco (PE)	P. Horta	14 Jun 2006	Cint21FN	PEUFR 48567	JQ950524
C. intertextum	Florianópolis (Gravatá Beach), SC	M.F. Oliveira-Carvalho & P. Horta	22 Sep 2006	Cint25SC	PEUFR 48554	JQ950525
C. intertextum	Buzios (Forno Beach), RJ	H. Verbruggen	16 Sep 2010	HV2741	GENT HV2741	JQ950544
C. intertextum	Buzios (Forno Beach), RJ	H. Verbruggen	16 Sep 2010	HV2767	GENT HV2767	JQ950539
C. isthmocladum	João Pessoa (Bessa Beach), Paraíba (PB)	M.F. Oliveira-Carvalho & P. Horta	01 Jun 2005	Cist11PB	PEUFR 48504	JQ950526
C. isthmocladum	Ilha de Itaparica (Mar Grande Beach), BA	M.F. Oliveira-Carvalho & M.C. Accioly	18 Sep 2005	Cist15BA	PEUFR 48498	JQ950527
C. pernambucensis	Cabo de Santo Agostinho (Paiva Beach), PE	M.F. Oliveira-Carvalho	28 Apr 2005	Crep1PE	PEUFR48573	JQ950513
C. pernambucensis	Ilha de Sto. Aleixo, PE	M.F. Oliveira-Carvalho & S.M.B. Pereira	31 Jan 2005	Crep22PE	PEUFR 48574	JQ950514
C. profundum	Vitória (costa, prof. 25 m), ES	S.M.P.B. Guimarães & G.A. Filho	09 Nov 2005	Cist3ES	PEUFR 48496	JQ950528
C. spongiosum	Búzios (Forno Beach), RJ	M.F. Oliveira-Carvalho & V. Cassano	27 Jul 2006	Cspo12RJ	PEUFR 48542	JQ950529
C. spongiosum	Arraial do Cabo (Mar Grande Beach), RJ	V. Cassano	11 Aug 2006	Cspo14RJ	PEUFR 48541	JQ950531
C. spongiosum	Vila Velha (Baleia Beach), ES	D. Barata	27 Feb 2006	Cspo13ES	PEUFR 48543	JQ950530
C. spongiosum	Buzios (Praia do Forno), RJ	H. Verbruggen	16 Sep 2010	HV2772	GENT HV2772	JQ950538
C. taylorii	Canal de São Sebastião, SP	M.F. Oliveira-Carvalho & M.M. Mosca	14 Jul 2006	Ctay2SP	PEUFR 48522	JQ950532
C. taylorii	Cabo Frio (Peró Beach), RJ	V. Cassano	28 Jul 2006	Ctay19RJ	PEUFR 48523	JQ950533
C. taylorii	Salvador (Jauá Beach), BA	M.F. Oliveira-Carvalho & M.C. Accioly	19 Nov 2005	Ctay07BA	PEUFR 48525	JQ950534
C. taylorii	Salvador (Guarajuba Beach), BA	M.F. Oliveira-Carvalho & M.C. Accioly	17 Sep 2005	Ctay09BA	PEUFR 48514	JQ950535
C. taylorii	Salvador (Itapoá Beach), BA	M.F. Oliveira-Carvalho & M.C. Accioly	17 Sep 2006	Ctay20BA	PEUFR 48513	JQ950536
C. taylorii	Buzios (Forno Beach), RJ	H. Verbruggen	16 Sep 2010	HV2750	GENT HV2750	JQ950542
C. taylorii	Buzios (Forno Beach), RJ	H. Verbruggen	16 Sep 2010	HV2781	GENT HV2781	JQ950537

to a standard Invitrogen ladder (1 kb). Sequencing reactions were performed on approximately 40 ng of the purified PCR product using the 'BigDye™ Terminator Cycle Sequencing Ready Reaction' sequencing kit of Applied Biosystems (Foster City, USA). For sequencing, the 12-34F and 799-778 R primers were used to obtain forward and reverse reads, respectively. The DNA samples were then sequenced using an ABI PRISM™ 3100 Genetic Analyzer automated sequencer (Applied Biosystems).

Alignment and tree construction

Consensus sequences of exon 1 of *rbc*L were assembled from two or more sequences generated by the primers 12-34F (F: direct) and 799-778 R (R: reverse) using the BioEdit program (Hall, 1999). The chromatograms were checked if differences were noted between the F and R sequences, and the sequencing was repeated if the conflict could not be resolved. The sequences were compared with those available in the GenBank database using the BLASTN tool (Altschul *et al*., 1990). Identical sequences among the 32 *Codium* samples were identified based on a genetic distance matrix (BioEdit) and the resulting 10 unique consensus sequences of exon 1 of *rbc*L were aligned with those on GenBank using the ClustalW program within BioEdit (Hall, 1999). Following visual inspection of the alignment, sequences corresponding to the amplification primers and the 5′ and 3′ terminal regions were removed from the alignments, generating a matrix of 48 sequences and 729 positions, without any gaps. A sequence of *Bryopsis plumosa* (GenBank accession AB038480) was included as an outgroup (Supplementary Table S1).

All phylogenetic analyses were performed with MEGA 5 (Tamura *et al*., 2011). An appropriate model of sequence evolution for maximum likelihood (ML) analysis was selected using the AICc criterion with MEGA's built-in model testing suite. A ML tree was inferred using the selected GTR + G + I model using nearest neighbour interchange tree rearrangements. A neighbour-joining (NJ) distance-based tree was constructed (Saitou & Nei, 1987) using a Tamura–Nei distance matrix. A maximum parsimony (MP) tree was inferred by a heuristic search. Bootstrap resampling was carried out with 100 replicates for ML and 1000 replicates for NJ and MP (Felsenstein, 1985). All analyses were run once with only *Codium* sequences and a second time with the *Bryopsis* outgroup sequence. Two additional distance-based trees were generated to illustrate species delimitation in one of the focal clades. These analyses included 10 *rbc*L exon 1 sequences, listed in the results, and were carried out by NJ and UPGMA clustering based on Tamura–Nei distance matrices with 1000 bootstrap replicates. In our interpretations, bootstrap values up to 70% were considered low, from 71% to 90% moderate, and above 90% high for all analyses.

Results

The sequences obtained for the *rbc*L exon 1 of 32 *Codium* samples collected along the coast of Brazil yielded 10 distinct haplotypes. Two samples of *C. decorticatum* obtained from Santa Catarina state and five from Rio de Janeiro State had identical sequences. The same situation was observed with the nine samples of *C. intertextum* collected along the Brazilian coast from Santa Catarina State to Bahia, and a specimen from the island of Fernando de Noronha. Four samples of *C. spongiosum* collected off the southeastern coast of Brazil yielded identical sequences. Seven samples of *C. taylorii* demonstrated a divergence of no more than four nucleotides. The two samples of *C. isthmocladum* from Bahia and Paraíba presented a single nucleotide divergence. A single sequence was obtained for *C. profundum*, and the two samples of a sprawling species from Pernambuco State that could not be readily identified yielded two identical sequences.

Initial analyses of 126 *rbc*L sequences including ours and those available on GenBank allowed the selection of 39 sequences representative of the principal lineages and the elimination of those that were very short or represented long branches that might distort the tree (Holland *et al*., 2003). Sequences of species related to our Brazilian samples were retained and the resulting matrix of 48 sequences was subjected to phylogenetic analysis, resulting in the ML tree presented in Fig. 1. The different analysis methods yielded similar trees that differed mainly in poorly supported regions. Because the analysis with *Bryopsis* as the outgroup rooted the tree along the branch towards *C. cranwelliae* + *C. megalophysum*, which may be a long-branch attraction artefact (see Discussion), we chose to present the midpoint-rooted tree inferred from *Codium* species only. The root position in this midpoint-rooted tree corresponds to the root position inferred with the molecular clock method by Verbruggen *et al*. (2007), and as such our interpretations are consistent with that paper.

The main monophyletic groupings were constant in all of the analyses performed. Clade A (Fig. 1) presented high bootstrap values (100%) in all analyses, and included two species from Brazil: *C. spongiosum*, which grouped with another sample of this species in all the analyses (97% support), and *C. intertextum*, which grouped with another specimen of the same species from Jamaica (91–95% support). In both these cases, the molecular data confirmed the species identification based on morphological characters: *C. spongiosum* had weakly adhering pulvinate thalli with utricles longer than 1000 µm and *C. intertextum* had strongly adhering crustose thalli with utricles shorter than 1000 µm (Oliveira-Carvalho *et al*., 2010). Other taxa, such as *C. arabicum*, *C. convolutum*, *C. hubsii* and *C. lucasii*, were also included in Clade A.

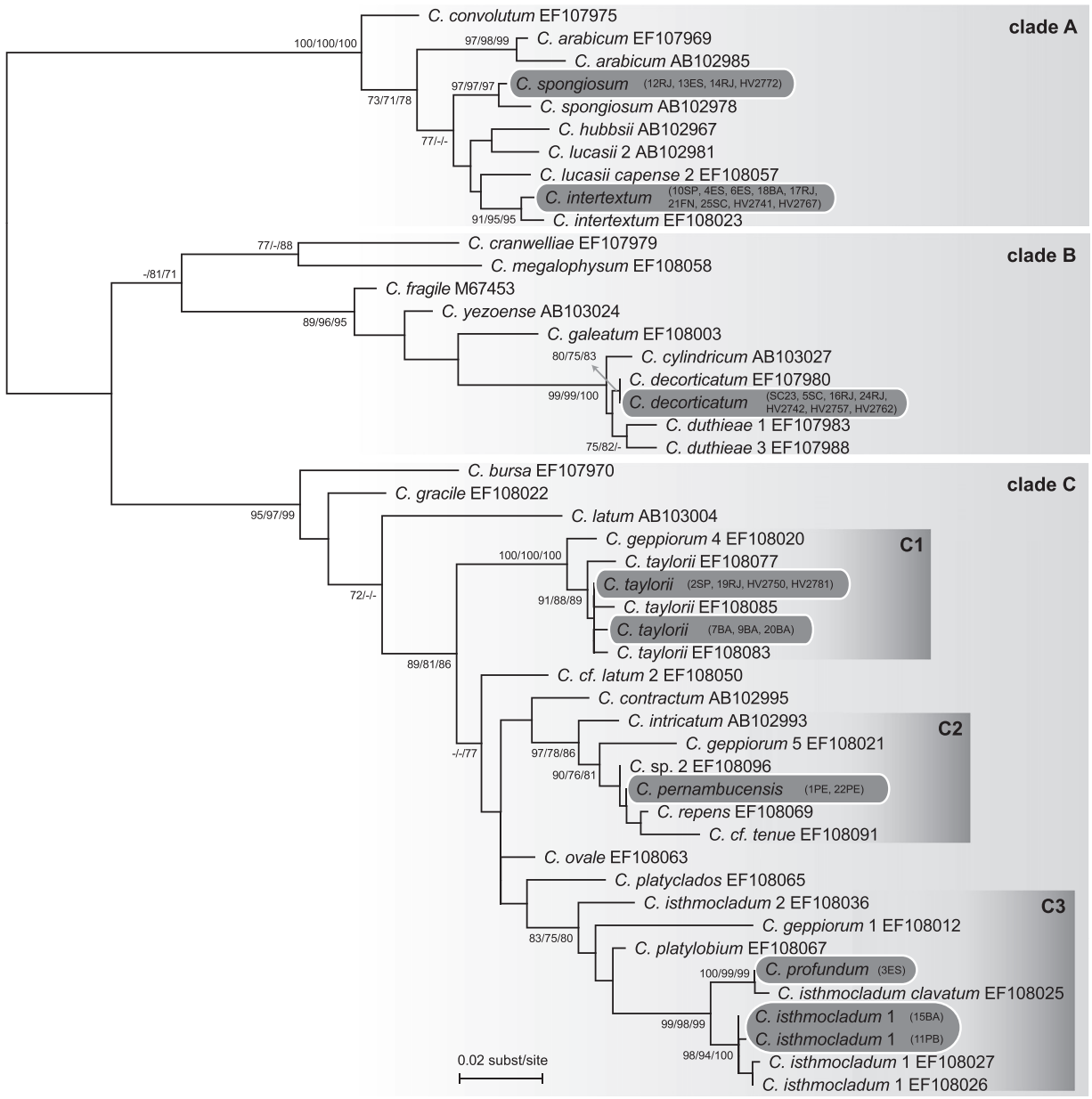


Fig. 1. The maximum likelihood (ML) tree constructed from an alignment of *rbc*L exon 1 sequences for *Codium*. Bootstrap values indicated on the branches are for ML/MP/NJ analyses, with only values > 70% being shown. The Brazilian samples whose sequences were determined in the present work are in grey boxes. The accession numbers for samples obtained from GenBank are indicated, and more details can be found in Table S1.

Another monophyletic grouping (Clade B) that had moderate to high bootstrap values in all analyses included our sequences of *C. decorticatum*, which grouped with another sample of the same taxa from the Atlantic coast of the USA. The upright branched thalli of *C. decorticatum* were characterized by wedge-shaped flattened dichotomies and had larger, broader utricles compared with other branched species (Oliveira-Carvalho *et al.*, 2010). Clade B also included *C. cylindricum*, *C. duthieae*, *C. fragile*, *C. galeatum* and *C. yezoense*.

A third monophyletic grouping (Clade C) was recovered in all of the analyses, with bootstrap support of 95–99%. Clade C was unresolved at its base, including the taxa *C. bursa*, *C. gracile*, *C. latum* and a large subclade containing the remainder of the species. Within this subclade, another unresolved set of lineages presented itself, including *C. ovale*, *C. contractum*, *C.* cf. *latum* and *C. platyclados* and two larger groups (Fig. 1).

Brazilian specimens were recovered in three of the monophyletic subclades (C1, C2 and C3) within Clade C. It should be noted that, while the three major clades (A, B, C) correspond to those in Verbruggen *et al.* (2007), the subclades recognized here are defined differently. The C1 subclade had bootstrap support of 90–100% in all of the analyses and included *C. taylorii* from Brazil (characterized by irregularly flattened and

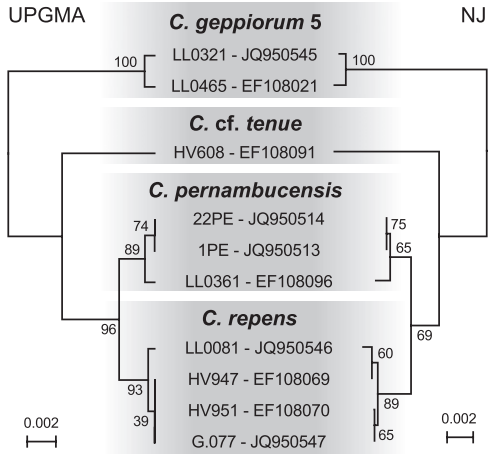


Fig. 2. Distance-based clustering trees of sequences in clade C2, illustrating the clear differences between the species *Codium geppiorum* 5, *C.* cf. *tenue*, *C. pernambucensis* and *C. repens*.

irregularly branched thalli), which grouped with the other samples of the same species from the Caribbean region and Florida (USA); the C1 subclade also included the species *C. geppiorum* 4 (EF108020). The C2 subclade included a Brazilian *Codium* species, to be described below as *C. pernambucensis*, which grouped with a specimen of uncertain affinities (*Codium* sp. 2: EF108096) and *C. repens* (both from the Caribbean region), as well as *C.* cf. *tenue* (from the Philippines). Other species included in subclade C2 are *C. geppiorum* 5 from Belize and *C. intricatum* from Japan. A detailed analysis of subclade C2 with several additional specimens (Fig. 2) shows that the *Codium* sp. 2 sequence (LL0361 = EF108096) barely differs from the Brazilian *C. pernambucensis* samples and forms a tight cluster with it, suggesting that they are conspecific. The C3 subclade had bootstrap support of 75–83% and included two Brazilian species: *C. profundum*, which clustered with another sample from Anguilla (EF108025, 99–100% support), and *C. isthmocladum* 1, whose two Brazilian haplotypes grouped with other samples of the same species from the Caribbean region (94–100% bootstrap). *Codium profundum* and *C. isthmocladum* 1 are both upright, branched species; while *C. isthmocladum* has regularly dichotomously branching cylindrical branches and thickened utricle apices, the branches of *C. profundum* are more subcylindrical and irregularly branched, and its utricles have thin apices. Other species in Clade C3 were *C. platylobium*, *C. geppiorum* 1 and *C. isthmocladum* 2.

Discussion

A previous study on the molecular phylogenetics and evolution of *Codium* (Verbruggen *et al.* 2007) concluded that intensive analyses of regional *Codium* diversity, based on detailed morphological observations combined with analysis of molecular markers, are needed to refine our knowledge of the taxonomy and distribution of *Codium* species. Such studies would be particularly valuable for the Southern Atlantic Ocean, as previous work had included very few species from South America and not a single species had been collected in Brazil. In combination with a recently published morphological treatment (Oliveira-Carvalho *et al.*, 2010), the present work yields a detailed regional study for the warm-temperate to tropical coasts of the Western South Atlantic, spanning the Brazilian coast between the states of Santa Catarina and Paraíba (29° 15′ 59.43″ S, 49° 41′ 41.20″ W to 6° 29′ 11.36″ S, 34° 58′ 05.08″ W). Besides advancing knowledge of *Codium* species diversity in Brazil, our results show the utility of molecular data for species identification and delimitation, lead to proposals for revising the taxonomy of the genus, and yield insight into the phylogenetic distribution of morphological types of *Codium*.

Species identification and delimitation

Identification of *Codium* specimens is currently based primarily on morphological characters and can be troublesome due to the large number of species already described in the genus, the considerable phenotypic plasticity that they exhibit, and the limited knowledge of their geographical distribution (Silva, 1951; Pedroche *et al.*, 2002; Verbruggen *et al.*, 2007). Various authors have therefore proposed to use DNA barcodes as a complementary method for species identification and taxonomy in the genus (Shimada *et al.*, 2004; Maggs & Kelly, 2007; Verbruggen *et al.*, 2007). Molecular sequences of exon 1 of the *rbc*L gene have proven useful for the identification and delineation of species, and have provided the basis for an important first step towards resolving phylogenetic relationships among species. The phylogenetic framework has subsequently been used to evaluate the utility of morphological characters traditionally used for species identification, the validity of biogeographical hypotheses, the occurrence of cryptic species, and the identification of invasive species (Maggs & Kelly, 2007; Verbruggen *et al.*, 2007).

In the present study, fairly good correspondence was found between identifications based on morphology (presented by Oliveira-Carvalho *et al.*, 2010) and those from molecular sequence data: for *C. decorticatum*, *C. intertextum*, *C. isthmocladum* 1, *C. spongiosum* and *C. taylorii* the morphological identifications were unambiguously confirmed by the *rbc*L sequence data. For two

other species, however, there were conflicts between the molecular and morphological data that warrant further discussion. First, our specimen of *C. profundum* clustered with a specimen (EF108025) from Anguilla (Lesser Antilles) and several other published sequences from Caribbean material. These samples were identified as *C. isthmocladum* subsp. *clavatum* by Verbruggen *et al.* (2007). Given the close similarity of our *C. profundum* sequence to the *C. isthmocladum* subsp. *clavatum* sequences, it seems safe to assume that these samples are conspecific. These results could be interpreted in two ways. First, it could be that the conflict between identifications is simply a result of recent changes in the taxonomy of the species in question. The epithet *profundum* was first used in 2003 for deep-water specimens from Bermuda, Florida, the Gulf of Mexico, Brazil, and the Canary Islands (Chacana *et al.*, 2003). The entity remained a *nomen nudum* until it was validated in a recently published note (Silva & Chacana, 2010). It was already known before our study that *C. profundum* and *C. isthmocladum* subsp. *clavatum* are morphologically similar, as evidenced by Chacana *et al.*'s (2003) comment that 'At one time during the development of our concept of *C. profundum* we considered this taxon to be closely related to *C. isthmocladum* subsp. *clavatum* [. . .]. Pertinent molecular data, however, are not yet available'. So, our molecular results could be interpreted as evidence that *C. profundum* is indeed conspecific with *C. isthmocladum* subsp. *clavatum* and that the latter should be considered a synonym of *C. profundum*. However, a second possible interpretation needs to be considered. Verbruggen *et al.* (2007) showed that *C. isthmocladum* is a complex of at least three distinct species, which are all included in subclade C3 of our phylogeny (*C. isthmocladum* 1, *C. isthmocladum* 2 and *C. isthmocladum* subsp. *clavatum*). It is not known in sufficient detail how the morphologies of these three entities relate to one another and to *C. profundum*. The fact that the three entities were identified as *C. isthmocladum* suggests that they are morphologically closely related, but it is not known whether the morphologies overlap completely (cryptic species) or whether the species can be separated on morphological grounds once the right morphological features have been identified (pseudocryptic species). Because of this uncertainty, we consider that no firm taxonomic conclusions should be drawn until molecular sequences are obtained for the type of *C. profundum*.

The second case where molecular and morphological data did not match up unambiguously concerns the entity from Pernambuco that could not readily be identified. This is included in our phylogeny as *C. pernambucensis*, in subclade C2. Although the ML phylogeny in Fig. 1 may suggest otherwise, the sequence of the entity from Pernambuco is nearly identical to a previously published sequence of *Codium* sp. 2 (EF108096). These two sequences differ by a single nucleotide and we postulate that the reason that these two closely related haplotypes do not form a monophyletic cluster in the ML phylogram is that this analysis uses an unconstrained model of sequence evolution in which rates and times are not distinguished. In such analyses, if sequences are considered to be similar or identical to hypothetical ancestral sequences, the resulting trees will show these sequences sitting on an internal node with a very short branch length. In our specific case, the two very similar sequences (EF108096 and *C. pernambucensis*) are apparently both inferred to be close or identical to hypothetical ancestral sequences, with the result that these two sequences are recovered as a paraphyletic assemblage with respect to the *C. repens* + *C.* cf. *tenue* clade. Trees based on distance analyses (UPGMA and NJ) of a broader set of samples in subclade C2 clearly show that the two samples cluster together (Fig. 2). In combination with the morphological similarities between them, the two should be considered to be conspecific. *Codium* sp. 2 was one of the species-level entities included in the study of Verbruggen *et al.* (2007) that could not be identified to the species level. The entangled habit and branch thickness of *Codium* sp. 2 fit the description of *Codium edule*, but this species is only known from the Indo-Pacific region. The Atlantic counterpart is *C. repens*, but this is distinct in its DNA sequences (Fig. 2). Both *C. edule* and *C. repens* are part of the '*geppiorum* complex', which is characterized by procumbent thalli with intertwined anastomosing branches (Silva, 1960; Jones & Kraft, 1984; Chang *et al.*, 2002) and is known to harbour high numbers of unrecognized species-level clusters (Verbruggen *et al.*, 2007). Based on the morphological data presented by Oliveira-Carvalho *et al.* (2010) and the molecular data presented here, we are of the opinion that the entity from Pernambuco deserves recognition at the species level, and we provide a formal description for the new species *C. pernambucensis* at the end of the manuscript.

Our analyses indicated that members of the '*geppiorum* complex', including *C. repens* and *C. pernambucensis* are widely distributed in clade C, being present in all three subclades (C1, C2 and C3). *Codium geppiorum* is traditionally thought to be an Indo-Pacific species that is taxonomically close to *C. repens* from the Atlantic region (Silva, 1960; Jones & Kraft, 1984; Chang *et al.*, 2002). According to Chang *et al.* (2002), the taxa of the '*geppiorum* complex' remain poorly delimited, and they are frequently confused due to the size

continuum of their utricles and of other morphological characters. Our molecular results confirm that the present concept of *C. geppiorum* is untenable, and it is likely that the characterization of the various genetic lineages within this morphospecies will be one of the toughest challenges facing *Codium* taxonomists. With the description of *C. pernambucensis* we take a first step towards resolving the problem but at the same time realize that most of the work still lies ahead and will require a more global scope.

A discussion of species delimitation based on DNA sequences also warrants a brief treatment of the usefulness of different DNA markers for this purpose. So far, only the first exon of *rbc*L has been used for species delimitation in *Codium* (Shimada *et al*., 2004, 2007; Verbruggen *et al*., 2007). While the initial analysis of Shimada *et al*. (2004) indicated *rbc*L to have great promise as a DNA barcoding marker, subsequent analyses showed that it may be too slowly evolving to distinguish between recently diverged species, which are particularly found in Clade C (Verbruggen *et al*., 2007). This is also confirmed in the present study, as the low sequence divergences among species in subclade C2 made the interpretation of species boundaries difficult. While the distance analyses in Fig. 2 show a clear distinction between *C. pernambucensis*, *C. repens* and *C.* cf. *tenue*, this is not clear in the ML analysis in Fig. 1, where the two *C. pernambucensis* sequences (one as *Codium* sp. 2) do not form a cluster. Although this is not a major obstacle for our interpretations, it would be preferable to use a faster evolving, more variable marker. Recent experiments carried out by the DNA barcoding community have shown that *tuf*A is more variable than *rbc*L and that this gene should be chosen as the green algal DNA barcode (Saunders & Kucera, 2010). As more comprehensive reference datasets for this gene are being built, we contribute a *tuf*A sequence of the new species *C. pernambucensis* for future comparisons via GenBank (JQ966947).

In summary, species identifications based on morphological characters were accurate for most Brazilian species, but problems remain in the '*isthmocladum* complex'. Although we were able to distinguish the Brazilian species *C. pernambucensis* from similar species in the '*geppiorum* complex', this complex remains to be resolved at the global scale.

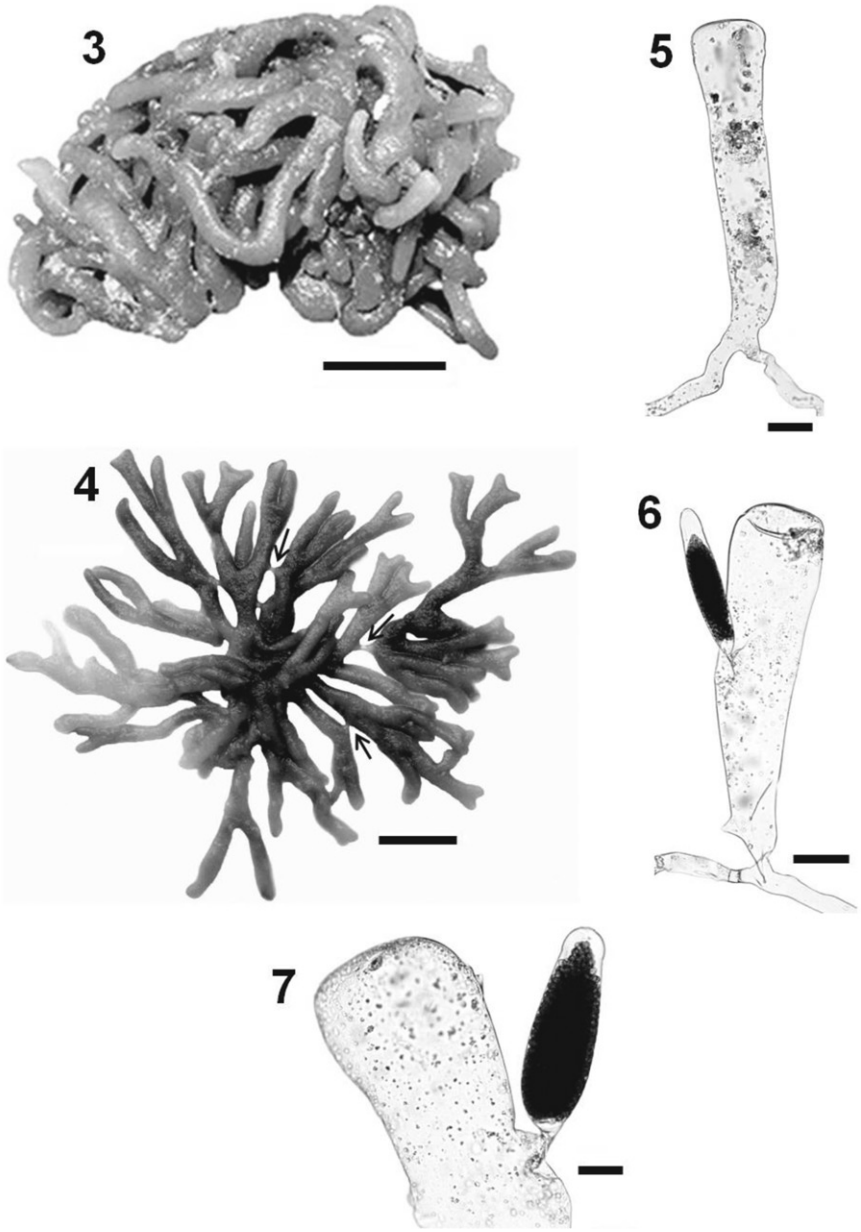
Oliveira-Carvalho *et al*. (2010) have recently provided a morphological key and comparative table, which were also used to identify the samples sequenced in this study. Not surprisingly, therefore, the key and table correspond well with the molecular work presented here, with the exception that the species called '*Codium* sp.' in Oliveira-Carvalho *et al*. (2010) is now described as *C. pernambucensis*.

Phylogenetic considerations

Our molecular phylogenies, inferred with *Bryopsis* as an outgroup, recovered the root of *Codium* within Clade B, more precisely along the branch joining *C. cranwelliae* and *C. megalophysum*. A similar result was previously obtained by Verbruggen *et al*. (2007), who provide an in-depth analysis of the problems concerning the root of the *Codium* phylogeny. Our analyses suggest that, despite our efforts to reduce the potential for long branch attraction by removing some short sequences and sequences producing long branches in preliminary analyses, the problem remains. It appears that, despite the fact that *Bryopsis* is solidly resolved as the sister genus of *Codium* (Woolcott *et al*., 2000; Lam & Zechman, 2006; Verbruggen *et al*., 2009), it is too distant to accurately root the tree. Future studies may improve the situation by including additional *Bryopsis* sequences, thereby breaking up the long branch towards the outgroup (Smith, 1994; Shavit *et al*., 2007; Verbruggen & Theriot, 2008). For now, we have presented a midpoint-rooted tree consistent with the tree presented by Verbruggen *et al*. (2007), where the oldest branch was determined by a molecular clock model.

The molecular phylogeny can be subdivided into three principal lineages. Clade A is represented by species with a prostrate habit, without upright branched parts. The utricles encountered in the species of this clade are generally grouped and narrow. Clade B consists of species with an upright habit, generally cylindrical branches and dichotomous branching, although the early-branching *C. cranwelliae* and *C. megalophysum* have spherical thalli. The species in this group have large individual utricles. Clade C is largely composed of branched species with cylindrical or slightly flattened branches and individual utricles of intermediate size. It has a lot of thallus shape variation, including the sprawling species that comprise the '*geppiorum* complex'.

The Brazilian samples appear in all three of the principal lineages in the phylogeny, so despite the relatively low species diversity in the country, these species represent diverse phylogenetic lineages. The three major clades include species from the world's three main oceans (Atlantic, Pacific and Indian). By far the largest global diversity of *Codium* species is found in the Indo-Pacific region, and Atlantic species are mostly found in clades derived from Indo-Pacific ancestry, indicating an Indo-Pacific origin and diversification of the genus, with the dispersal of species to the Atlantic



Figs 3–7. *Codium pernambucensis*. **3.** General aspect of the plant freshly collected from the intertidal zone of Santo Aleixo Island, Pernambuco State. Thallus procumbent, tufts densely interwoven, similar to balls. **4.** General aspect of the thallus after fixation. Anastomosed branches (arrow) restricted to the median portion of thallus. **5.** Individual, clavate utricle with slightly subtruncate apex. **6.** Reproductive clavate utricle. **7.** Tip of the utricle, showing gametangium and hair scars. Figures 3 and 4 are reproduced from Oliveira-Carvalho *et al*. (2010) with the permission of the publishers (www.borntraeger-cramer.de). Scale bars = 3 cm (Fig. 3), 2 cm (Fig. 4), 100 μm (Figs 5, 6) and 50 μm (Fig. 7).

region on different occasions (Verbruggen *et al*., 2007). However, some species, such as *C. decorticatum*, *C. intertextum, C. isthmocladum* and *C. pernambucensis*, seem to have remained restricted to the Atlantic Ocean. On the other hand, *C. spongiosum* and *C. taylorii* appear to be cosmopolitan species present in both the Atlantic and Indo-Pacific regions.

Taxonomic treatment

As discussed above, the molecular and morphological features of the *Codium* entity from Pernambuco State warrants its description as a new species. The entity was described in detail (as *Codium* sp.) by Oliveira-Carvalho *et al*. (2010) and we will restrict ourselves to the formal description and a summary of the main features.

Codium pernambucensis *Oliveira-Carvalho & S.M.B. Pereira, sp. nov.*

(Figs 3–7)

Description: Thallus sprawling, light green, with dichotomous or unequal branching. Branches

subcylindrical or slightly flattened. The thalli are characterized by the exceptional prevalence of anastomosis between branches, especially in the median region of the thallus, forming densely tangled tufts similar to balls of wool yarn are fixed to the substratum by several rhizoidal tufts. Medullary filaments 15–40 (23 ± 8) μm in diameter. Utricles club-shaped, subcylindrical, rarely pear-shaped, 100–280 (197 ± 50) μm in diameter and 610–900 (727 ± 78) μm long. Utricle apices rounded or somewhat truncated, with thin apical walls 5–10 (7 ± 1) μm thick. The utricles bear two to four hairs or hair scars per utricle 70–90 (82 ± 8) μm below the apex of the utricle. When present, one or two gametangia have been observed per utricle. They are lanceolate-ovoid to fusiform, 60–130 (97 ± 22) μm in diameter and 260–330 (285 ± 23) μm long, borne on a short pedicel 290–340 (304 ± 13) μm below the apex of the utricle. Reference sequences for molecular identification are JQ950513, JQ950514 and EF107947 (*rbc*L exon 1) and JQ966947 (*tuf*A).

Holotype: PEUFR 48574; Coll. S.M.B. Pereira & M.F. Oliveira-Carvalho, 31 January 2006.

Specimens examined: PEUFR 48191; Coll. M.F. Oliveira-Carvalho & G.M.P. Dias, 25 December 2004. PEUFR 48575; Coll. S.M.B. Pereira & M.F. Oliveira-Carvalho, 25 May 2005.

Type locality: Ilha de Santo Aleixo (08° 36′ S and 35° 01′ E), Serinhaém, Pernambuco, Brazil.

Etymology: The species epithet '*pernambucensis*' refers to the Brazilian State Pernambuco, where this new species was collected.

Acknowledgements

The authors would like to acknowledge funding provided by the Fundação de Amparo à Ciências e Tecnologia do Estado de Pernambuco (FACEPE–Projeto Pronex), Fundação de Amparo a Pesquisa do estado de São Paulo (FAPESP), Conselho Nacional do Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior–CAPES (doctoral fellowship to first author), the Research Foundation Flanders (grant G.0142.05 and postdoctoral fellowship to HV), and the Australian Research Council (FT110100585). We sincerely thank all collectors listed in Table 1 as well as Mark and Diane Littler (Smithsonian Institution) and Suzanne Fredericq (University of Louisiana Lafayette) for providing samples, Silvia R. Blanco and Rosário Petti (University de São Paulo), and Sofie D'hondt (Ghent University) for technical help.

Supplementary material

The following supplementary material is available via the Supplementary Content tab of the article's online page at http://dx.doi.org/10.1080/09670262.2012.718363

Supplementary Table S1. Sequences of the exon 1 of *rbc*L of *Codium* and *Bryopsis* obtained from the GenBank database, with their respective access numbers, collection localities, and references.

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