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Mesophyllum erubescens (Corallinales, Rhodophyta)—so many species in one epithet

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Abstract

The name *Mesophyllum erubescens* has been applied to protuberant rhodolith specimens which sometimes occur abundantly, as well as to encrusting specimens in tropical and temperate waters in the Western Pacific, Indian and Western Atlantic Oceans. A DNA sequence, representing about 20% of the *rbc*L gene, was obtained from the 140 year old holotype specimen collected in the Fernando de Noronha Archipelago by the Challenger Expedition. This sequence was identical to field-collected topotype specimens as well as to specimens ranging south along the coast of Brazil. Sequences for *psb*A from these same Brazilian specimens and specimens from the east coast of Mexico were identical or differed by 1 base pair. In contrast, specimens called *M. erubescens* based on morpho-anatomical characters in the Pacific Ocean differed from Western Atlantic Ocean specimens by 2.5–13.1%, indicating that these represent numerous distinct species. All reports of non-geniculate coralline species said to be widely distributed across different oceans or in different biogeographic provinces based on morpho-anatomical characters need to be verified by DNA sequences.

Keywords: Cryptic species, DNA Barcoding, Hapalidiaceae

Introduction

Lemoine (1928) proposed *Mesophyllum* Me. Lemoine (1928: 251) for non-geniculate coralline species with a coaxial arrangement (cells of adjacent filaments arranged in arching tiers) of medullary cells. This generic characterization was accepted for 60 years until Woelkerling (1988) examined its validity in *Mesophyllum*, *Clathromorphum* Foslie (1898: 4) and *Synarthrophyton* R.A. Townsend (1979: 252), pointing out that all three genera included species that lacked the coaxial arrangement and that *Mesophyllum* and *Synarthrophyton* contained species that had both coaxial and non-coaxial areas of the thallus. Woelkerling and Harvey (1992) therefore proposed using the ontogeny and anatomy of spermatangial conceptacles to delimit these genera as follows: 1) in *Mesophyllum* and *Clathromorphum* spermatangia form on unbranched filaments, whereas in *Synarthrophyton* both branched and unbranched filaments bear spermatangia; 2) in *Mesophyllum* and *Synarthrophyton*, protective cells overlay spermatangial initials during early stages of development, unknown in the generitype species of *Clathromorphum*; and 3) spermatangial conceptacle roof development is centripetal in *Mesophyllum* and *Synarthrophyton*, but vertical in *Clathromorphum*. Athanasiadis *et al.* (2004) proposed a different suite of characters to distinguish *Mesophyllum* from other Melobesioideae genera, particularly *Leptophytum* W.H. Adey (1966: 323) and *Synarthrophyton* including: 1) the historical character of a predominantly co-axial hypothallus, 2) unbranched filaments bearing spermatangia (Woelkerling & Harvey 1992), and 3) a new character, the dumbbell-shaped carposporangial conceptacles. Thus, we have different suites of morpho-anatomical characters proposed to distinguish *Mesophyllum* from related genera in Hapalidiaceae.

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TABLE 1. List of specimens used in molecular analyses including herbarium voucher numbers, collection data and sequenced markers with corresponding GenBank accession numbers; – indicates marker was not sequenced.

Voucher		Location	Date	Collectors	*GenBank* accession numbers		
					UPA	*rbc*L	*psb*A
TRH C15-3212	*Mesophyllum erubescens*	Fernando de Noronha, Brazil	August/September 1873	H.N. Ridley, T.S. Lea & G.A. Ramage	-	KP050698	-
FBCS 12791	*Mesophyllum erubescens*	Santiaguillo/VER, Mexico	16 October 2012	P. Avila	-	KP085636	KM983032
FBCS 12795	*Mesophyllum erubescens*	Santiaguillo/VER, Mexico	16 October 2012	P. Avila	-	KP085637	KM983033
FBCS 12872	*Mesophyllum erubescens*	Topatillo/VER, Mexico	17 October 2012	P. Avila	-	KP085638	KM983034
FLOR 14895	*Mesophyllum erubescens*	Risca do Meio/CE, Brazil	18 April 2012	M. Sissini	KM877270	-	-
FLOR 14896	*Mesophyllum erubescens*	Fernando de Noronha, Brazil	08 August 2013	P. Horta	KM877271	-	KM983035
FLOR 14897	*Mesophyllum erubescens*	Fernando de Noronha, Brazil	08 August 2013	P. Horta	KM877272	-	-
FLOR 14898	*Mesophyllum erubescens*	Fernando de Noronha, Brazil	21 November 2011	F. Schener & D. Burgos	KM877273	KP085623	KM983036
FLOR 14899	*Mesophyllum erubescens*	Camaçari/BA, Brazil	23 May 2013	B. Torrano, C. Azevedo & T. Vieira-Pinto	KM877274	KP085624	KM983037
FLOR 14900	*Mesophyllum erubescens*	Rasas Island/ES, Brazil	29 March 2012	M. Sissini	KM877275	KP085625	KM983038
FLOR 14901	*Mesophyllum erubescens*	Rasas Island/ES, Brazil	29 March 2012	M. Sissini	KM877276	-	KM983039
FLOR 14902	*Mesophyllum erubescens*	Rasas Island/ES, Brazil	29 March 2012	M. Sissini	KM877277	-	KM983040
FLOR 14903	*Mesophyllum erubescens*	Escalvada Island/ES, Brazil	30 March 2012	E. Mazzei & H. Pinheiro	KM877278	-	-
FLOR 14904	*Mesophyllum erubescens*	Rasas Island/ES, Brazil	29 March 2012	M. Sissini	KM877279	-	-
FLOR 14905	*Mesophyllum erubescens*	Trindade Island, Brazil	16 June 2012	M. Sissini	KM877280	-	KM983041
FLOR 14906	*Mesophyllum erubescens*	Trindade Island, Brazil	25 June 2012	M. Sissini	KM877281	-	-
FLOR 14907	*Mesophyllum erubescens*	Trindade Island, Brazil	06 August 2012	M. Sissini	KM877282	-	-
FLOR 14908	*Mesophyllum erubescens*	Trindade Island, Brazil	25 August 2012	M. Sissini	KM877283	-	-
FLOR 14909	*Mesophyllum erubescens*	Trindade Island, Brazil	24 July 2012	M. Sissini	KM877284	KP085626	KM983042
FLOR 14910	*Mesophyllum erubescens*	Ilhabela/SP, Brazil	02 February 2012	P. Horta	KM877285	-	-
FLOR 14911	*Mesophyllum erubescens*	Queimada Grande Island/SP, Brazil	19 April 2011	M. Sissini & B. Torrano-Silva	KM877286	KP085627	-
FLOR 14912	*Mesophyllum erubescens*	Queimada Grande Island/SP, Brazil	19 April 2011	M. Sissini & B. Torrano-Silva	KM877287	-	-
FLOR 14913	*Mesophyllum erubescens*	Queimada Grande Island/SP, Brazil	19 April 2011	M. Sissini & B. Torrano-Silva	KM877288	-	-
FLOR 14915	*Mesophyllum erubescens*	Ilhabela/SP, Brazil	02 February 2012	P. Horta	KM877289	-	-
FLOR 14916	*Mesophyllum erubescens*	Ilhabela/SP, Brazil	02 February 2012	P. Horta	KM877290	-	KM983043

…continued on the next page

TABLE 1. continued

Voucher		Location	Date	Collectors	*GenBank* accession numbers		
					UPA	*rbc*L	*psb*A
FLOR 14917	*Mesophyllum erubescens*	Ilhabela/SP, Brazil	02 February 2012	P. Horta	KM877291	KP085628	KM983044
FLOR 14918	*Mesophyllum erubescens*	Palmas Island/SP, Brazil	29 August 2011	B. Torrano-Silva, A. Medeiros & C. Iha	KM877292	-	-
FLOR 14919	*Mesophyllum erubescens*	Arvoredo Island/SC, Brazil	28 November 2012	M. Sissini & L. Ferreira	KM877293	-	KM983045
FLOR 14920	*Mesophyllum erubescens*	Arvoredo Island/SC, Brazil	28 November 2012	M. Sissini & L. Ferreira	KM877294	-	KM983046
FLOR 14921	*Mesophyllum erubescens*	Arvoredo Island/SC, Brazil	28 November 2012	M. Sissini & L. Ferreira	KM877295	-	-
FLOR 14922	*Mesophyllum erubescens*	Arvoredo Island/SC, Brazil	28 November 2012	M. Sissini & L. Ferreira	KM877296	-	-
FLOR 14923	*Mesophyllum erubescens*	Arvoredo Island/SC, Brazil	28 November 2012	M. Sissini & L. Ferreira	KM877297	-	-
FLOR 14924	*Mesophyllum erubescens*	Deserta Island/SC, Brazil	04 August 2011	E. Bastos	KM877298	KP085629	KM983047
FLOR 14925	Unidentified Hapalidiaceae	Risca do Meio/CE, Brazil	18 April 2012	M. Sissini	KM877299	KP085630	-
FLOR 14926	Unidentified Hapalidiaceae	Arvoredo Island/SC, Brazil	28 November 2012	M. Sissini & L. Ferreira	KM877300	KP085631	-
FLOR 14927	Unidentified Hapalidiaceae	Papagaio Island/RJ, Brazil	27 January 2013	M. Sissini & C. Martins	KM877301	KP085632	-
FLOR 14928	Unidentified Hapalidiaceae	Arvoredo Island/SC, Brazil	28 November 2012	M. Sissini & L. Ferreira	KM877302	KP085633	-
FLOR 14929	Unidentified Hapalidiaceae	Arvoredo Island/SC, Brazil	28 November 2012	M. Sissini & L. Ferreira	KM877303	KP085634	-
FLOR 14930	Unidentified Hapalidiaceae	João Pessoa/PB, Brazil	09 April 2012	L. Lucena	KM877304	KP085635	-

Fifty species are currently recognized as belonging to *Mesophyllum* (Guiry & Guiry 2014). Among the eight species reported from the tropical and subtropical Western Atlantic Ocean (Wynne 2011), four were described in detail (Athanasiadis 1999, Nunes *et al*. 2008, Farias 2009). Among them, *M. erubescens* (Foslie 1900b: 9) Me. Lemoine (1928: 252) is the species most studied in this region (Figueiredo & Steneck 2000, Nunes *et al*. 2008, Bahia *et al*. 2010, Horta *et al*. 2011), being unquestionably ecologically important (Horta *et al*. 2008, Scherner *et al*. 2010, Pascelli *et al*. 2013) and under threat due to growing exploitation of South Atlantic rhodolith beds (Riul *et al*. 2008) among other stressors including coastal pollution and climate change (Wilson *et al*. 2004, Turra *et al*. 2013).

Mesophyllum erubescens was originally described from Fernando de Noronha Archipelago (Foslie 1900b, as *Lithothamnion erubescens*) located 600 km off the northeast coast of Brazil. More than one century after the species was described, it was studied along the subtropical Brazilian coast and in the Caribbean Sea (Horta *et al*. 2011). From a molecular perspective, material under that name in the Western Pacific had its plastid (*psb*A) and nuclear (SSU) DNA characterized (Broom *et al*. 2008, Kato *et al*. 2011, Bittner *et al*. 2011) with Brazilian material characterized by a single SSU rDNA sequence (Bailey & Chapman 1996). The Brazilian sequence, however, was from São Sebastião area (São Paulo state), within the South Atlantic Warm Temperate Province (Horta *et al*. 2001), 2700 km distant from Fernando de Noronha, a typical tropical environment. None of these studies included DNA sequence data from type or topotype material.

Herein, we characterize by *rbc*L sequence the holotype of *Lithothamnion erubescens* Foslie (1900b: 4), basionym of *M. erubescens*, and compare that sequence with sequences from topotype specimens and specimens from along the Brazilian coast morpho-anatomically identified as *M. erubescens*. Using primarily UPA and *psb*A sequences from these same field-collected specimens whose *rbc*L sequences matched type material, we show that all specimens outside the tropical Atlantic morpho-anatomically identified as *M. erubescens*, except two from Hawai'i, do not belong to this species. The implications of these results for phylogeography and conservation of other non-geniculate coralline species said to be widely distributed in different ocean basins or in different biogeographic provinces are discussed.

Materials and methods

Study area:—Specimens were collected by SCUBA diving in 12 locations along the Brazilian coast and in two oceanic islands, Fernando de Noronha and Trindade. Three specimens originating from two localities in Veracruz (Santiaguillo and Topatillo reefs), Mexico (SW Gulf of Mexico) were also included (Fig. 1).

Specimens:—Immediately after collection, specimens were cleaned to remove epiphytes and associated fauna, and stored appropriately for morphological and molecular studies. For molecular analyses, material was dried in the shade and stored in silica gel. Specimen data including field codes, locations of sampling and sequenced markers are in Table 1. For morphological analysis, material was fixed in 4% formaldehyde and transferred after 24 hours to a solution of ethanol (70%) and glycerol (10%). Specimens were deposited in the Herbarium FLOR of Federal University of Santa Catarina (Brazil) and in the Herbarium FBCS at the University of Baja California Sur (Mexico).

DNA extraction, amplification and sequencing of fresh material:—Small pieces of calcareous algae (4 mm^3) were selected under an optical magnifier and ground to a fine powder in liquid nitrogen in a mortar and pestle. DNA extraction was performed using the NucleoSpin Plant II kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol. Molecular markers were PCR-amplified in a final volume of 50 µL: 1x PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 mM of each primer (forward and reverse), 5-10 ng of total DNA and 1.25 U Taq DNA polymerase (Invitrogen, Carlsbad, USA). The PCR cycles and primers varied according to the marker. Amplifications of UPA were performed using the primers of Presting (2006) and the cycle used was 94 °C for 2 min, 35 cycles at 94 °C for 20 sec, 55 °C for 30 sec, 72 °C for 30 sec, and a final extension at 72 °C for 10 min. Amplifications of *psb*A were performed using the primers of Yoon *et al*. (2002) and the cycle used was 94 °C for 2 min, 35 cycles at 94 °C for 30 sec, 47 °C for 1 min, 72 °C for 2 min, and a final extension at 72 °C for 7 min. PCR was performed in a gradient thermal cycler (Techne TC-512, Techgene Techne, Burlington, USA) and after the reaction, DNA fragments were verified by electrophoresis on a 0.7%

agarose gel. Amplified products were purified with the GFX ™ PCR and Gel Band Purification DNA kit (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's protocol. Sequencing reactions were performed with BigDyeTM Terminator v3.1 Sequencing Kit (Applied Biosystems, Carlsbad, California USA), according to the manufacturer's protocol, using the same PCR primers specific for each molecular marker and the same PCR thermocycler. For *psb*A sequencing, three internal primers were also used: 500F and 600R (Yoon *et al*. 2002) and 550R (5'-TTRTGTTCRGCYTGRAATAC-3' (developed by M.N. Sissini, B.N. Torrano-Silva, T.V. Pinto and M.C. Oliveira). In total, 40 cycles at 96 °C for 10 sec, 54 °C for 20 sec and 60 °C for 4 min. These DNA fragments were precipitated with 1 µL of EDTA 125 mM, 1 µL of sodium acetate 3M, 25 µL of 100% ethanol and washed in 35 µL of 70% ethanol. Samples were analyzed on the automatic DNA sequencer ABI 3730 Genetic Analyzer (Applied Biosystems, Carlsbad, California, USA).

DNA extraction, amplification and sequencing of *M. erubescens* holotype:—A fragment about 3 mm^3 was removed from the holotype of *Lithothamnion erubescens* (TRH C15-3212, Fig. 2). DNA extraction, amplification and sequencing were performed according to Gabrielson *et al*. (2011). A new internal forward *rbc*L primer, F1150Cor 5'-GGTATACATTGTGGACAAATGC-3' was used in combination with reverse *rbc*S (Freshwater & Rueness 1994). The same primer pair was used in the sequencing reaction. To avoid possible cross contamination, the type material was sequenced at University of North Carolina, in USA while the field collected material was processed and sequenced at the Marine Algae Laboratory at University of São Paulo, Brazil.

Alignment:—Sequences were aligned and edited in BioEdit version 5.0.6 (Hall 1999). Chromatograms were visually inspected for validation of ambiguous nucleotides before generating a consensus sequence. Multiple alignments were generated for each marker (excluding the regions of the PCR primers) using *Clustal W* (Thompson *et al.* 1994) available in *BioEdit*. For UPA, a matrix of 37 sequences, 346 base pairs (bp) in length was constructed from sequences generated in this study plus eight sequences available in *GenBank* including *Gracilaria dotyi* Hoyle (1977: 85) (EF426613) as outgroup. For *psb*A the matrix had 71 sequences, 445 bp long, of which 55 were from *GenBank*; *G. textorii* (Suringar 1868: 259) De Toni (1895: 27) (DQ095842) was used as outgroup. For *rbc*L, a matrix of 23 sequences was constructed with partial sequences (293 bp), of which 6 were from GenBank; *Gracilaria textorii* (AY049325) as outgroup, in order to compare field-collected sequences with the type sequence.

Cluster and phylogenetic analysis:—For UPA, *psb*A and *rbc*L Neighbor-Joining (NJ) trees were constructed using distance method in MEGA 5 program (Tamura *et al.* 2011) with 2000 bootstrap (BS) replications. Phylogenetic analyses were performed on *psb*A and *rbc*L datasets using evolutionary models selected by jModeltest 2.1.4 (Darriba *et al*. 2011). Maximum Likelihood (ML) was performed using MEGA5 with 2000 BS replicates. Bayesian analysis was performed in MrBayes v3.2.2 (Ronquist *et al*. 2012), using two runs with four Markov chains ($4 \cdot 10^6$ generations). Chains were initiated in random trees, sampled every 100 generations, and the initial generations were discarded. Only BS values greater than or equal to 70% for NJ and ML were plotted with 70 to 89% considered moderate support and 90 to 100% high support. Sequences generated for this study were deposited in GenBank.

Morphological study:—Permanent slides and stubs used for observation under light microscopy and scanning electron microscopy, respectively, were prepared according to the procedures described in Horta (2002) and Moura *et al*. (1997). Growth form terminology follows Woelkerling & Harvey (1993); anatomical terminology follows Woelkerling (1988), Keats & Chamberlain (1994) and Athanasiadis (2004). Morpho-anatomical information of the type material was from Keats & Chamberlain (1994).

Results

Molecular analysis:—In this study, 61 sequences were newly generated from 38 field-collected specimens: 29 of UPA, 16 of *psb*A and 16 of *rbc*L. The UPA marker was amplified and sequenced for all samples in order to provide the initial overview of specimen clustering. After analyzing the clusters of UPA sequences, representatives from different clusters and different localities were sequenced for the phylogenetic markers: *psb*A and *rbc*L. With respect to *rbc*L, some of the Brazilian field-collected samples morpho-anatomically

identified as *M. erubescens* and the Atlantic Mexican sequences were identical to the holotype sequence of *M. erubescens* and resolved in a clade with high support (100% BS for NJ and ML, 0.99 of *posteriori* probability (PP) for Bayesian analysis) as shown in Fig. 3. Six other Brazilian specimens (FLOR 14925, FLOR 14926, FLOR 14927, FLOR 14928, FLOR 14929, FLOR 14930) were grouped in three other distinct clades (Fig. 3). Regarding UPA, the analysis revealed a main group strongly supported (100% BS), composed of 29 Brazilian specimens characterized in this study (Fig. 4). Sister to this clade was a clade with specimens described as *M. erubescens* from Hawai'i. The divergence between the UPA sequences from Brazil and Hawai'i was 0.29% (1 bp). Regarding *psb*A, the phylogram in Fig. 5 showed that Hapalidiaceae constituted a monophyletic group with moderate support (84% BS for NJ and ML) and 1.00 PP for Bayesian analysis. Monophyly of the genus *Mesophyllum* was not recovered since specimens morphologically placed in *Phymatolithon repandum* (Foslie 1904: 4) Wilks & Woelkerling (1994: 190) and *M. erubescens* form a strongly supported clade (100% BS for NJ and ML and 1.00 PP for Bayesian analysis).

TABLE 2. Variation found for *psb*A molecular marker, represented by percentage (%) and number of base of pair (bp) differences for specimens morphologically identified as *M. erubescens*. Seqs = number of sequences in matrix.

	seqs	%	bp
Brazil Tropical Province vs. Brazil Warm Temperate Province	13	0.3	1
Brazil Tropical Province vs. Mexico	9	0.0	0
Brazil Warm Temperate Province vs. Mexico	10	0.3	1
Brazil vs. Japan, Vanuatu and Fiji	15	2.5–3.2	11–14
Brazil vs. New Zealand 1	24	8.6–8.8	38–39
Brazil vs. New Zealand 2	19	7.5–8.8	33–39
Brazil vs. New Zealand 3	16	12.9–13.1	57–58
Brazil vs. New Zealand 4	14	11.7–12.0	52–53
Brazil vs. New Zealand 5	14	11.7–12.0	52–53
New Zealand 1 vs. New Zealand 2	17	1.6–3.2	7–14
New Zealand 1 vs. New Zealand 3	14	8.1–8.6	36–38
New Zealand 1 vs. New Zealand 4	12	8.4–9.0	37–40
New Zealand 1 vs. New Zealand 5	14	11.7–12.0	52–53
New Zealand 2 vs. New Zealand 3	9	8.4–9.5	37–42
New Zealand 2 vs. New Zealand 4	7	8.6–9.5	38–42
New Zealand 2 vs. New Zealand 5	7	8.4–9.0	37–40
New Zealand 3 vs. New Zealand 4	4	2.7–3.0	12–13
New Zealand 3 vs. New Zealand 5	4	6.3	28
New Zealand 4 vs. New Zealand 5	2	5.9	26
Japan vs. Vanuatu	2	3	13
Vanuatu vs. Fiji	2	2.7	12
Japan vs. Fiji	2	5	22
Vanuatu vs. Vanuatu	2	7.5	33

Specimens placed morphologically in *M. erubescens* appeared in several clades. However, the Brazilian representatives constituted a monophyletic clade with 99% BS for NJ, 98% for ML and 1.00 PP for Bayesian analysis. Sequences from Mexico grouped with Brazilian sequences. The divergence between sequences from the Tropical Province (including samples from Mexico, Trindade Island, Bahia and Fernando de Noronha Archipelago) and WarmTemperate Province (São Paulo and Santa Catarina) was only one single nucleotide polymorphism (SNP), and specimens from Espírito Santo in the Transition Zone had both haplotypes (see Fig. 6).

Mesophyllum erubescens sequences from Japan and Vanuatu were sister to Brazilian and Mexican specimens with high BS (98% for NJ and 96% for ML) and a PP of 0.98.

Considering the divergence between the Tropical and Temperate Provinces was low (0.23%), it can be inferred that the Brazilian specimens corresponded to a single widely distributed species. However, the differences between the sequences of the Brazilian material and the sequences from Japan and Vanuatu (2.5 to 3.2%) suggested the existence of distinct species.

Sequences of *M. erubescens* from New Zealand grouped into at least two major clades, with sequences of *M. macroblastum* (Foslie) W.H. Adey (1970: 25) and *M. printzianum* Woelkerling & A.S. Harvey (1993: 593). Further, another sequence of *M. erubescens* (DQ167891) from New Zealand showed 100% identity with *P. repandum* (FJ361395 and FJ361683) also from New Zealand.

Molecular marker variation:—Sequences for the *rbc*L and UPA markers were identical between Brazil plus Tropical Mexico and Brazil Warm Temperate Provinces and for *psb*A differed by only one nucleotide (Fig. 6). Table 2 presented the variation found among clades of *psb*A sequences from specimens morphologically identified as *M. erubescens*. Sequences from Brazilian material diverged from sequences from Japan, Vanuatu and Fiji by 11-14 nucleotides (2.5% to 3.2%), while this number was much higher when compared to New Zealand 3 (12.9% to 13.1%) and New Zealand 4 and 5 (11.7% to 12.0%). Within New Zealand, a high genetic variation (1.6% to 12.0%) was observed, with 7-53 divergent nucleotides, indicating the existence of cryptic species.

***Mesophyllum erubescens* (Foslie) Me. Lemoine (1928: 252).**
Basionym: *Lithothamnion erubescens* Foslie (1900: 9–10).

Type:—BRAZIL. Fernando de Noronha Archipelago: Chaloup Bay, *14 August-24 September 1873, leg. Ridley, Lea, Ramage* (holotype: TRH! C15-3212).

Material examined: BRAZIL. Santa Catarina: Arvoredo Island, *28 November 2012, leg. M. Sissini & L. Ferreira*. São Paulo: Queimada Grande Island, *19 April 2011, leg. M. Sissini, B. Torrano*; Cabras Island, Ilhabela, *6 February 2012, leg. P. Horta*. Espírito Santo: Rasas Islands, Guarapari, *29 March 2012, leg. M. Sissini*. Trindade Island, *16 June 2012, 25 June 2012, 24 July 2012, 6 August 2012, leg. M. Sissini*. Bahia: Praia do Jauá, Camaçari, *23 May 2013, leg. B. Torrano, C. Azevedo, T. Vieira-Pinto*. Pernambuco: Boca do Inferno, Fernando de Noronha Archipelago, *21 November 2011, leg. F. Scherner, D. Burgos*. Cagarras, Fernando de Noronha Archipelago, *8 August 2013, leg. P. Horta*. Ceará, *Risca do Meio, 18 April 2012, leg. M. Sissini*.

Taxa excluded from synonymy:—*Lithothamnion erubescens* f. *madagascarense* Foslie (1901a: 3), *Lithothamnion erubescens* f. *haingsisianum* Weber-van Bosse & Foslie (1901b: 4), *Lithothamnion erubescens* f. *subflabellatum* Foslie (1904: 31), *Lithothamnion madagascarense* (Foslie 1901a: 3) Foslie (1906: 19), *Mesophyllum madagascarense* (Foslie 1901a: 4) W.H. Adey (1970: 25).

Habit and ecological observations:—Thalli varied from epilithic and encrusting (Figs. 7A-F) to epizoic on corals, to free-living rhodoliths in beds at Santa Catarina and around Trindade Island in association with *Lithophyllum* spp. and *Lithothamnion* spp. Encrusting forms were found in the intertidal (Jauá Beach, Bahia) and free forms up to 25 meters deep at Risca do Meio (CE) and Cagarras (FN). Thalli were violet brown, with protuberances varying from fruticose to warty, with protuberances often branched, basally cylindrical with distal portions slightly flattened, 1–2 mm in diameter and 1.5–4.0 mm high, with branches frequently fused.

FIGURE 1. Collection locations for *M. erubescens*. BA: Bahia; CE: Ceará; ES: Espírito Santo; FN: Fernando de Noronha; IT: Trindade Island; SC: Santa Catarina; SP: São Paulo; VER: Veracruz.

Vegetative structure:—Thalli pseudoparenchymatous; internal organization monomerous in crustose portions and radial in protuberances, consisting of a single system of branched filaments that formed a core running more or less parallel to the substrate and a more peripheral region in which portions of core filaments or their derivatives curved outwards towards the thallus surface and terminated in a single layer of epithallial cells rounded to oval, but not flared, 5–14 mm in diameter and 2–4 mm long (Fig. 8B). Isolated trichocytes present (Fig. 8A). Adjacent filaments joined by cell fusions (Fig. 8C); secondary pit connections not observed. Perithallial cells containing starch grains (Fig. 8D).

Reproductive structures:—Tetrasporangial conceptacles multiporate (Fig. 9A), protruding and flat at the roof (Fig. 9B), without differentiation into a peripheral rim and without a central, sunken pore plate (Fig. 9D); conceptacle roofs 5–7 cells and 30–45 mm thick above the chamber; pore canals occluded by an apical plug (Fig. 9C). Pore canals lined by 3–4 celled filaments with a basal cell more elongate than other roof cells, 10–17 µm long (Fig. 9E). Conceptacle chambers elliptical, 231–349 mm in diameter and 126–238 mm high, usually without a central columella. Tetrasporangia/bisporangia scattered across the chamber floor; each mature sporangium 63–84 mm long, containing four zonately arranged tetrasporangia or two bisporangia (Fig. 9F). Gametangial thalli monoecious. Spermatangial conceptacles uniporate, slightly raised in relation to the surrounding surface (Fig. 10A); chamber domed 205–210 µm in diameter and 133–138 high (Fig. 10B); spermatangial systems simple on the floor walls and roof of the chamber with spermatangial filaments unbranched. Carposporangial conceptacle uniporate, dumbbell-shaped and markedly raised in relation to the surrounding surface (Fig. 10C); chamber 349–446 µm in diameter and 366–516 µm high (Fig. 10D).

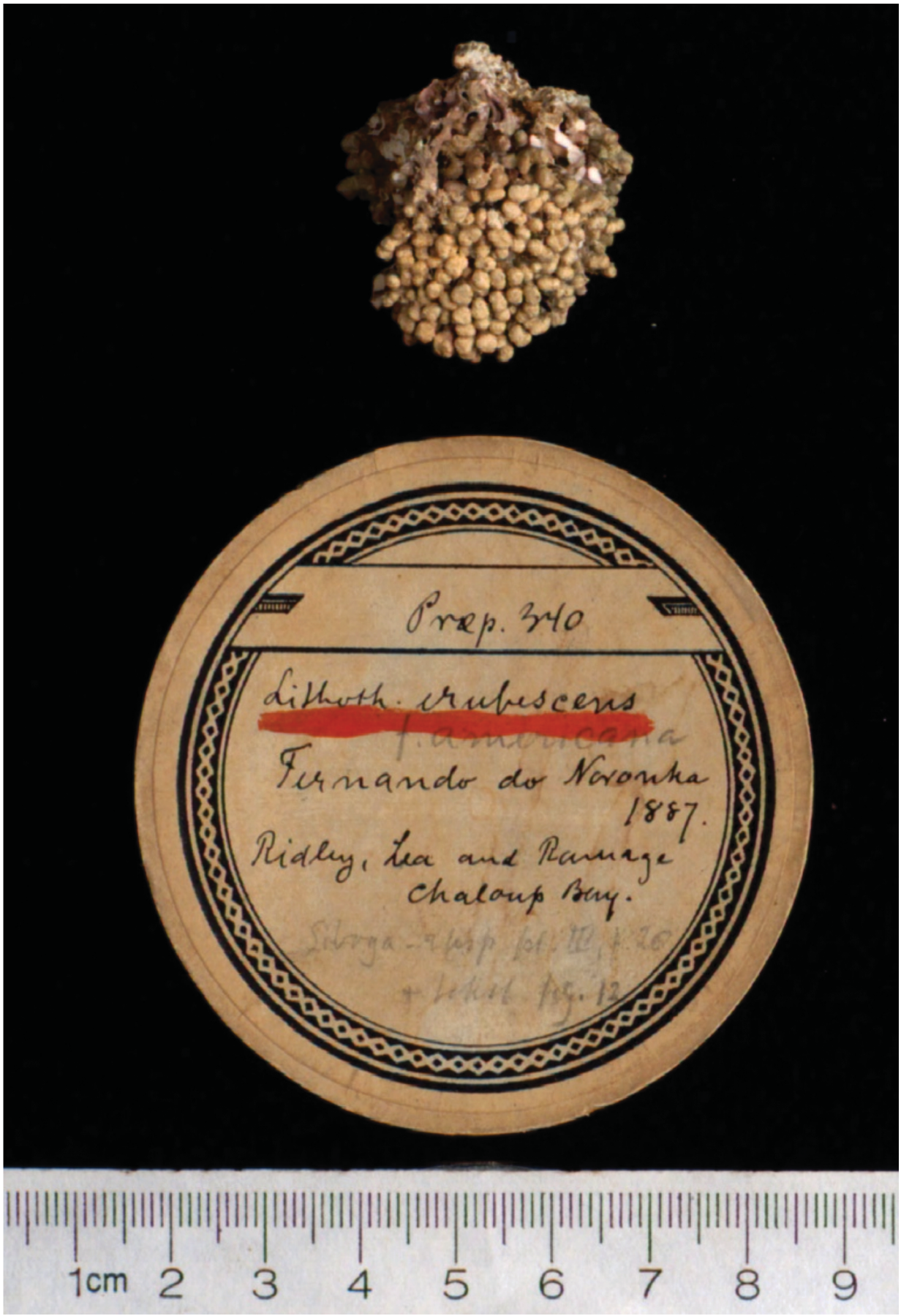

FIGURE 2. Holotype of *Lithothamnion erubescens* Foslie (TRH C15-3212), basionym of *Mesophyllum erubescens*.

Distribution along the Brazilian coast:—Santa Catarina (present study, Horta *et al.* 2011, and Pascelli *et al.* 2013); São Paulo (present study); Espírito Santo (present study); Trindade Island (present study); Bahia (present study and Figueiredo & Steneck 2000, Figueiredo *et al*. 2007, Nunes *et al.* 2008, Bahia *et al*. 2010); Fernando de Noronha (present study and Foslie 1900a) and Ceará (present study).

Comments:—Foslie (1900b) described *Lithothamnion erubescens* based on specimens collected during August and September 1873 at Chaloup Bay, Fernando do Noronha Islands, on the Challenger Expedition. The algae of the expedition were described in a series of papers by G. Dickie, including those from the Fernando do Noronha Archipelago (Dickie 1874 and see Oliveira 1974). Dickie (1874) described these specimens under the name *Lithothamnion mamillare* (Harvey 1849: 109) Areschoug (1852: 521). Oliveira (1974), examined Dickie's material deposited at the British Museum (Natural History) and called this species *Goniolithon mamillare* (Harvey 1849: 109) Foslie (1900b: 16), but later referred to specimens collected along Brazilian coast (Oliveira 1977) as *Neogoniolithon mamillare* (Harvey) Setchell & L.R. Mason. Foslie (1900b) briefly described the habit, habitat, vegetative anatomy and sporangial conceptacles of his new species. As Woelkerling (1993: 86) pointed out, Foslie (1901b) later referred to the type form as *L. erubescens* f. *americana*, but this is superfluous for *L. erubescens* f. *erubescens*. Printz (1929, pl. XV, Figs. 15, 16, 20 & 21) illustrated three specimens from Foslie's collection (Figs. 16 and 20 are the same specimen photographed from the side and top, respectively), only one of which currently is in the type specimen box, and this apparently was also true when the type specimen was described and illustrated by Keats & Chamberlain (1994). Figure 2 shows the holotype specimen that matches one of the specimens photographed in Printz (1929, Figs. 16, 20) and a fragment of which was sequenced. By their description, this was the same specimen examined by Keats and Chamberlain (1994).

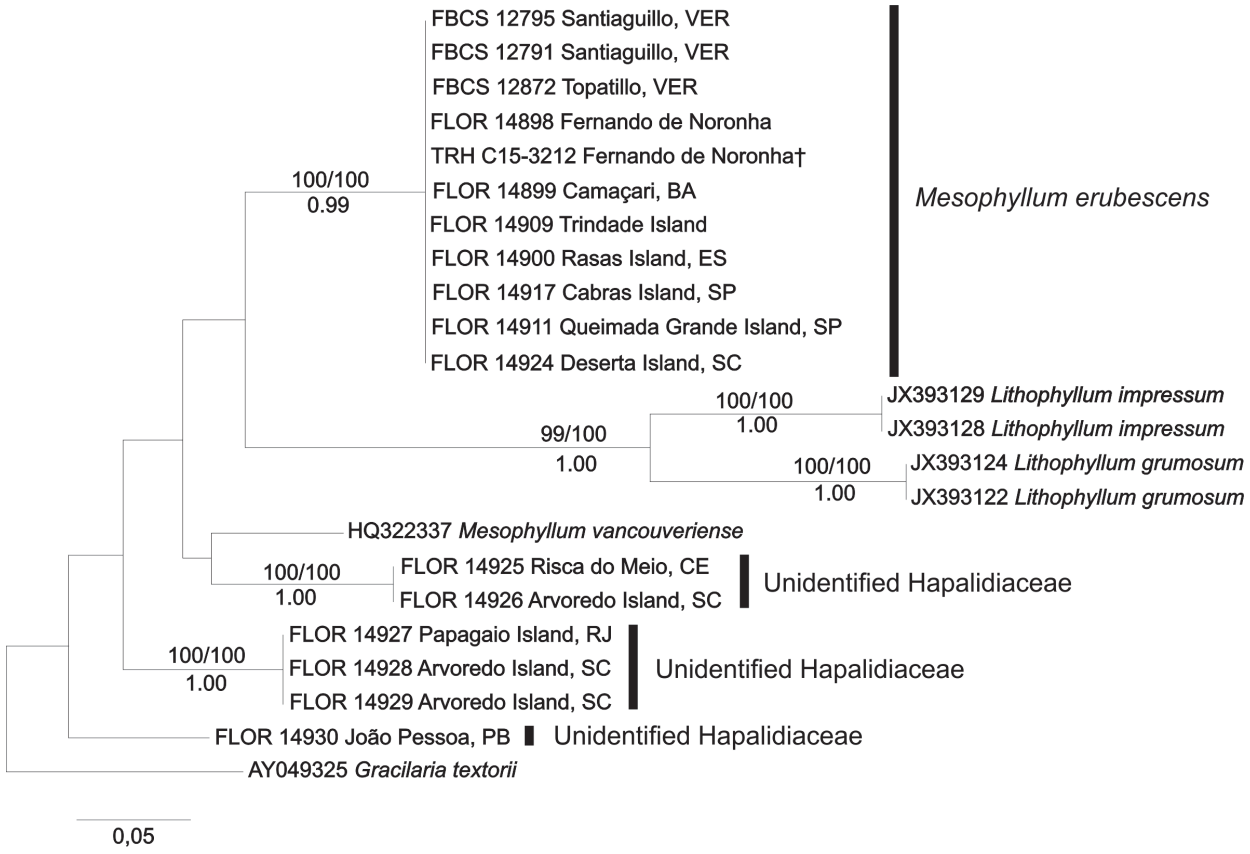

FIGURE 3. Maximum likelihood phylogram based on *rbc*L dataset. Numbers above branches are Neighbor-Joining (NJ)/ Maximum likelihood (ML) bootstrap values, numbers below branches are *Posteriori* probability (PP) for Bayesian analysis. Scale bar represents number of substitutions.†indicates sequence from type material.

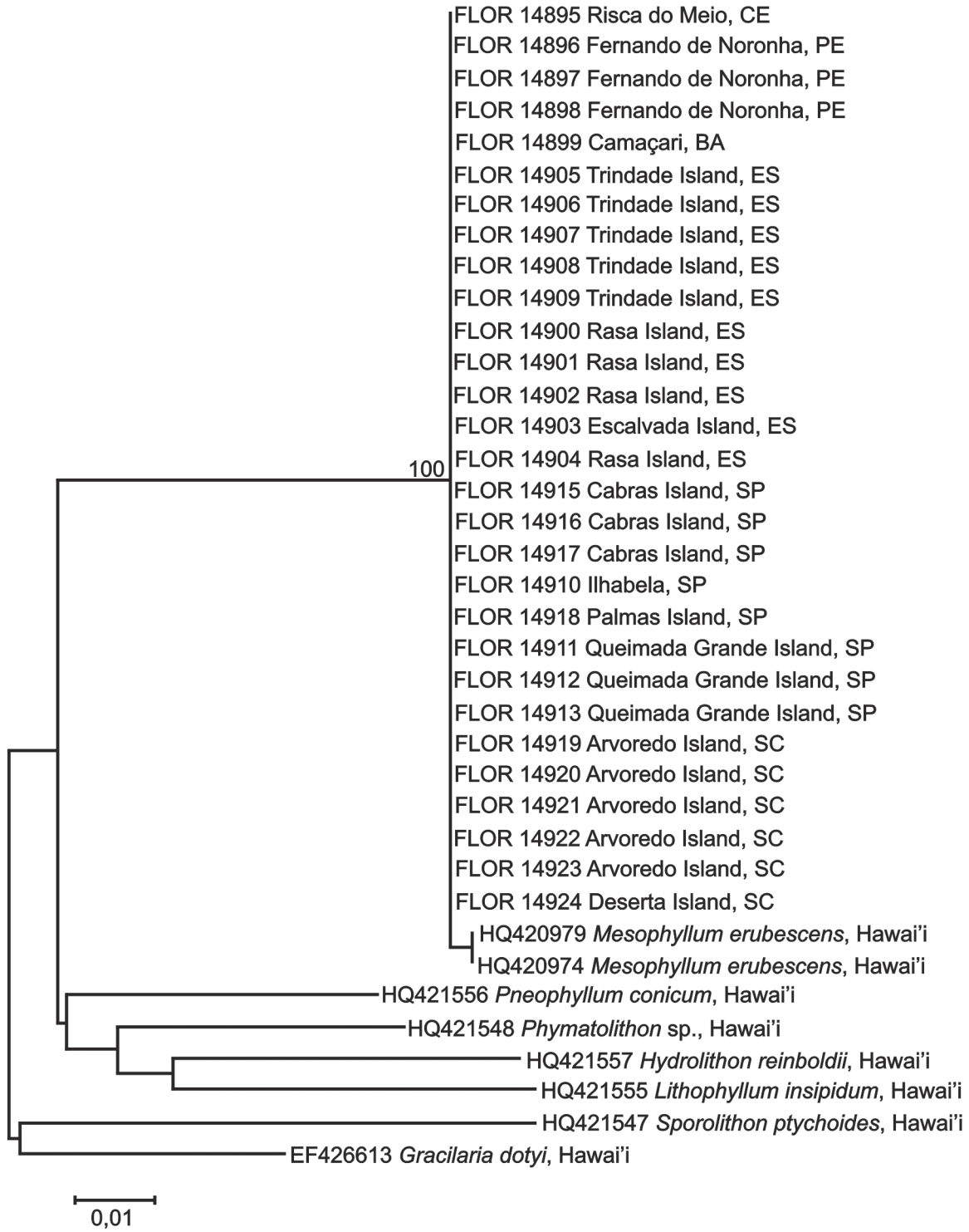

FIGURE 4. Neighbor-Joining phylogram inferred from UPA dataset. Percentage bootstrap support shown above branches; only values over 70% were plotted. Scale bar represents number of substitutions.

Discussion

Phylogenetic analyzes based on multiple markers SSU, *rbc*L, and *psb*A (Bittner *et al*. 2011, Peña *et al*. 2011, herein) indicate that *Mesophyllum* is very likely a polyphyletic genus. *Mesophyllum erubecens*, based on specimens from Brazil that match the type specimen, is not in the same clade as *M. lichenoides* (Ellis 1768: 407) Me. Lemoine (1928: 252), the generitype species from the NE Atlantic (Bittner *et al*. 2011, Peña *et al*. 2011).

Given that Woelkerling and Harvey (1992) and Athanasiadis *et al*. (2004) have proposed different suites of morpho-anatomical features to characterize *Mesophyllum*, it is perhaps not surprising that DNA sequence analyses indicate polyphyly for the genus. A resolution of this problem awaits the sequencing of confirmed specimens of the generitypes of the related genera *Clathromorphum*, *Synarthrophyton*, and *Phymatolithon* Foslie (1898: 4). Only when we resolve the relationships among these genera based on DNA sequences, will we be able to understand what morpho-anatomical characters, if any, are useful in segregating these taxa.

The partial 293 bp *rbc*L sequence, representing about 20% of the *rbc*L gene, generated from the holotype specimen of *M. erubescens*, was identical in sequence to recently collected topotype specimens of the same species (140 years separates these collections), unequivocally confirming the application of this name to this and other Brazilian and Atlantic Mexican material. Saunders and McDevit (2012) questioned whether previous studies that sequenced algal type specimens (e.g., Hughey *et al*. 2001, Gabrielson 2008) had sufficient controls to exclude modern exogenous laboratory contamination as a reason for their success in amplifying 19th C and early 20th C type specimens. Saunders and McDevit (2012) raised doubts because they experienced serious contamination issues and were unable to obtain any sequence data from a 19th C archival specimen. Hughey and Gabrielson (2012), however, independently, in separate laboratories, and using the same 19th C archival material along with controls advocated by Saunders and McDevit (2012), were able to sequence several different markers (Hughey & Gabrielson 2012) useful in identifying the specimen. Although not always possible, the best control is to amplify type material in a lab where specimens of the target species never have been present, and this was the case for *M. erubescens*—type material was extracted, amplified and sequenced in North Carolina, USA, whereas recently collected material, including topotype specimens, was processed in São Paulo, Brazil.

In Brazil, the distribution of *M. erubescens* is expanded west to Ceará from Fernando de Noronha Archipelago, and additional localities are added between Ceará in the north and Santa Catarina in the south. *Mesophyllum erubescens* occurs in the biogeographic provinces proposed by Horta *et al*. (2001), from the Tropical Province localities of Ceará and the Fernando de Noronha Archipelago in the north through the Transition Zone, Espírito Santo state, to the Warm Temperate Province in the south, including the localities of São Paulo and Santa Catarina. Based on sequencing to date, the two haplotypes segregate according to the biogeographic provinces proposed by Horta *et al*. (2001) with one haplotype only present in Tropical waters, both haplotypes present in the Transition Zone of Espírito Santo and the other present in warm temperate waters. In Espírito Santo, the transition from tropical to warm temperate waters, as well as a wide variety of habitats, could explain the high diversity of marine species, from macroalgae (Oliveira 1969, 1977, Horta *et al*. 2001) to reef fishes (Floeter *et al*. 2001) or corals (Leão *et al*. 2003).

Populations of *M. erubescens* from the Atlantic Mexican coast have identical *psb*A sequences as the Brazilian Tropical Province specimens. Despite the ca. 6000 km that separate them and the large outflow of fresh water and sediment from the Amazon and Orinoco rivers that have emptied into Atlantic Ocean over the last 10 million years (Hoorn 1994), gene flow between these populations appears to be occurring. Now that *M. erubescens* has been confirmed to occur along the Mexican east coast, it should be looked for along the east coasts of countries of Central America and the Atlantic coast of other South American countries. The affinity between the marine flora of Brazil and the Caribbean Sea has been recognized since Taylor (1960), however, phylogeographic studies documenting the connectivity between these populations are lacking. Sequences of other markers more variable than *psb*A, for example COI, may give more insight into the distributions of haplotypes along the coast of Brazil, and between Brazil and the Caribbean Sea/Gulf of Mexico.

Despite the morpho-anatomical similarites of Pacific and Atlantic Ocean specimens both called *M. erubescens*, it is clear, based on *psb*A sequence divergence values (Table 2), that nearly all of those specimens from the Pacific not only are not *M. erubescens,* but that they represent many different species. In New Zealand alone, at least five species are passing under this name (Fig. 11). This was foreshadowed by the results of Kato *et al*. (2011) who included sequences from both Japanese and New Zealand specimens identified as *M. erubescens*, but it could have been argued that these specimens were just misidentified without comparison to type material. Now, however, it is only Brazilian and Atlantic Mexican specimens of *M. erubescens* that are clearly linked to type material by identical DNA sequences. Specimens from Japan, New Zealand and likely other Pacific Ocean localities belong to other species and not to *M. erubescens* despite their morpho-anatomical similarities. Likewise, we have not accepted the heterotypic synonyms typically listed for *M. erubescens* (Guiry & Guiry 2014), since all of them have their type localities in the tropical Indo-Pacific, not the western Atlantic Ocean. The 1 bp difference between Brazilian and Hawai'ian specimens identified as *M. erubescens* needs confirmation with less conservative markers, such as *psb*A, *rbc*L and COI.

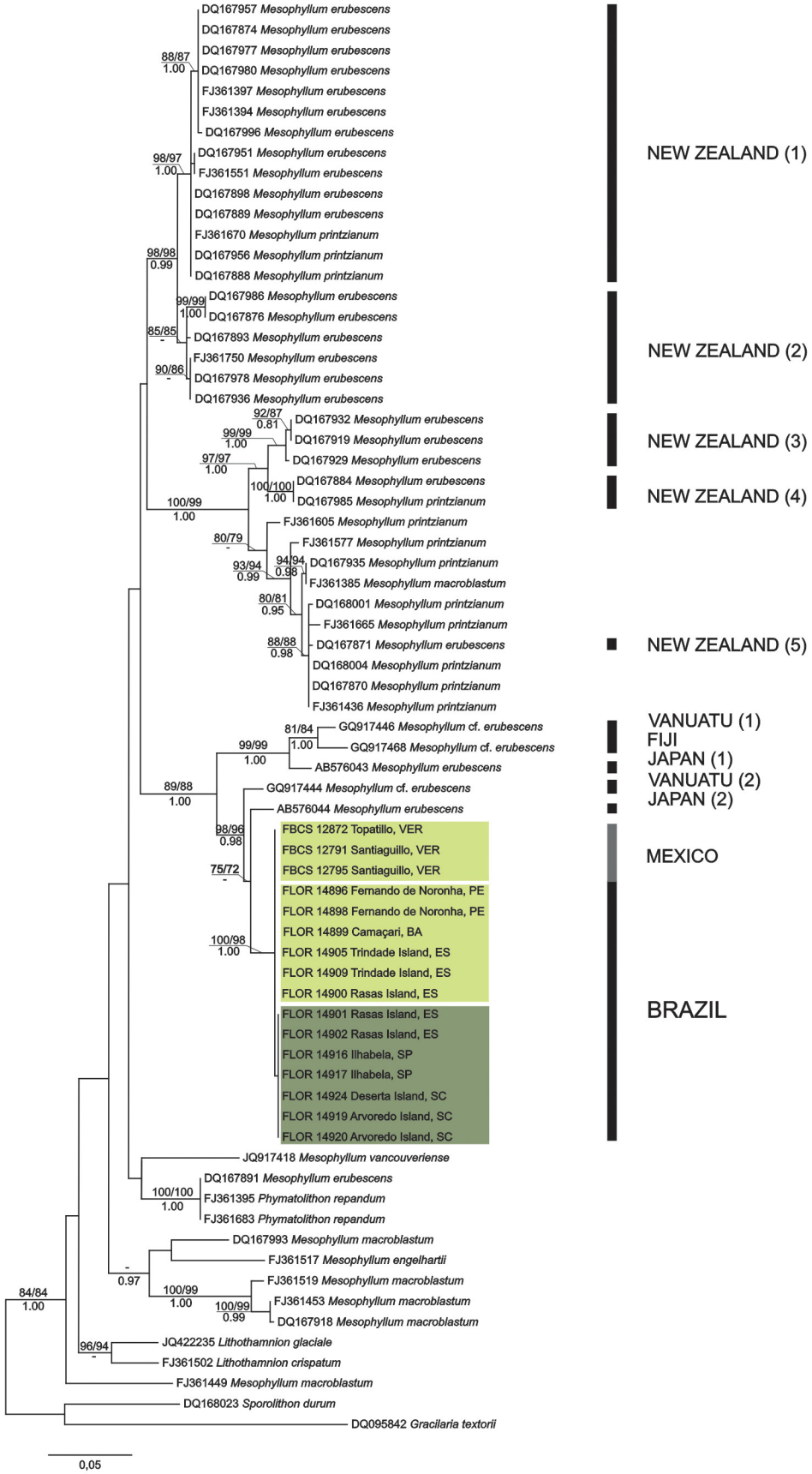

FIGURE 5. Maximum likelihood phylogram based on *psb*A data. Numbers above branches are Neighbor-Joining (NJ)/ Maximum likelihood (ML) bootstrap values, numbers below branches are *Posteriori* probability (PP) for Bayesian analysis. Scale bar represents number of substitutions.

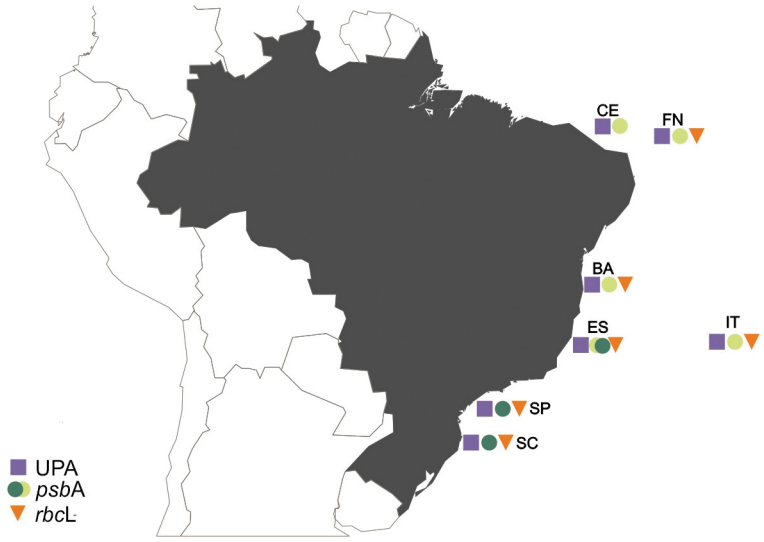

FIGURE 6. Locations of sequenced specimens of *M. erubescens* along Brazilian coast. Symbols represent molecular markers and colors represent haplotypes. Note that two haplotypes (light green and dark green circles) occur in Espírito Santo state. CE: Ceará; FN: Fernando de Noronha; BA: Bahia; ES: Espírito Santo; IT: Trindade Island; SP: São Paulo; SC: Santa Catarina.

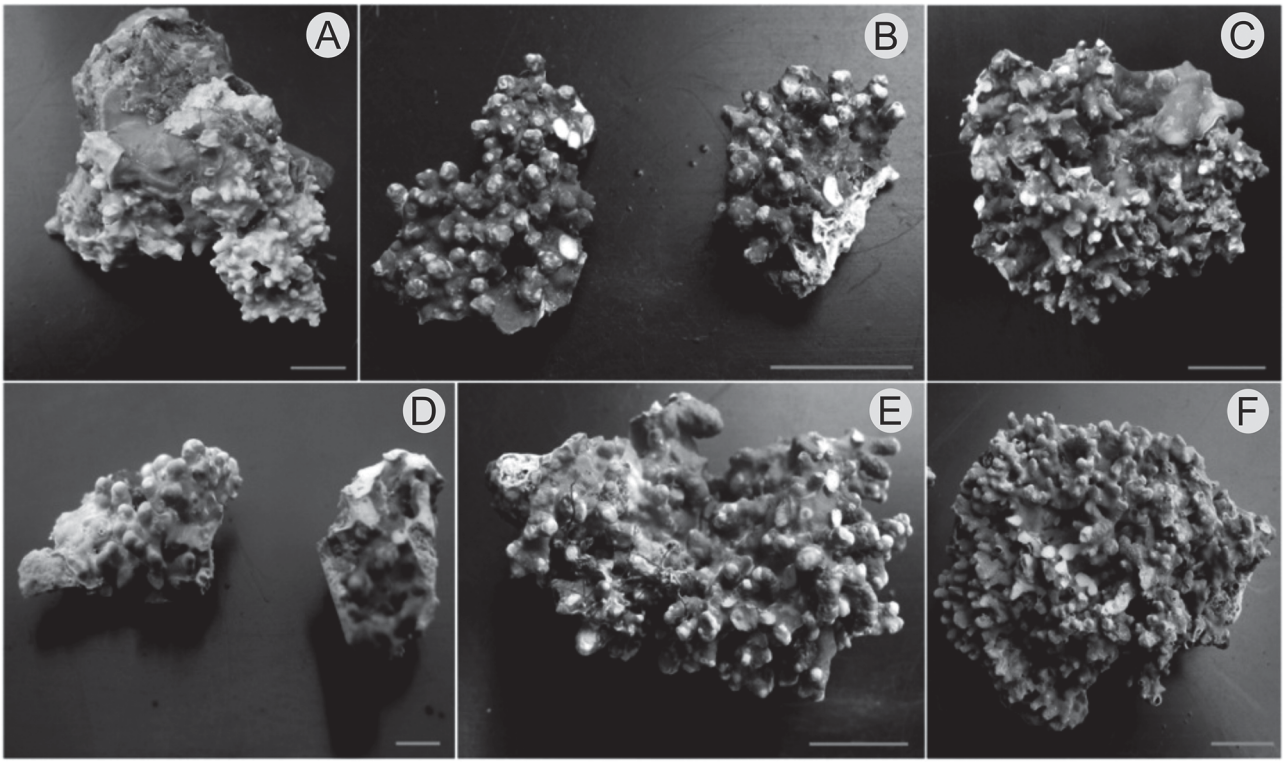

FIGURE 7. Habits of *Mesophyllum erubescens* specimens analyzed in present study. A. Rasas Islands, ES. B. Jauá, BA. C. Queimada Grande Island, SP. D. Cagarras, FN. E. Arvoredo Island, SC. F. Trindade Island. Scale bar: 1 cm.

These results from *Mesophyllum erubescens*, along with those of Kato *et al*. (2013) on *Neogoniolithon fosliei* (Heydrich 1897: 58) Setchell & L.R. Mason (1943: 90) in Japan, illustrate a two-fold problem with using only morpho-anatomical features to apply names to non-geniculate corallines. First, DNA sequencing is revealing multiple, cryptic species passing under a single name in the same geographic area (e.g., southern Japan, Kato *et al*. 2013; central New Zealand, Broom *et al*. 2008)—species that have not been segregated by morpho-anatomical

characters (Harvey *et al*. 2005). When morpho-anatomical characters cannot segregate species in a local area, applying names correctly becomes even more difficult. Only by sequencing type material can names be applied with some certainty (Hughey *et al*. 2001, Hughey & Gabrielson 2012). Second, morpho-anatomical characters have a long-standing and ongoing tradition of being used to place non-geniculate coralline species in synonymy, even when their type localities are very distant geographically—in different ocean basins and/or in different biogeographic provinces (*e.g.,* Penrose 1992, Basso *et al*. 2011). Kato *et al*. (2013) faced this problem with the synonymy of *N. frutescens* (Foslie 1900a: 9) Setchell & L. R. Mason (1943: 91) (type locality: Funafuti, Tuvalu) and *N. brassica-florida* (Harvey 1849: 110) Setchell & L.R. Mason (type locality: Algoa Bay, Cape Province, South Africa), under *N. fosliei* (type locality: El Tor, Egypt). They recommended that all three species be recognized and that gross morphological differences (encrusting species versus those with protuberances or branches) not be used to place species in synonymy. We concur with this recommendation and further caution the use of even some anatomical characters to lump specimens from different ocean basins and/or biogeographic provinces in the same species. For example, Brazilian *M. erubescens* specimens have tetrasporangial conceptacles with elongate basal pore canal cells (Nunes *et al.* 2008, Horta *et al.* 2011, present study, Fig. 9E) as do specimens called *M. erubescens* in New Zealand (*e.g.,* Harvey *et al*. 2005, Farr *et al*. 2009). This feature, along with others, led Harvey *et al*. (2005) and Farr *et al*. (2009) to call these specimens *M. erubescens*, but by DNA sequence these specimens clearly do not belong to *M. erubescens* (Fig. 11). Therefore, we strongly recommend that all reports of *Mesophyllum* species in Brazil, whose names are based on type specimens not from the Western Atlantic, to be reassessed with DNA sequence data.

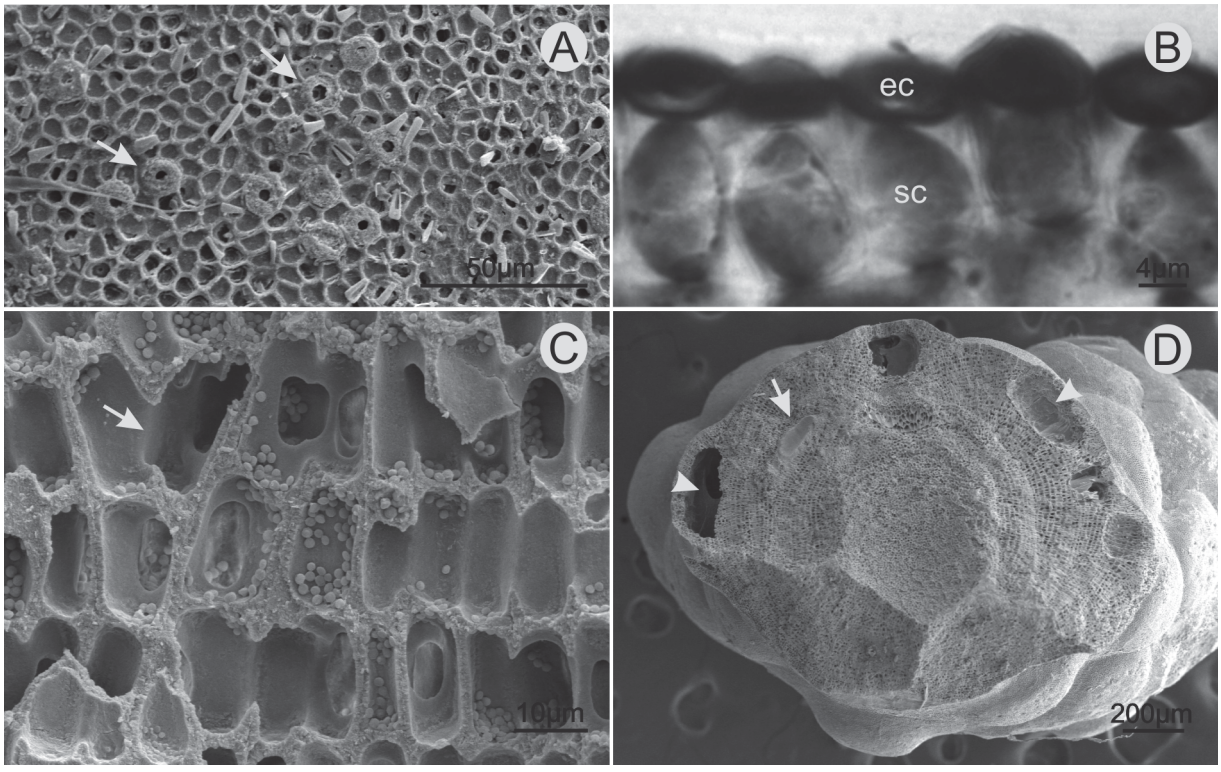

FIGURE 8. Vegetative aspects of *M. erubescens*. A. Superficial view of epithallial cells with scattered trichocytes (arrows). B. Transversal section of epithallial cells (ec) flattened to rounded, but not flared and subepithallial cells (sc) more elongated than the subsequent ones. C. Arrow showing cell fusion in perithallus. D. Transversal section of protuberance, highlighting scattered superficial (arrow head) and buried conceptacles (arrow).

Conclusion

Mesophyllum erubescens, based on DNA sequences from the holotype specimen as well as topotype material, is not a widely distributed species across different ocean basins or biogeographic provinces, but is restricted to the tropical and warm temperate regions of the western Atlantic Ocean, including the Caribbean Sea and southern Gulf

of Mexico. Additional sequencing with other markers is needed to confirm the two haplotypes and their distributions in the Western Atlantic. Because the morpho-anatomical features traditionally used to identify this species worldwide are not congruent with the molecular data, those features cannot be considered diagnostic and should be disregarded. Multiple, morpho-anatomically similar species in Japan and New Zealand are passing under *M. erubescens* and need to be reviewed. We recommend that the long-standing tradition of placing non-geniculate coralline species in synonymy based only on morpho-anatomical characters not be continued unless DNA sequence data supports such placement. This study of *M. erubescens* is an exemplar of an integrated approach to clarify non-geniculate coralline algal diversity and their distributions.

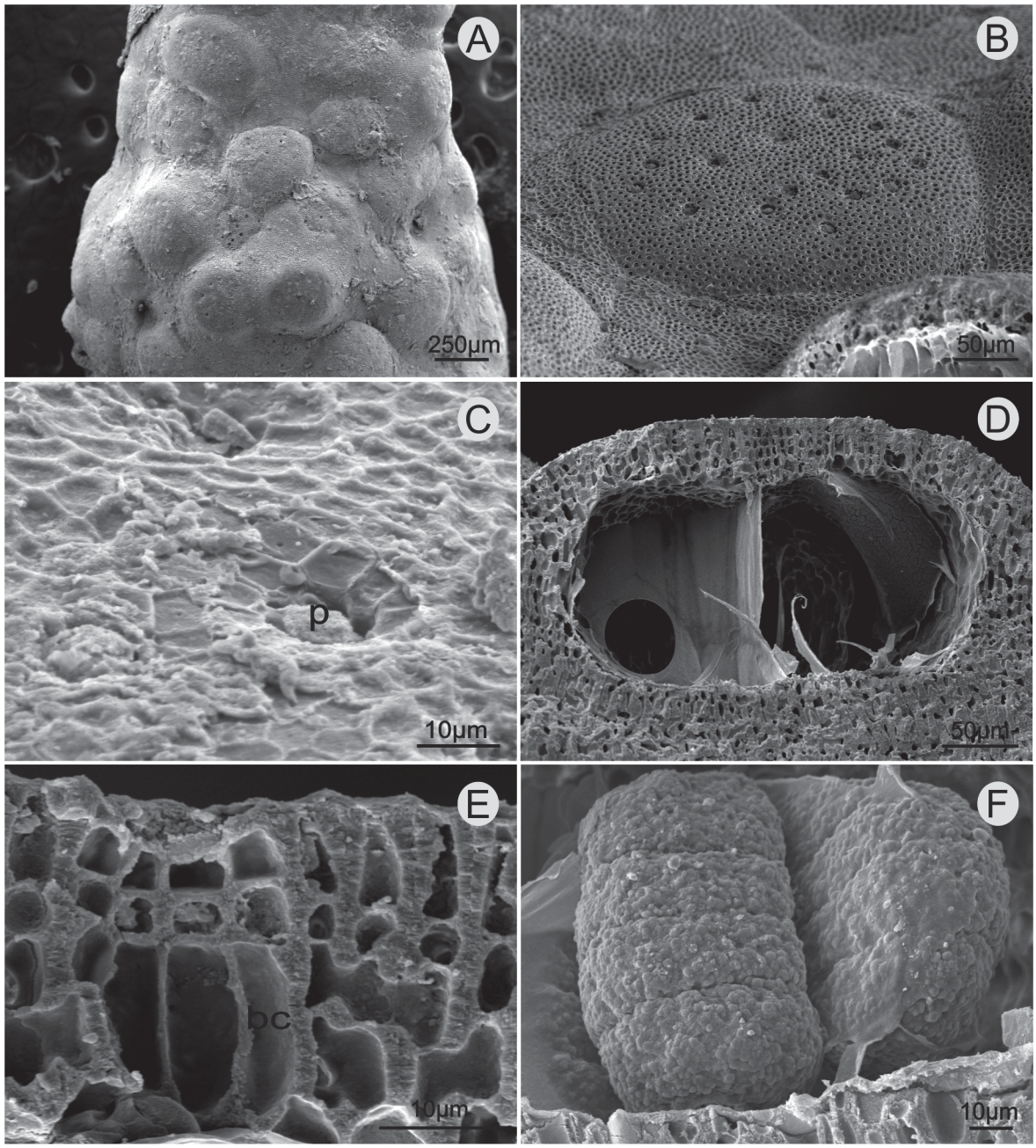

FIGURE 9. Reproductive features of *M. erubescens* seen in SEM. A. Superficial view of multiporate tetrasporangial conceptacles. B. Multiporate conceptacle with flat roof. C. Surface view of pore (p) slightly sunken. D. Longitudinal section of tetrasporangial conceptacle. E. Cells that surround pore canal, with basal cell (bc) more elongate. F. Zonately divided tetrasporangia.

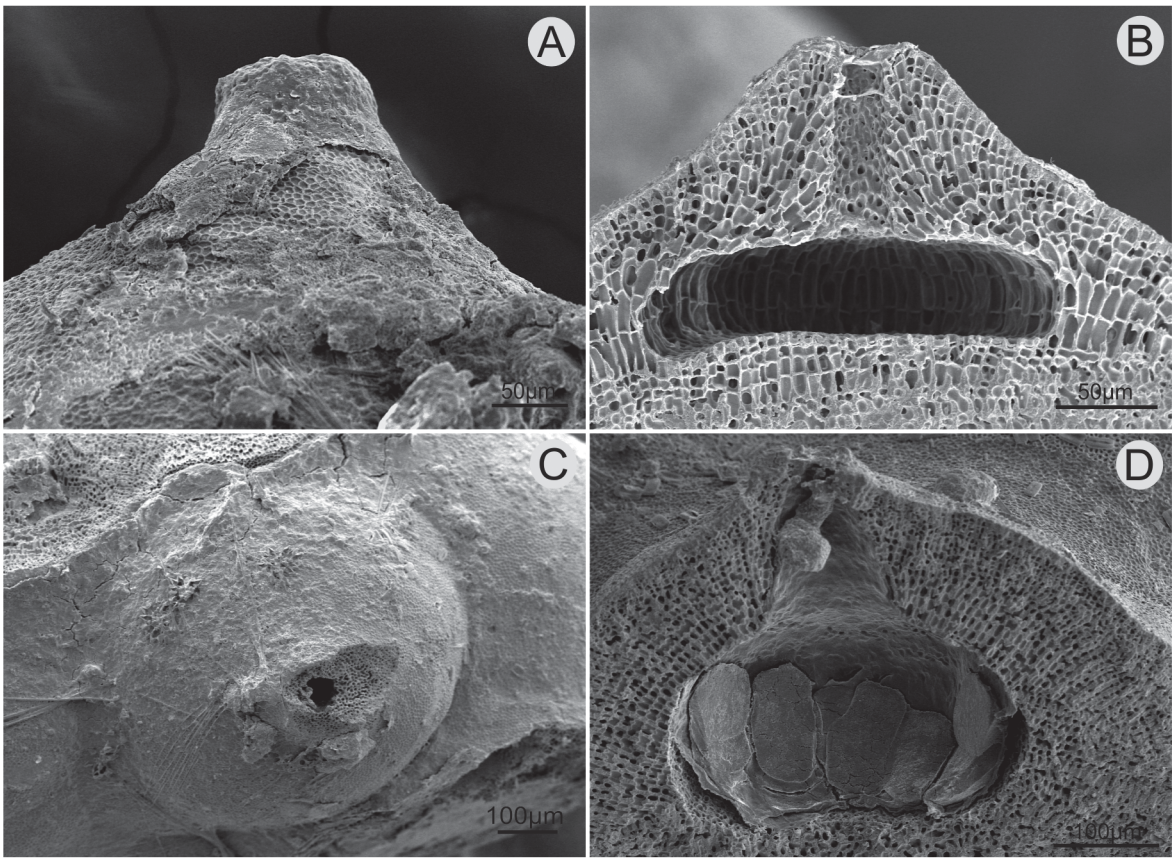

FIGURE 10. Gametangial conceptacles of *M. erubescens* seen in SEM. A. Male conceptacle in superficial view. B. Longitudinal section of male conceptacle. C. Female conceptacle in superficial view. D. Longitudinal section of female conceptacle with carposporangia.

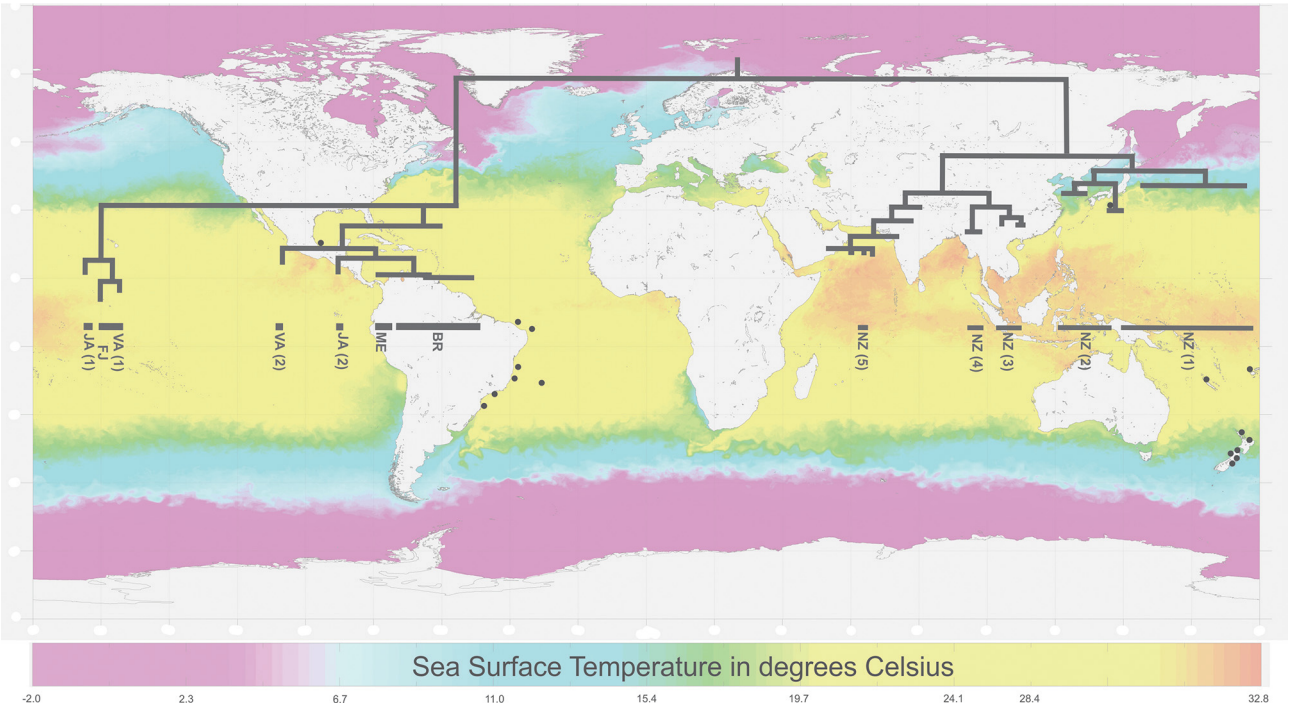

FIGURE 11. Map of sea surface temperature in May 2014 (adapted from www.ospo.noaa.gov) showing localities where there are *psb*A sequences for specimens morpho-anatomically identified as *M. erubescens*. Superimposed is the *psb*A phylogram (unscaled) showing the existence of multiple species under the same epithet.

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