



***Lithophyllum* species from Brazilian coast: range extension of *Lithophyllum margaritae* and description of *Lithophyllum atlanticum* sp. nov. (Corallinales, Corallinophycidae, Rhodophyta)**

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Abstract

Coralline algae (Corallinophycidae, Rhodophyta) are primarily characterized by the impregnation of calcium carbonate (calcite) in their cell walls. To determine the systematic position of genera and species, researchers are increasingly combining anatomical studies with comparisons of DNA sequences. In the present study we have described the main representatives of the genus *Lithophyllum* in intertidal and subtidal habitats from the southern coast of Brazil based on anatomical studies, supported by molecular markers. Analyses were conducted on specimens collected from five sites along the coast of southern Brazil. Anatomical studies were carried out by light and scanning electron microscopy. Molecular studies were based on DNA barcoding markers (*cox1*; UPA) and SSU rDNA. Using comparative anatomical features, corroborated by the molecular data, we identified two taxonomic entities from the Brazilian coast: *Lithophyllum margaritae*, and we propose the new species *Lithophyllum atlanticum*.

Key words: *cox1*, Corallinales, DNA-barcoding, *Lithophyllum*, new species, taxonomy, SSU rDNA, UPA

Introduction

The Lithophylloideae Setchell (1943: 134) includes *Amphiroa* Lamouroux (1812: 185), *Ezo Adey*, Masaki & Akioka (1974: 331), *Lithothrix* Gray 1867, *Lithophyllum* Philippi (1837: 387), *Paulsilvella* Woelkerling, Sartoni & Boddi 2002, *Tenarea* Bory de Saint-Vincent (1832) and *Titanoderma* Näegeli (1858: 532) (Guiry & Guiry 2014). All species in this subfamily possess secondary pit connections. *Amphiroa* and *Lithothrix* possess genicula; the remaining genera, including *Lithophyllum*, lack genicula (Woelkerling 1988). According to Woelkerling & Campbell (1992), *Lithophyllum* also does not produce haustoria, and does not have a thallus composed of flattened branches with an isobilateral internal organization. *Lithophyllum* species are distributed worldwide in all oceans (Guiry & Guiry 2014); nevertheless, the taxonomical knowledge of this group in the subtropical western Atlantic coast is still scarce (Horta *et al.* 2011). Taylor (1960) first reported *Lithophyllum* pointing out the occurrence of *L. pustulatum* (Lamouroux) Foslie (1904:8) on the rocky shores of the southeastern Brazilian coast. More recently species of *Lithophyllum* were cited in some studies in tropical and subtropical environments such as coral reefs, rocky shores (Figueiredo & Steneck 2002, Tâmega & Figueiredo 2005, Nunes *et al.* 2008) and rhodolith beds (Figueiredo & Steneck 2002, Rocha *et al.* 2006, Horta *et al.* 2008, Riul *et al.* 2009, Villas-Boas *et al.* 2009). However, most of these studies focused on the geological and ecological significance of non-geniculate coralline algae. Detailed comparative systematics through morphological and anatomical studies are presented by Nunes *et al.* (2008) and Villas-Boas *et al.* (2009), in which the latter also describes *L. depressum* Villas-Boas, Figueiredo & Riosmena-Rodriguez (2009: 245), based on anatomical features from a shallow rhodolith bed in southeastern Brazil.

Given the morphological plasticity and the dependence of diagnostic characteristics based on the reproductive structures (Woelkerling *et al.* 1983, Riosmena-Rodriguez *et al.* 1999), alternative methods are necessary to

investigate the systematics of *Lithophyllum* species and other non-geniculate coralline red algae. Therefore, a DNA-based approach in combination with detailed anatomical characterization is fundamental for accurate systematic evaluation of this group (Vidal *et al.* 2003). Herein, we used three markers to assess the diversity among *Lithophyllum* species in the Southern Brazilian coast: *cox1* (the 5' region of the mitochondrial cytochrome c oxidase subunit I gene, Saunders 2005), UPA (the Universal Plastid Amplicon, Sherwood & Presting 2007, Sherwood *et al.* 2008, Sherwood *et al.* 2010) and the nuclear encoded SSU (Bailey & Chapman 1998, Bailey 1999). Our goal is to describe a new species of the genus *Lithophyllum* from the Brazilian coast and to extend the range distribution of *Lithophyllum margaritae* based on morphological, anatomical and molecular analyses.

Materials and methods

Collections. Specimens examined were collected from November 2009 to December 2010 along the subtropical western Atlantic coast located between the Paraná and Rio Grande do Sul states. In Paraná, samples were collected in the subtidal of Currais Archipelago. In Santa Catarina State, samples were collected from the intertidal rocky shore, at Ponta das Canas beach and in the subtidal from the rhodolith bed in Arvoredo Marine Biological Reserve and also from sandy bottom in Xavier Island. In the Rio Grande do Sul encrusting specimens were collected from the intertidal rocky shore in Guarita beach. Subtidal collections were taken by SCUBA dives. Collections have been incorporated in the Herbarium of University of Santa Catarina—FLOR (Thiers 2014) (Table 1).

TABLE 1. Collection data for newly generated sequences and sequences from the referenced studies used in this study. Thallus form, “e” for encrusting and “r” for rhodolith.

Taxa (Sample ID) thallus form	Locality	Voucher	GenBank Accession No.		
			UPA	<i>cox1</i>	SSU
<i>Lithophyllum atlanticum</i> (1) r	Brazil, SC, Arvoredo Island 27°16.32'S, 48°22.74'W	FLOR 14568	KP192371	-	KP192386
<i>Lithophyllum atlanticum</i> (2) r	Brazil, SC, Arvoredo Island 27°16.32'S, 48°22.74'W	FLOR 14567	KP192372	KP192393	KP192387
<i>Lithophyllum atlanticum</i> (3) r	Brazil, SC, Arvoredo Island 27°16.32'S, 48°22.74'W	FLOR 14569	KP192373	KP192394	KP192388
<i>Lithophyllum atlanticum</i> (7) e	Brazil, RS, Torres, Guarita Beach 29°21.63'S, 49°44.98'W	FLOR 14566	KP192374	-	KP192389
<i>Lithophyllum atlanticum</i> (14) e	Brazil, SC, Xavier Island 27°36.34'S, 48°23.13'W	FLOR 14575	KP192375	-	-
<i>Lithophyllum atlanticum</i> (C2) e	Brazil, SC, Ponta das Canas beach 27°24.89'S, 48°26.78'W	FLOR 14574	KP192376	KP192395	-
<i>Lithophyllum atlanticum</i> (C6) e	Brazil, SC, Ponta das Canas beach 27°24.89'S, 48°26.78'W	FLOR 14576	KP192377	KP192396	KP192390
<i>Lithophyllum margaritae</i> (4) r	Brazil, SC, Arvoredo Island 27°16.32'S, 48°22.74'W	FLOR 14570	KP192378	KP192397	KP192391
<i>Lithophyllum margaritae</i> (5) r	Brazil, SC, Arvoredo Island 27°16.32'S, 48°22.74'W	FLOR 14571	KP192379	KP192398	KP192392
<i>Lithophyllum margaritae</i> (28) r	Brazil, SC, Arvoredo Island 27°16.32'S, 48°22.74'W	FLOR 14580	KP192380	-	-
<i>Lithophyllum margaritae</i> (31) r	Brazil, SC, Arvoredo Island 27°16.32'S, 48°22.74'W	FLOR 14581	KP192381	-	-
<i>Sporolithon</i> sp.1 (4P) r	Brazil, PB, João Pessoa, 7°09,18'S, 34°47.35'W	IBC 1519	KP192382	-	-
<i>Sporolithon</i> sp.1 (5P) r	Brazil, PB, João Pessoa, 7°09,18'S, 34°47.35'W	IBC 1520	KP192383	-	-
<i>Sporolithon</i> sp.1 (R2) r	Brazil, PE, Aleixo Island, 8°36.42'S, 35°01.23'W	IBC 1522	KP192384	-	-
<i>Sporolithon</i> sp.1 (R2) r	Brazil, PE, Aleixo Island, 8°36.42'S, 35°01.23'W	IBC 1523	KP192385	-	-
<i>Amphiroa</i> sp.	Australia	Bailey & Chapman 1998	-	-	U62115
<i>Amphiroa foliacea</i>	Hawaii	Sherwood <i>et al.</i> 2010	-	HQ422626	-
<i>Amphiroa fragilissima</i>	Guatemala	Bittner <i>et al.</i> 2011	-	GQ917303	-

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TABLE 1. (Continued)

Taxa (Sample ID) thallus form	Locality	Voucher	GenBank Accession No.		
			UPA	<i>cox1</i>	SSU
<i>Amphiroa fragilissima</i>	USA	Bailey & Chapman 1998	-	-	U60744
<i>Amphiroa valanoides</i>	Hawaii	Sherwood <i>et al.</i> 2010	HQ421023	HQ422698	-
<i>Lithophyllum sp.</i>	Vanuatu	Bittner <i>et al.</i> 2011	-	GQ917263	-
<i>Lithophyllum sp.</i>	Fiji	Bittner <i>et al.</i> 2011	-	GQ917282	-
<i>Lithophyllum sp.</i>	Fiji	Bittner <i>et al.</i> 2011	-	GQ917277	-
<i>Lithophyllum sp.</i>	New Zealand	Broom <i>et al.</i> 2008	-	-	EF628242
<i>Lithophyllum cf. bamleri</i>	Fiji	Bittner <i>et al.</i> 2011	-	GQ917281	-
<i>Lithophyllum incrustans</i>	France	Bittner <i>et al.</i> 2011	-	GQ917250	-
<i>Lithophyllum incrustans</i>	UK	Bailey 1999	-	-	AF093410
<i>Lithophyllum insipidum</i>	Hawaii	James, S. A. unpublised	-	-	DQ628977
<i>Lithophyllum insipidum</i>	Hawaii	Sherwood <i>et al.</i> 2010	-	HQ423068	-
<i>Lithophyllum insipidum</i>	Hawaii	Sherwood <i>et al.</i> 2010	HQ421555	HQ423075	-
<i>Lithophyllum insipidum</i>	Hawaii	Sherwood <i>et al.</i> 2010	HQ420970	HQ422710	-
<i>Lithophyllum insipidum</i>	Hawaii	Sherwood <i>et al.</i> 2010	HQ420966	-	-
<i>Lithophyllum kotschyanum</i>	Hawaii	Sherwood <i>et al.</i> 2010	HQ421024	-	-
<i>Lithophyllum kotschyanum</i>	Hawaii	Sherwood <i>et al.</i> 2010	-	HQ423072	-
<i>Lithophyllum cf. pygmaeum</i>	New Caledonia	Bittner <i>et al.</i> 2011	-	GQ917268	-
<i>Lithophyllum stictaeforme</i>	New Zealand	Broom <i>et al.</i> 2008	-	-	EF628241
<i>Lithothrix aspergillum</i>	Canada	Hind & Saunders 2013	-	JQ615866	-
<i>Lithothrix aspergillum</i>	Canada	Hind & Saunders 2013	-	JQ615865	-
<i>Mesophyllum erubescens</i>	Hawaii	James, S. A. unpublised	-	-	DQ629012
<i>Pneophyllum cf. conicum</i>	Hawaii	James, S. A. unpublised	-	-	DQ628994
<i>Sporolithon ptychoides</i>	Hawaii	Sherwood <i>et al.</i> 2010	-	HQ422711	-
<i>Titanoderma prototypum</i>	Hawaii	Sherwood <i>et al.</i> 2010	-	HQ423070	-
<i>Titanoderma pustulatum</i>	UK	Bailey 1999	-	-	AF093409

Light and scanning electron microscopy. All specimens used for both light and electron microscopy were previously fixed in 4% formaldehyde/seawater. Permanent slides for light microscopy were prepared following procedures adapted from Moura *et al.* (1997) and Horta (2002). Stubs for Scanning Electron Microscopy (SEM) were prepared following Chamberlain (1993) and analyzed using a SEM (JEOL JSM-6390LV). Voltage used ranged from 15–25 kV, seeking the best resolution. Conceptacle measurements follow Chamberlain (1983). Growth form terminology follows Woelkerling *et al.* (1993), and anatomical terminology follows Woelkerling (1988).

Molecular studies

Samples included in this study, including specimen voucher numbers and GenBank accession numbers for newly generated sequences, as well as for specimens from the referenced studies, are presented in Table 1. Sequences from GenBank were included in this study in order to provide phylogenetic context. Four sequences of *Sporolithon* sp. were included in the UPA analysis to test the distinction between *Lithophyllum* specimens and specimens with a similar thallus habit found along the Brazilian coast.

DNA Extraction and PCR amplification. Specimens were manually cleaned to eliminate epiphytes and other contaminants, dried in silica gel, and ground to a powder in liquid nitrogen. Total DNA was extracted using the kit “NucleoSpin Plant II” (Macherey-Nagel, Düren, Germany) according to the manufacturer’s instructions. Mitochondrial *cox1* was amplified and sequenced using the primers GazF1 and GazR1 and protocols as described by Saunders (2005), plastidial UPA was amplified and sequenced using the primers p23Sv_f1 and p23Sv_r1 and protocols as described by Sherwood & Presting (2007) and nuclear SSU was amplified and sequenced using the primers and protocols as described by Bailey and Champan (1998).

Alignment. Sequences were assembled and edited in BioEdit version 5.0.6 (Hall 1999), and chromatograms were checked to confirm the validity of ambiguous nucleotides. UPA and *cox1* sequences were aligned manually with MacClade 4.08 (Maddison & Maddison 2000) and SSU sequences were initially aligned in MacClade 4.08 then exported and aligned using the CLUSTAL W (Thompson *et al.* 1994) program in MEGA 5.2.2 (Tamura *et al.* 2011). Ambiguous regions in the SSU alignment were cropped to the nearest conserved region. A UPA matrix was constructed with 20 sequences and was 370 bp in length. A *cox1* matrix of 23 sequences and 664 bp in length was constructed. The SSU rDNA matrix was 1612 bp in length and included 17 sequences, with sequences for *Mesophyllum erubescens* (Foslie) Me. Lemoine (1928: 252) and *Pneophyllum cf. conicum* (Dawson) Keats, Y.M. Chamberlain & Baba (1997: 264), used as outgroups.

Phylogenetic Analysis. Neighbor-joining analyses (NJ) for UPA and *cox1* data were conducted in MEGA 5.2.2 (Tamura *et al.* 2011) using the Maximum Composite Likelihood model with 2,000 bootstrap replicates to assess branch support. Bayesian analysis was conducted on SSU rDNA using the MrBayes 3.2.1 program (Huelsenbeck and Ronquist 2001). The appropriate evolutionary model was selected in MrModeltest 2.2 (Nylander 2004). Two parallel runs of four MCMC chains consisting of three heated chains and one cool chain were carried out with 4×10^6 generations and resampling every 1,000 generations resulting in a total of 80,002 trees. We discarded the first 50,000 generations as "burn-in" and a consensus tree was built using the remaining data. Sequences generated on this study were deposited on Genbank (for more details see Table 1).

Results

In this study two species were identified using the modern anatomical concepts following Woelkerling and Campbell (1992) that circumscribe the genus *Lithophyllum*, as follows: (1) cells of contiguous vegetative filaments joined by secondary pit-connections, (2) fusions between vegetative cells absent or not observed, (3) lack of genicula and haustoria, (4) lack of isobilateral organization, (5) tetrasporangial conceptacles uniporate. Also, molecular data were used as further evidence to distinguish the specimens in this study from the previously reported molecular entities. We have found *Lithophyllum margaritae* (Figs. 1–10; Table 2), which has had its distributional range extended from the Mexican Pacific to the Atlantic, and *Lithophyllum atlanticum* sp. nov. (Figs. 11–19; Table 2).

Lithophyllum margaritae (Hariot) Heydrich (1901: 530).
FIGURES 1–10.

Basionym:—*Lithothamnion margaritae* Hariot (1895:167)
Type Locality:—MEXICO. La Paz Bay-Gulf of California.

Homotypic and heterotypic synonyms follow Riosmena-Rodriguez *et al.* (1999).

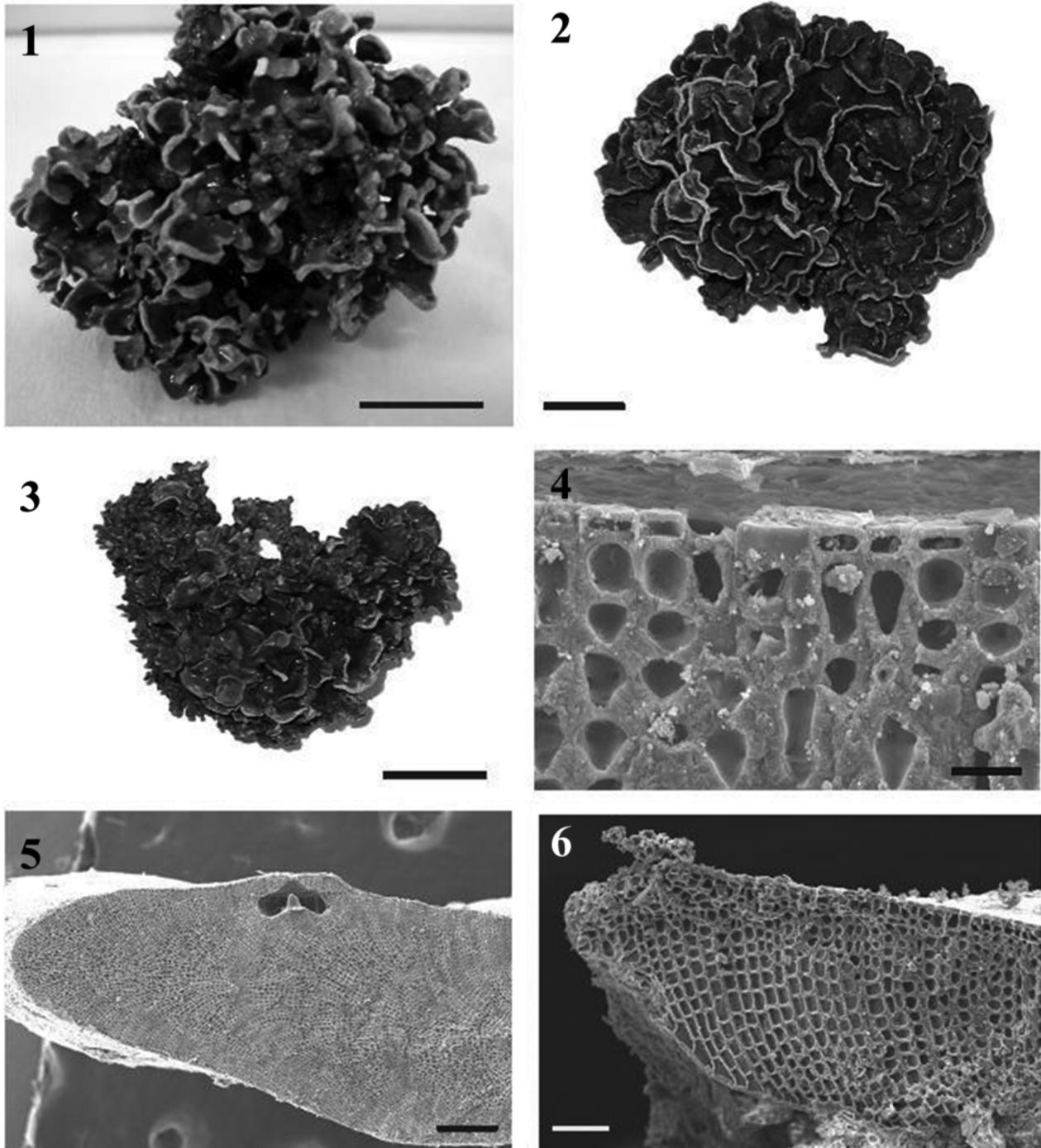
Distribution:—Gulf of California, Mexico (Riosmena-Rodriguez *et al.* 1999); São Paulo (personal communication P. A. Horta, unpublished data) and Santa Catarina, Brazil (present study).

Specimens studied:—BRAZIL, Santa Catarina: Florianópolis, Arvoredo Island, 10–15 m, 27°16.32'S, 48°22.74'W, 20 November 2009, FLOR 14570–14571. 20–13 July 2010, FLOR 14580–14581.

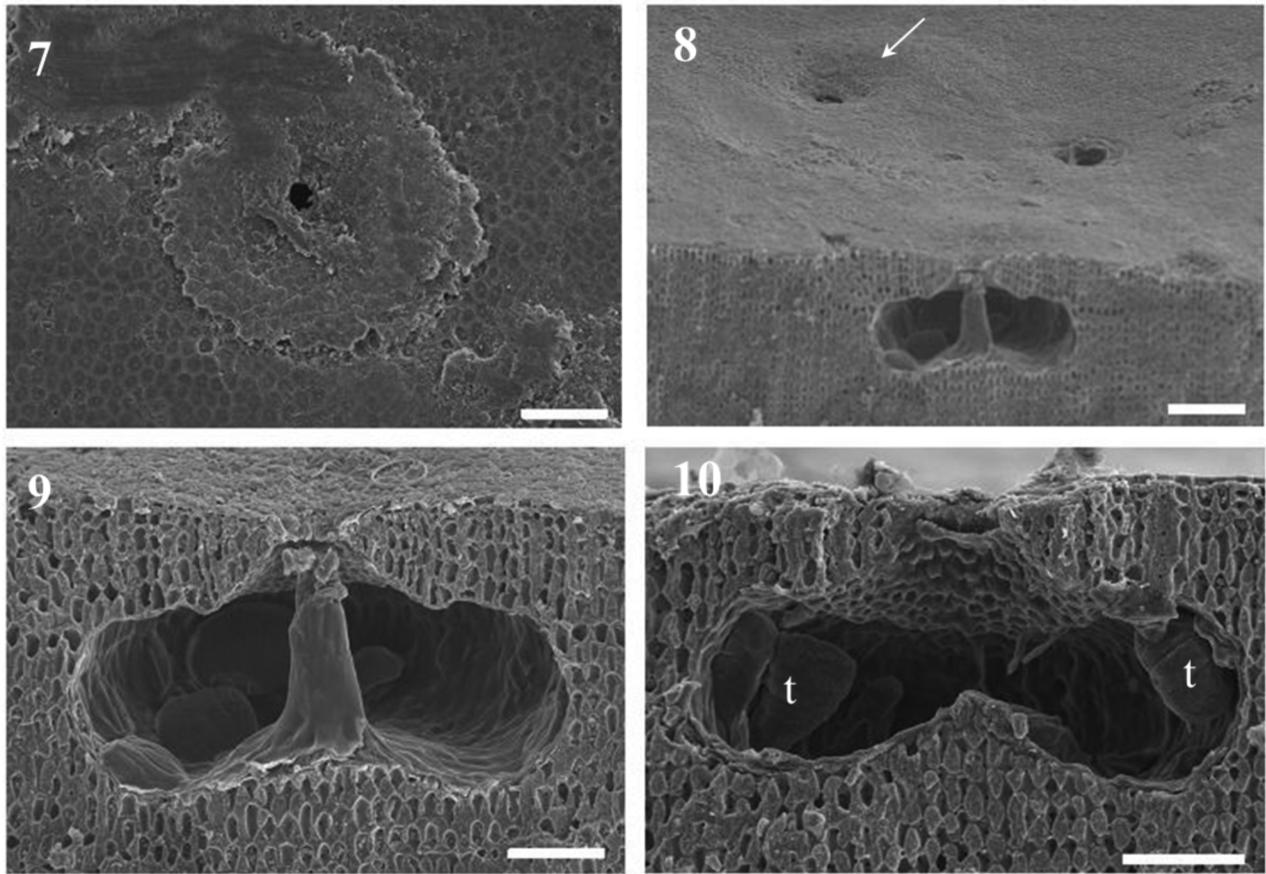
Vegetative features: Plants non-geniculate, purple to pink, rhodolith-forming with encrusting young parts. Rhodoliths with irregular shapes, ranging from fruticose to foliose, measuring 3–8 cm in diameter (Figs. 1–3). Pseudoparenchymatous internal thallus construction with dimerous organization in the crustose parts (growth margins) and monomerous organization in the protuberances with a central core of branched filaments with elongate cells (Figs. 5 and 6). Rectangular epithallial cells with flattened walls measuring from 1.5–5 μm in height and 5–13 μm in diameter, subepithallial cells 4–13 μm in height and 4–8 μm in diameter. Perithallial cells 7–16 μm in height and 3–10 μm in diameter, cells of adjacent filaments joined only by secondary pit-connections (Fig. 4). Cell fusions and trichocytes were not observed.

Reproductive features: Tetrasporangia born in uniporate conceptacles (Fig. 7) flat or slightly sunken in relation to the thallus surface (Fig. 8). Chambers of these conceptacles are 180–260 μm in diameter and 70–95 μm in height (Fig. 9). The filaments of the conceptacle roof are 3–5 cells thick (including the epithallial cell) and the

filaments surrounding the conceptacle are 10–14 cell layers thick from the epithelial cell to the chamber base (Fig. 9 and 10). Chambers often presenting calcified central columella which, in some conceptacles, project into the pore channels (Fig. 9). Buried conceptacles were observed in older portions of the thallus. Pore channels delimited by cells, but in SEM view not occluded by them; zonate tetrasporangia 40–60 μm in height and 15–35 μm in diameter (Fig. 10). Gametophytes not observed.



FIGURES 1–6. Vegetative features of *Lithophyllum margaritae* (FLOR14570, FLOR14580). Fig. 1: Fruticose growth form. Figs.2–3: Foliose growth form. Fig. 4: Cross-section showing epithelial cells with flattened walls. Fig. 5: Cross-section showing monomerous organization. Fig. 6: Cross-section showing dimerous organization in young portions of the thallus (growth margin–arrow). Scale bars: Fig. 1 = 2 cm; Fig. 2 = 2 cm; Fig. 3 = 2 cm; Fig. 4 = 10 μm ; Fig. 5 = 200 μm ; Fig. 6 = 50 μm .

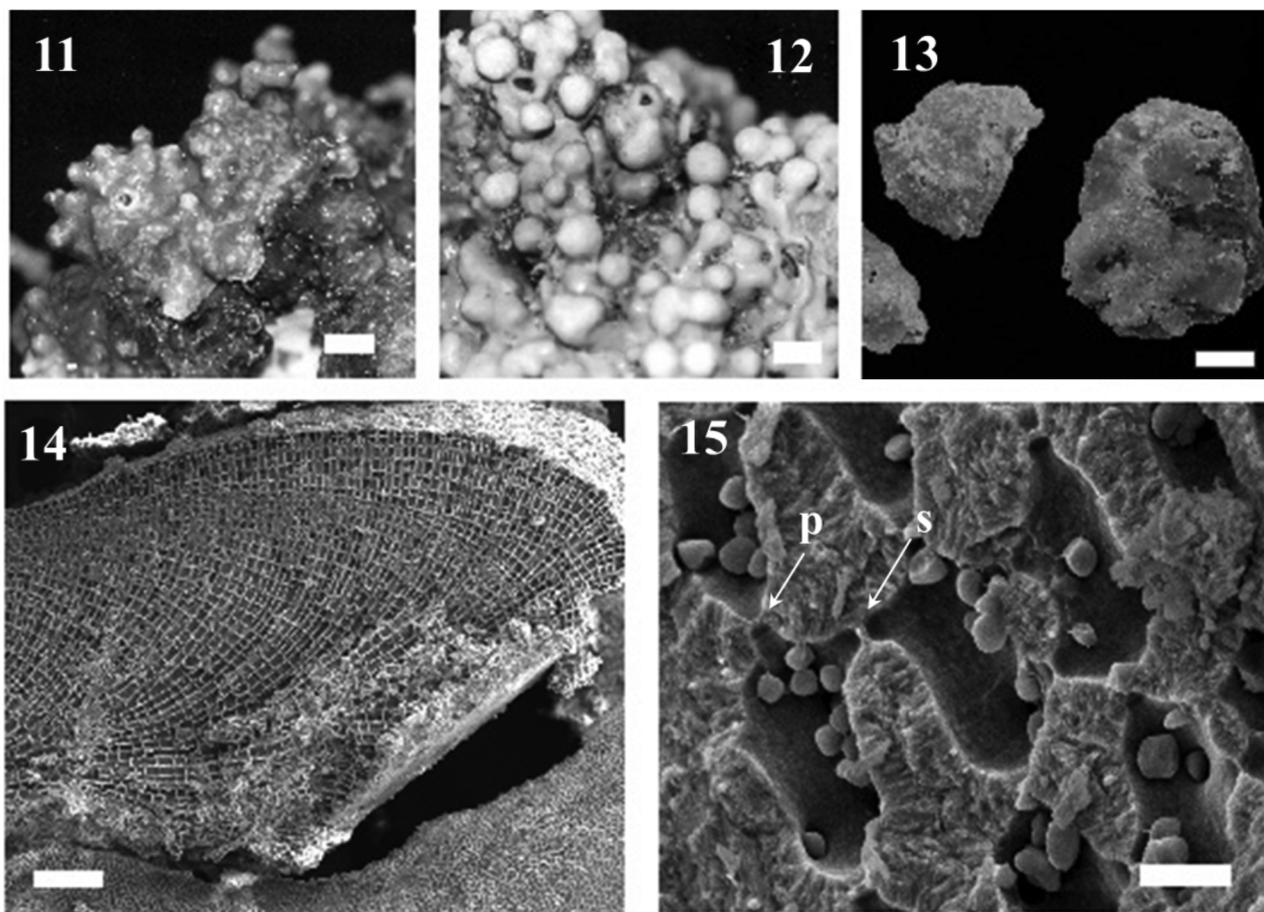


FIGURES 7–10. Reproductive features of *Lithophyllum margaritae* (FLOR14581). Fig. 1: Surface view of a uniporate tetrasporangial conceptacle. Fig. 8: Cross-section and surface view showing a conceptacle in the same plane and another slightly sunken in relation to the thallus surface (arrow). Fig. 9: Tetrasporangial conceptacle with calcified central columella. Fig. 10: Conceptacle with zonate tetrasporangia (t). Scale bars: 7 = 50 μm ; Fig. 8 = 100 μm ; Fig. 9 = 50 μm ; Fig. 10 = 50 μm .

TABLE 2. Comparison of species of *Lithophyllum* that share characters with Brazilian species (present study)–1) Keats 1997; 2) Keats 1997- according to scale; 3) Athanasiadis 1999; 4) Riosmena-Rodriguez 1999; 5) Furnari *et al.* 1996; 6) Nunes *et al.* 2008 7) Farr *et al.* 2009–according to scale ; 8) Harvey *et al.* 2009; 9) Villas-Boas *et al.* 2009. Value in parenthesis indicate minimum and maximum observed outliers.

	<i>L.</i> <i>margaritae</i> Present study	<i>L.</i> <i>atlanticum</i> Present study	<i>L.</i> <i>stictaeforme</i> (3;5,6;9)	<i>L.</i> <i>margaritae</i> (4)	<i>L.</i> <i>cabiochiaie</i> (5)	<i>L.</i> <i>incrustans</i> (1;3;5)	<i>L.</i> <i>johansenii</i> (1;7;9)	<i>L.</i> <i>pustulatum</i> (7)	<i>L.</i> <i>insipidum</i> (2)	<i>L.</i> <i>corallinae</i> (8)	<i>L.</i> <i>depressum</i> (9)
Tetrasporangial conceptacle chamber diameter (μm)	180–220	(295) 315–345	200–450 (475)	150–450	290–335 (390)	230–360	95–145 (216)	185–300	200–220	160–260 (340)	232–252
Tetrasporangial conceptacle chamber height (μm)	70–95	(75) 90–130	120–182	50–150	86–140	91–210	-	94–109	85–100	55–95 (135)	120–150
Number of cells in filaments of conceptacle roof (including epithallial cell)	3–5	4–7	5–9	3–4	5–8	6–17	2–4	1–2	4–7	2–4	6–8
Number of cells of filaments surrounding conceptacle (from epithallial cell to chamber base)	10–14	9–13	14–15	11–12	-	13–15	6–8(10)	3–4 (6)	6–9	6–11	12–14

Ecological observations. The specimens were found only as free-living rhodoliths in a rhodolith bed in the Arvoredo's Marine Biological Reserve (Florianópolis, Santa Catarina) at depths ranging from 10–15 m; no differences were observed in the samples during winter and summer seasons.



FIGURES 11–15. Vegetative structures of *Lithophyllum atlanticum* (FLOR 14567, FLOR 19569, FLOR 14573, FLOR 14577). Fig. 11: Warty growth form. Fig. 12: Lumpy growth form. Fig. 13: Encrusting growth form. Fig. 14: Cross-section showing monomerous organization in the growth margin. Fig. 15: Cross-section showing primary (p) and secondary (s) pit-connections. Scale bars: Fig. 11 = 5 mm; Fig. 12 = 5 mm; Fig. 13 = 5 mm; Fig. 14 = 20 μ m; Fig. 15 = 100 μ m.

***Lithophyllum atlanticum* Vieira-Pinto, Oliveira et Horta sp. nov.**

FIGURES 13–19.

Type Locality:—BRAZIL. Arvoredo Island, Santa Catarina. 27°16.32'S, 48°22.74'W.

Holotype: Tetrasporic plant (FLOR 14567)

Isotype: Tetrasporic plant (FLOR 14568)

Taxonomic Synonym in Brazil: *Lithophyllum stictaeforme* (Areschoug) Hauck 1877 (for Santa Catarina state).

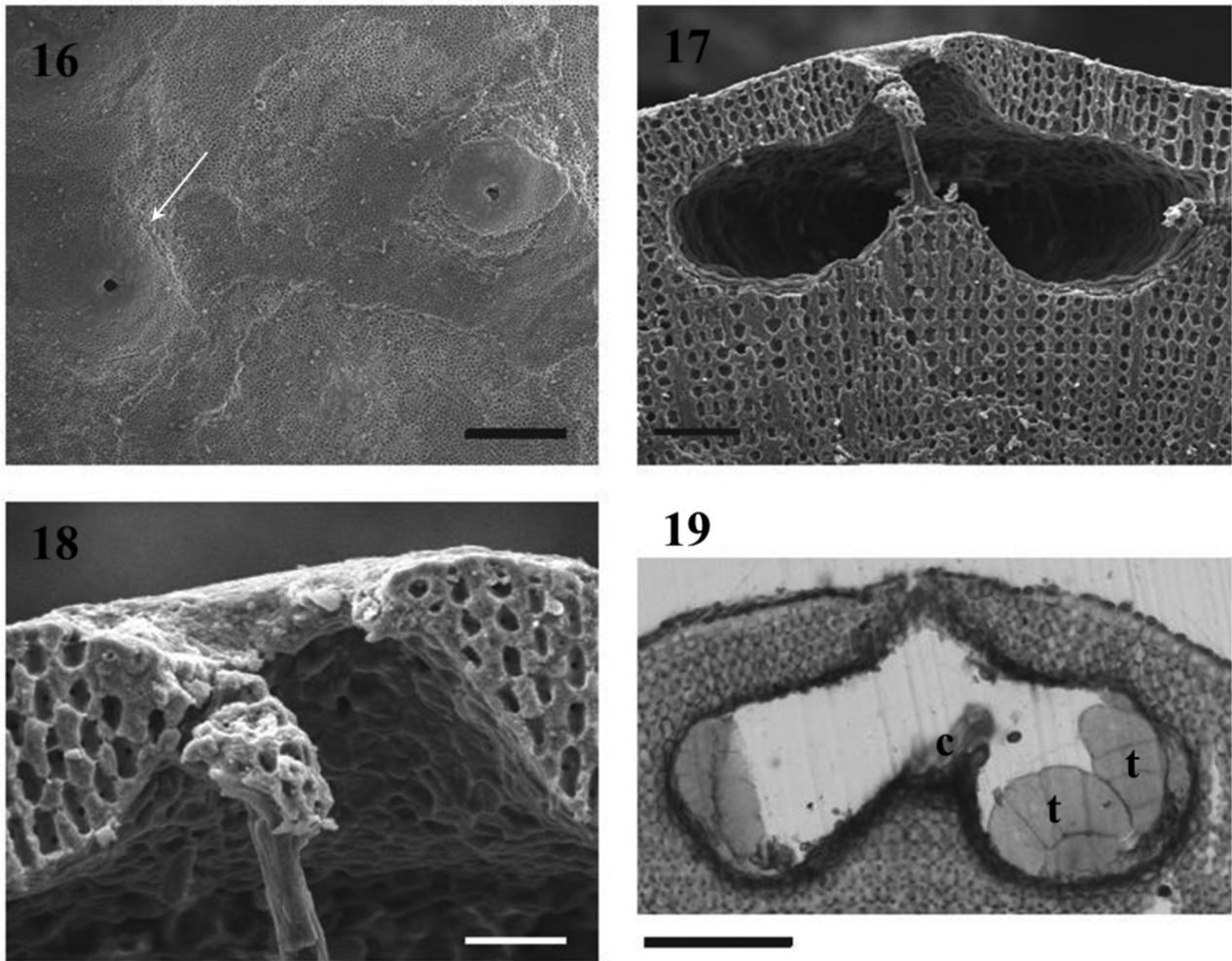
Etymology: *atlanticum* refers to the Atlantic Ocean.

Distribution: The species were found on the southern coast of Brazil in the States of Rio Grande do Sul, Santa Catarina and Paraná.

Diagnosis: The new species proposed here differs from other species of this genus mainly by presenting tetrasporangial conceptacles with 9–13 cells depth and 315–345 μ m diameter and 90–130 μ m height. GenBank accession numbers: UPA = KP192372; *cox1* = KP192393; SSU rDNA = KP192387.

Specimens studied:—BRAZIL. Santa Catarina: Arvoredo Island, 10–15 m, 27°16.32'S, 48°22.74'W, 13 July 2010, FLOR 14567-14569/14578-14579. Santa Catarina: Arvoredo Island, 10–15 m, 27°16.32'S, 48°22.74'W, 20

November 2009, FLOR 14570-14571. Santa Catarina: Florianópolis, Ponta das Canas Beach, 1m, 27°24.89'S, 48°26.78'W, 22 November 2010, FLOR 14574/14576. Rio Grande do Sul: Torres, 1m, 29°35.85'S, 49°73.32'W, 03 February 2010, FLOR 14566/14573. Paraná: Currais Archipelago 25°32.07'S, 48°19.52'W, March 2010, FLOR 14572/ 14577.



FIGURES 16–19. Reproductive features of *Lithophyllum atlanticum* (FLOR14567, FLOR14568, FLOR14566). Fig. 16: Surface view showing uniporate tetrasporangial conceptacles, in the same plane and another slightly raised in relation to the thallus surface (arrow). Fig. 17: Uniporate tetrasporangial conceptacle chamber; pore channel. Fig. 18: Detail of pore channel of the uniporate tetrasporangial conceptacle chamber. Fig. 19: Conceptacle with zonate tetrasporangia (t); non-calcified central columella (c). Scale bars: Fig. 16 = 200 μ m; Fig. 17 = 50 μ m; Fig. 18 = 20 μ m; Fig. 19 = 100 μ m.

Description

Vegetative features: Plants non-geniculate, can grow as rhodoliths, on sandy bottom, or as encrusting forms, slightly or fully attached to the substratum, found on rocky shores. Surface smooth or warty to lumpy, protuberances 3–5 mm in diameter and 5–10 mm in height (Figs. 11–13). Thallus pseudoparenchymatous with monomerous organization in older and younger portions (Fig. 14), and 1–2 layers of epithallial cells, 3–6 μ m in height and 6–11 μ m in diameter with flattened or concave distal walls. Subepithallial cells 6.5–13 μ m in height and 4.5 to 10 μ m in diameter. Perithallial filaments more-or-less perpendicular to hypothallial cells; perithallial cells 8 to 17 μ m in height and 5.5 to 11 μ m in diameter. Filaments are linked by secondary pit-connections (Fig. 15). Trichocytes were not observed.

Reproductive features: Tetrasporangia born in uniporate conceptacles, flush, slightly elevated or sunken in relation to the thallus surface (Fig. 16). Chambers of these conceptacles are 315–345 μ m in diameter and 90–130 μ m in height, the filaments of the conceptacle roof are 4–7 cell layers thick (including the epithallial cell) and the filaments surrounding the conceptacle are 9–13 cell layers thick from the epithallial cell to the chamber base (Figs.

17–18). Non-calcified central columella are frequently present (Fig. 19), zonate tetrasporangia are 46–68 µm in height and 15–33 µm in diameter (Fig. 19–t). Gametophytes not observed.

Ecological observations. This species was found forming rhodolith beds in the Marine Biological Reserve at 5–15 m depth. Encrusting forms found growing on rocky shores in epilithic and occasionally epizoic (mollusks) forms. In the intertidal region, specimens were found on the rocky shores and beaches located in Florianópolis city and surrounding islands off the coast, in Torres at Guartia beach, and in the Currais Archipelago at 5–10 m depth.

Molecular analyses

UPA

The UPA NJ analysis resulted in 15 newly generated sequences (Fig. 20). The NJ phylogram shows two groups with strong bootstrap support: the Lithophylloideae, collected in southeastern Brazil, represented by *Lithophyllum* and *Amphiroa*, and *Sporolithon* (Sporolithales) collected in northeastern Brazil. In the Lithophylloideae, encrusting and rhodolith forming specimens from Santa Catarina and Rio Grande do Sul of *L. atlanticum* (Arvoredo Island and Guarita Beach respectively) grouped with strong support. Another strongly supported clade includes 4 specimens of *L. margaritae* (Santa Catarina). Specimens of *L. inspidum* Adey, Townsend & Boykins (1982) from Hawaii formed another strongly supported group. UPA sequences of *L. atlanticum* and *L. margaritae* showed no intraspecific variation and the interspecific variation among *Lithophyllum* species ranged from 23–31 bp (6.2–8.4 %).

Cox1

The *cox1* NJ analysis resulted in 6 newly determined sequences (Fig. 21). Individuals of *L. atlanticum* (Santa Catarina) formed a clade with full support, including rhodolith forming and epilithic specimens. *L. margaritae* (Santa Catarina) sequences formed a clade with full support. None of the sequences of Lithophylloideae species from different locations of the world grouped with Brazilian specimens. Intraspecific variation of *cox1* sequences of *L. atlanticum* ranged from 0–1 bp (0–0.15%) while intraspecific variation of the 2 sequences of *L. margaritae* was 2 bp (0.3%). Interspecific variation among *Lithophyllum* species included in the analysis ranged from 64–118 bp (9.6–17.7%).

SSU rDNA

The results of the SSU rDNA analyses revealed seven sequences (1612 bp) of newly generated nuclear SSU rDNA (Fig. 22). The SSU rDNA phylogram shows results similar to the analyses of the other two markers. *Lithophyllum atlanticum* specimens grouped in a strongly supported clade separate from specimens identified as *L. margaritae*. Although the branching order of the major clades was only moderately supported (posterior probability (PP) = 0.6–0.68), several well supported lineages were observed. *Lithophyllum atlanticum* specimens comprised a monophyletic lineage. Sequences of *L. stictaeforme* and “*Lithophyllum* sp.” from New Zealand were sister to each other with full support, and did not group with *L. atlanticum* specimens from southern Brazil described in this study, indicating that this species is not conspecific with the specimens from the Brazilian coast. *L. in crustans* and *L. inspidum* (United Kingdom and Hawaii) comprised a clade sister to *L. stictaeforme* and *L. sp.* with moderate support (PP= 0.6). A moderately supported clade (PP= 0.85) within the Lithophylloideae included *L. margaritae* from Brazil and *L. kotschyannum* Unger (1858:22) from Hawaii as well as nested lineages comprised of *Titanoderma pustulatum* (Lamoroux) Nägeli (1858: 532) (United Kingdom), *Amphiroa* sp. (Australia) and *Amphiroa fragilisima* (Australia). Intraspecific variation of SSU rDNA sequences of *L. atlanticum* ranged from 0–3 bp (0–0.2%), while *L. margaritae* showed none. Among *Lithophyllum* species included in the analysis, interspecific variation ranged from 7–37 bp (0.4–2.3%), with 23–25 bp (1.4–1.55%) differences between *L. stictaeforme* and *L. atlanticum*.

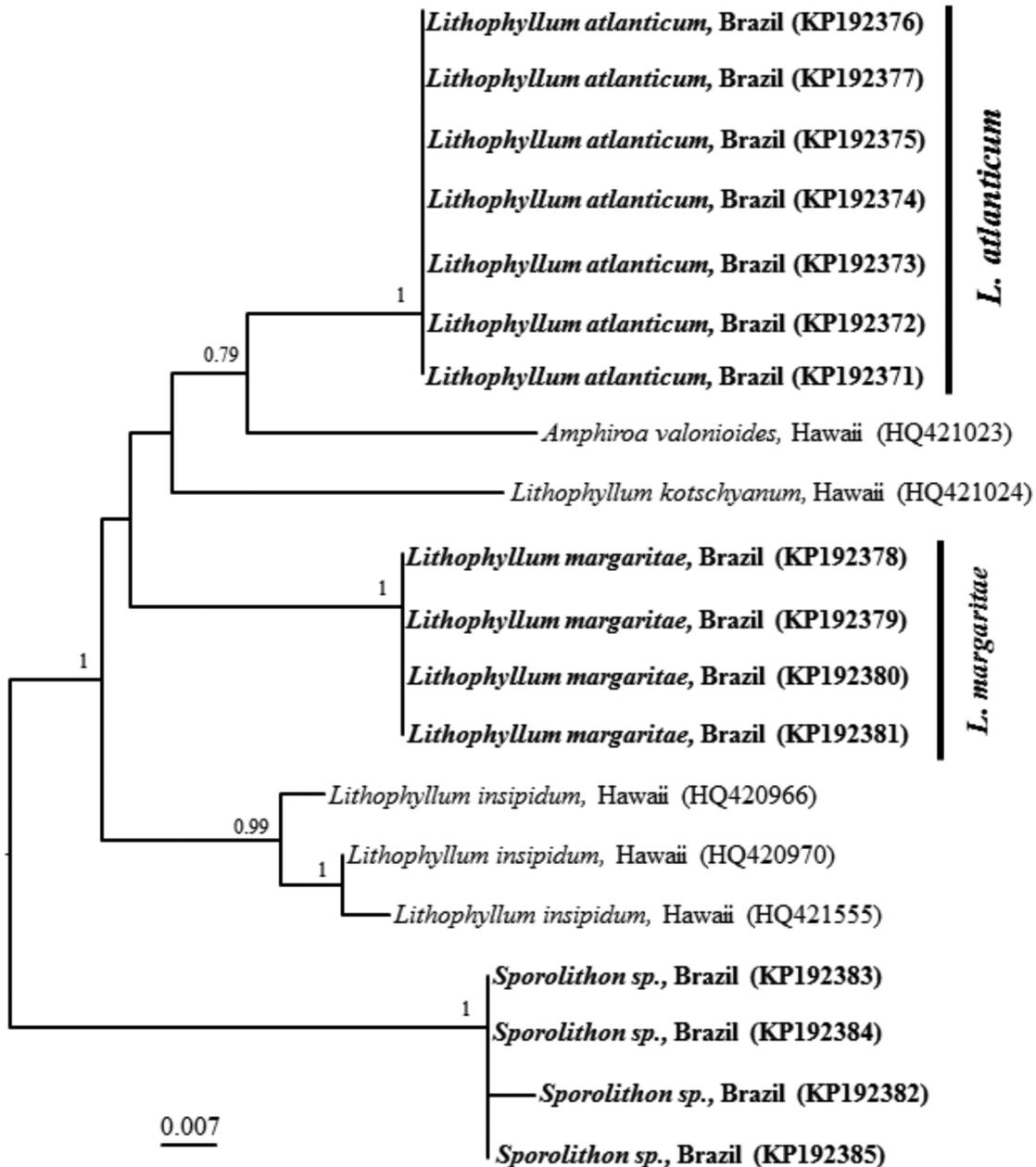


FIGURE 20. Unrooted neighbor-joining (NJ) phylogram of UPA sequences. Numbers at nodes indicate bootstrap values out of 2,000 replicates. Newly generated sequences shown in bold.

Discussion

Based on the results of the anatomical (Table 2) and molecular (Figs. 20–22) analyses, we propose the newly described species *Lithophyllum atlanticum* sp. nov. and report the presence of *L. margaritae* along the Brazilian coast. Our specimens were compared with similar taxa recently studied from Brazil and other parts of the world (Chamberlain *et al.* 1991, Woelkerling & Campbell 1992, Chamberlain 1996, Furnari *et al.* 1996, Woelkerling 1996, Chamberlain 1997, Keats 1997, Athanasiadis 1999, Riosmena-Rodriguez *et al.* 1999, Harvey *et al.* 2005, Farr *et al.* 2009, Harvey *et al.* 2009, Villas-Boas *et al.* 2009). DNA sequences are available for few species of this genus (Bailey 1999, Bittner *et al.* 2011), and for Corallinales in general, reinforcing that more molecular data are needed for a better characterization of this group. However, in this study, it was possible to separate the two species and also to clearly segregate *Lithophyllum stictaeforme* from the new species we propose in this study.

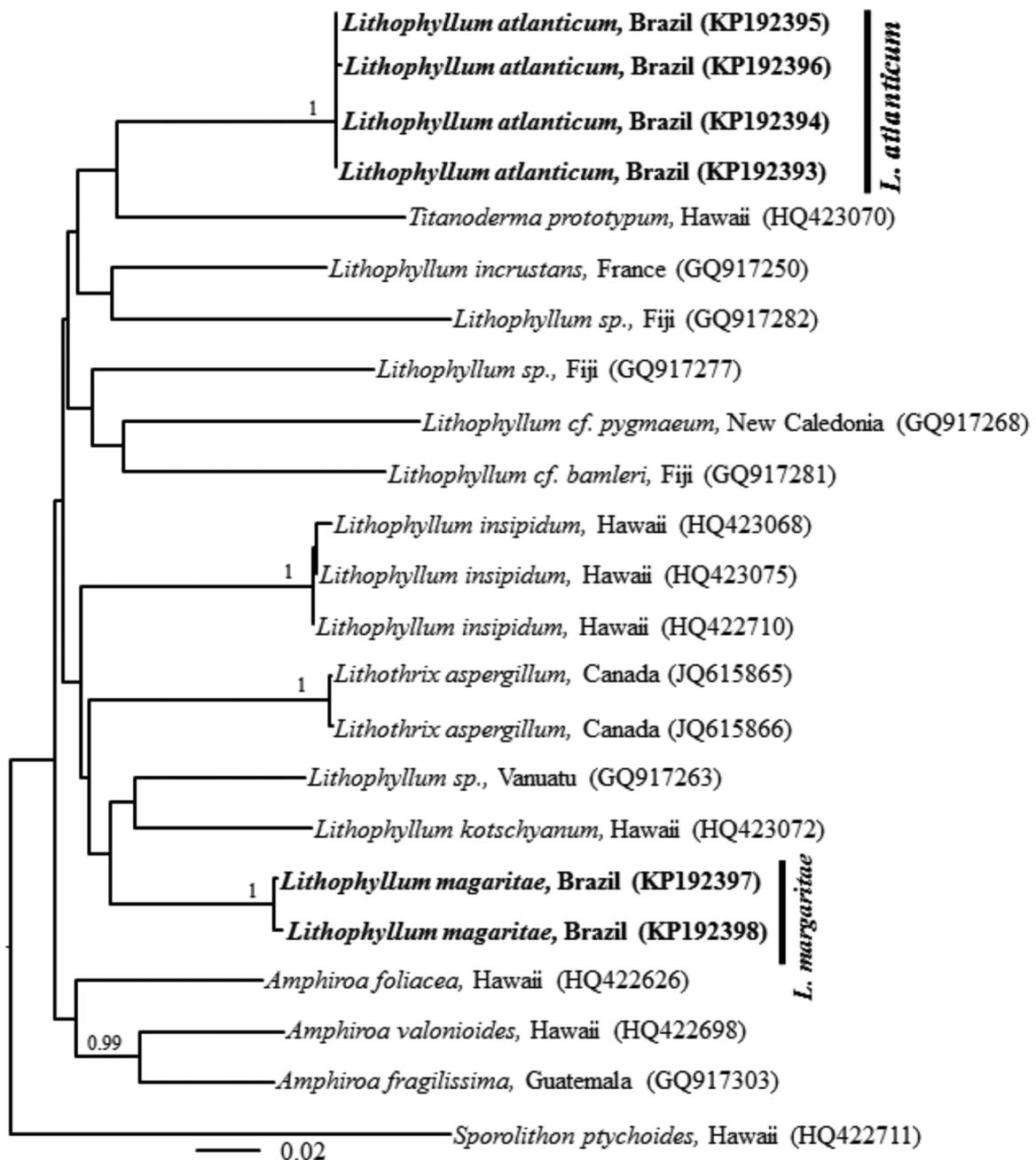


FIGURE 21. Unrooted neighbor-joining (NJ) phylogram of *cox1* sequences. Numbers at nodes indicate bootstrap values out of 2,000 replicates. Newly generated sequences shown in bold.

Lithophyllum atlanticum was found all along the southern Brazilian coast and, considering anatomical features and molecular data, it is proposed as a new species. Considering the species described so far for the Brazilian coast, *Lithophyllum stictaeforme* is one that most closely resembles *L. atlanticum*. *L. stictaeforme* (type locality Mediterranean Sea), is reported to have a widespread distribution and the main features which distinguish this species from the new species described here are shown in Table 2. Also, the divergence values (1.4–1.55%) between *L. stictaeforme* (specimen from NZ) and *L. atlanticum* in the SSU rDNA analyses provide further evidence to distinguish *L. atlanticum* as a new species. For example, Bailey (1999) reported 1.72% sequence dissimilarity between *Amphiroa fragilissima* and *Amphiroa sp.*, and congeneric sequence dissimilarities as low as 0.62% in other groups within the Corallinales. Furthermore, based on the geographical proximity and anatomical similarities with *L. atlanticum*, the specimens identified as *L. stictaeforme* found in other localities of Brazil

(Nunes *et al.* 2008, Villas-Boas *et al.* 2009), on the Northwest and Southwest Brazilian coast respectively, should be reexamined with a DNA-based approach to determine the genetic affinities among these two species. The results presented in this study reinforce that the use of molecular tools, especially DNA sequencing and barcoding, in combination with the morphological and anatomical characterization will be essential to understand the diversity of Corallinophycidae in Brazil and to unveil new species in this highly diverse group of red algae.

In the case of *Lithophyllum margaritae*, our findings corroborate that this species is widely distributed (Riosmena-Rodriguez *et al.* 1999) and that anatomical characteristics of the population from Baja California (Mexico) are very similar to those observed in the specimens analyzed in this study (as seen in Table 1). However, some differences were observed. For example, the dimerous organization observed for specimens in this study, especially at margins, is not described in the type specimen nor was observed in specimens from Baja California. In addition, these two populations differ in some aspects of the tetrasporangial conceptacles, which are quite smaller and present more cells from the thallus floor to the surface in the specimens from Brazil (Table 2). Therefore, DNA sequencing of type specimen of *Lithophyllum margaritae* is desirable in order to clarify the species boundaries between these closely related populations.

Overall, in this study we conclude that both the molecular and morphological evidence distinguishes *L. atlanticum* sp. nov. from other closely related species. However, to achieve a better understanding of species diversity within the Lithophylloideae from Brazil and throughout the world, ongoing DNA sequencing, especially for type specimens and of newly collected specimens from other parts of Brazil, is highly recommended.

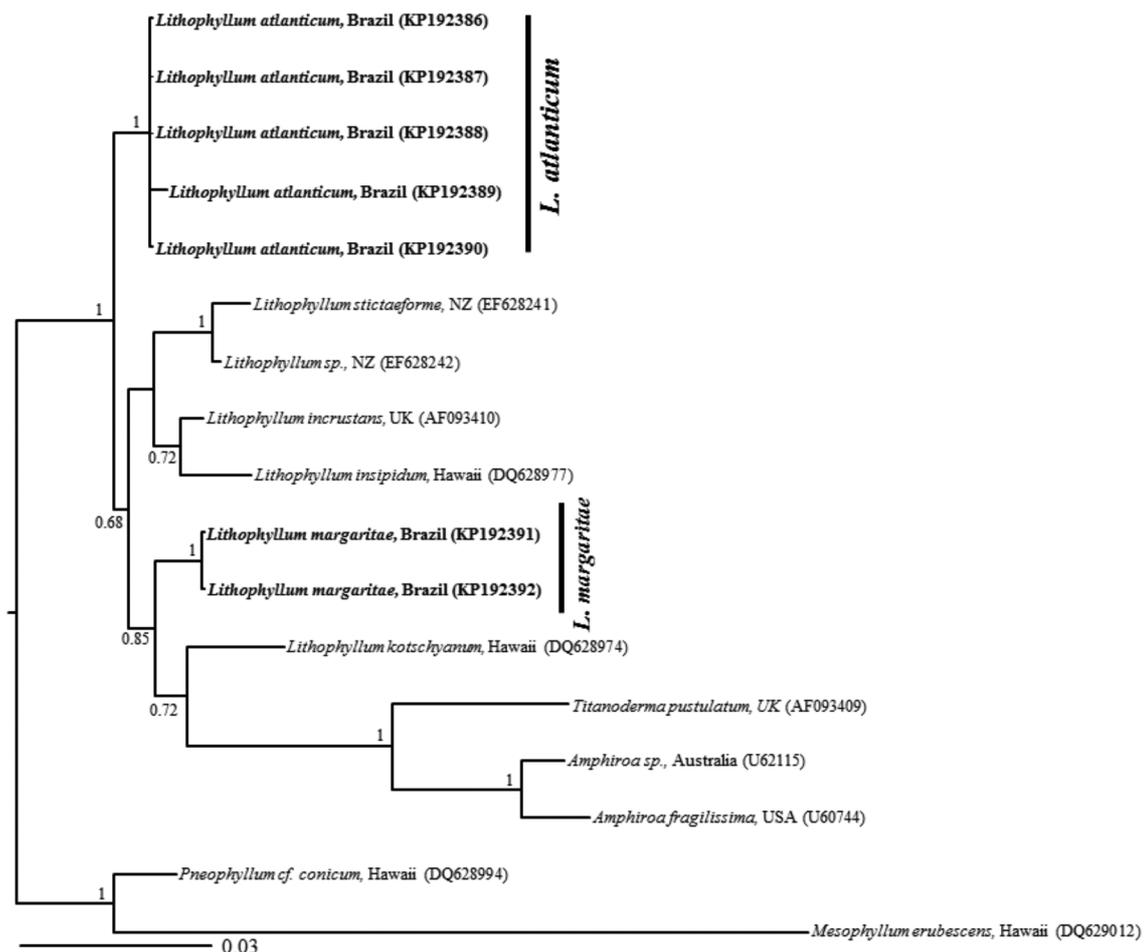


FIGURE 22. Phylogram inferred from Bayesian analysis of SSU rDNA. Numbers at nodes indicate Bayesian posterior probabilities. Newly generated sequences shown in bold.

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