



Systematic position of *Paulsilvella* in the Lithophylloideae (Corallinaceae, Rhodophyta) confirmed by molecular data

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Abstract

New specimens of *Paulsilvella huveorum* were collected in Brazil at Baía de Ilha Grande, Rio de Janeiro, and Sebastião Gomes reef, Bahia. These new collections represent a relevant range extension and new hosts for the species (*Amphiroa beauvoisii*, *Jania cubensis* and non-identified Hydrozoa and Bryozoa) and enabled the first DNA amplifications for *Paulsilvella*. The systematic position of *Paulsilvella* in the subfamily Lithophylloideae is confirmed based on SSU rDNA, *psbA* and *rbcL* molecular markers. Morphologically and anatomically the specimens are similar to the original description in which basal dimerous thalli with monomeric erect branches characterize the genus. But, the analyzed carposporangial conceptacles express the roof position varying from flush or above the thallus surface, the chambers always buried within tiers of columnar cells, suggesting that this feature is variable within the species and might also suggest that *P. huveorum* and the fossil *P. antiqua* should be considered as potential synonyms. We do not want to suggest this clump waiting for more collections from different geographical areas where new data may support our idea. Our results strongly suggest that the subfamily Lithophylloideae urgently needs to be reviewed to delimit genera based on molecular and morphological analysis because monomeric and dimeric thalli organization have evolved several times in the group.

Key words: Brazil, morphology, *Paulsilvella*, *Amphiroa*, *psbA*, *rbcL*, and SSU rDNA

Introduction

The current concept of Lithophylloideae Setchell (1943: 134) follows Cabioch (1972 and 1988), emended by Bailey (1999), in which secondary pit connections occur between neighbor cells, but fusions are not found—with the exception of two *Lithophyllum* Philippi (1837: 387) species with fused cells by Suneson 1943. This group includes six genera or genera complexes: *Amphiroa* J.V. Lamouroux (1812a: 185), *Ezo* Adey, Masaki & Akioka (1974: 331), *Lithophyllum/Titanoderma* Nägeli in Nägeli & Cramer (1858: 532), *Lithothrix* J.E. Gray (1867: 33), *Paulsilvella* Woelkerling, Sartoni & Boddi (2002: 359) and *Tenarea* Bory de Saint-Vincent, 1832: 207, in which *Amphiroa* and *Lithothrix* are geniculated, *Lithophyllum/Titanoderma* and *Tenarea* are completely crustose and *Paulsilvella* has a very unusual morphology among all other coralline algae: an encrusting inconspicuous base associated with erect branches presenting a monomerous form of growth (Woelkerling *et al.* 2002).

Paulsilvella was described as a new genus based on vegetative and reproductive anatomical data (Woelkerling *et al.* 2002). As a result of that work, *Paulsilvella* was positioned in the subfamily Lithophylloideae since there are undoubted secondary pit connections and cell fusions are absent. Moreover, one living species *Paulsilvella huveorum* Woelkerling, Sartoni & Boddi (2002: 359), was described and one fossil species *Paulsilvella antiqua* Woelkerling, Sartoni & Boddi (2002: 367) (from late Pleistocene, i.e., 126,000 to 11,500 years ago) was transferred to the genus. Living and fossil species were differentiated in possessing carposporangial and tetrasporangial conceptacles and roofs respectively flush or protruding, the former presenting the chambers buried within tiers of columnar cells. The modern species occurs as an epiphyte on the geniculate corallines *Amphiroa*, *Cheilosporum* (Decne.) Zanardini (1844: 187) and *Jania* J.V. Lamouroux (1812a: 186). *Amphiroa fragilissima* (Linn.) J.V. Lamouroux (1816: 298) was the commonest host (Woelkerling *et al.* op. cit.).

Species of *Paulsilvella* presented morphological features that positioned the new genus within Lithophylloideae in Corallinales P.C. Silva & H.W. Johansen (1986: 250), but modern systematic approaches should encompass both morphological and molecular approaches (Saunders 2008). The Lithophylloideae have long been proved monophyletic by molecular investigation (Bailey & Chapman 1996; Bailey & Chapman 1998; Vidal *et al.* 2003; Harvey *et al.* 2003; Broom *et al.* 2008; Bittner *et al.* 2011). The first phylogenetic studies on Corallinales which have included sufficient samples of Lithophylloideae to infer the internal relationships among genera were done by Bailey & Chapman (1998), which have shown that the geniculated taxa (*Amphiroa* and *Lithothrix*) formed a monophyletic group and *Lithophyllum* was basal to that clade. A subsequent approach by Bailey 1999 included one *Titanoderma* sequence, which surprisingly grouped with *Amphiroa* rather than with *Lithophyllum* specimens; and *Lithophyllum* was more closely related to *Lithothrix* samples than it was to *Titanoderma*. This has changed the hypothesis about the genicula emergence on the Lithophylloideae, suggesting that this morphological feature arose twice, supported by ontogenetical differences on *Amphiroa* and *Lithothrix* genicular cells. Two relevant inferences were made at 2003 by Harvey *et al.* and by Vidal *et al.*, in which *Amphiroa*, *Titanoderma* (and now also *Lithothrix*) kept on grouping together, prior to a separate *Lithophyllum* sister branch. Although complementary sequences on these datasets varied, the Lithophylloideae was always positioned as a sister clade to *Metagoniolithon* Weber-van Bosse, (1904: 86, 101), from the Metagoniolithoideae H.W. Johansen (1969: 47) subgroup, in Corallinaceae J.V. Lamouroux (1812b: 185). Bittner *et al.* (2011) have added many more samples to the matrix, resulting on the Lithophylloideae closer connexion to different groups from the “Mastophoroideae” Setchell (1943: 134)—the Mastophoroideae subgroup has shown to be non-monophyletic (Kato *et al.* 2011), but, since this group will be sparingly referred to in this work, and aiming to facilitate discussing, we will use the old acronym. In conclusion, all referred papers have shown Lithophylloideae monophyletic, but its relation to the Metagoniolithoideae and “Mastophoroideae” subgroups is unstable, varying according to: the number and molecular variability of samples included, as well as to the molecular markers used. At the present research we focused on the positioning of *Paulsilvella* in the Lithophylloideae. Therefore, the relationships among the subfamilies of the Corallinaceae are not prioritized.

Recent collections of *Paulsilvella* have been made at Baía de Ilha Grande (Rio de Janeiro) and Sebastião Gomes reef (Bahia), Brazil. This led us the opportunity to evaluate the systematic position of the genus using three molecular markers, including its position in the Lithophylloideae and inferring its evolutionary relation to other genera, and also to review the morphological and anatomical features of the collected material to add information to the species level.

Materials and methods

Collection

Samples from Ilha Grande island (Fig. 1) were obtained at 5–7 m depth by SCUBA diving. Algae turfs were preserved at a 70% ethanol solution to allow both DNA preservation and individuals subsampling. Samples from Sebastião Gomes reef (Fig. 1) were collected on tide pools from reef plateau area (0–0.5 m depth) at low tide, and preserved in 4% formalin in seawater. Representatives were deposited at the Phycological Herbarium of University of São Paulo (SPF; *Index Herbariorum* <http://sciweb.nybg.org/science2/IndexHerbariorum.asp>).

Morphological approach

Morphological identification of articulated coralline species follows Moura & Guimarães (2005) and *Paulsilvella* individuals follow Woelkerling *et al.* (2002). Anatomical concepts follow Harvey *et al.* (2005) and growth forms follow Woelkerling *et al.* (1993). Metacrilatoglicol (Leica®) resin embedded sample fragments were cut to 5–7 µm sections (Moura *et al.* 1997). For morphological final excerpt, each variable was measured 10 times.

Molecular procedures

We have elected a group of molecular markers to infer positioning of *Paulsilvella* in the Lithophylloideae based on previous published evidences on molecular relationships on the Corallinaceae. The *psbA* is the preferred marker used by Bittner *et al.* 2011, being also short and informative, in agreement with Broom *et al.* 2008; the SSU rDNA has a long history of usage on phylogeny inferences including the Corallinophycidae L. Le Gall & G.W.

Saunders (2007: 1129), therefore *Paulsilvella* SSU rDNA sequences will be very relevant to help on future complete datasets on Lithophylloideae; *rbcL* has traditionally been used for phylogeny inferences in Florideophyceae (Freshwater *et al.* 1994; 1999; Yoon *et al.* 2006; Le Gall & Saunders, 2010), but has never been tried on an exclusively Corallinophycidae investigation except with the Corallinoideae (Gabrielson *et al.* 2011; Martone *et al.* 2012). The host species *Jania rubens*, *Amphiroa* sp. and *A. fragilissima* were also sequenced to rule out possible cross contaminations with *P. huveorum*. The Universal Plastid Amplicon (UPA), a short and easy to obtain marker, was also sequenced as it has been proposed as universal DNA barcode for photosynthetic organisms (Presting 2006).

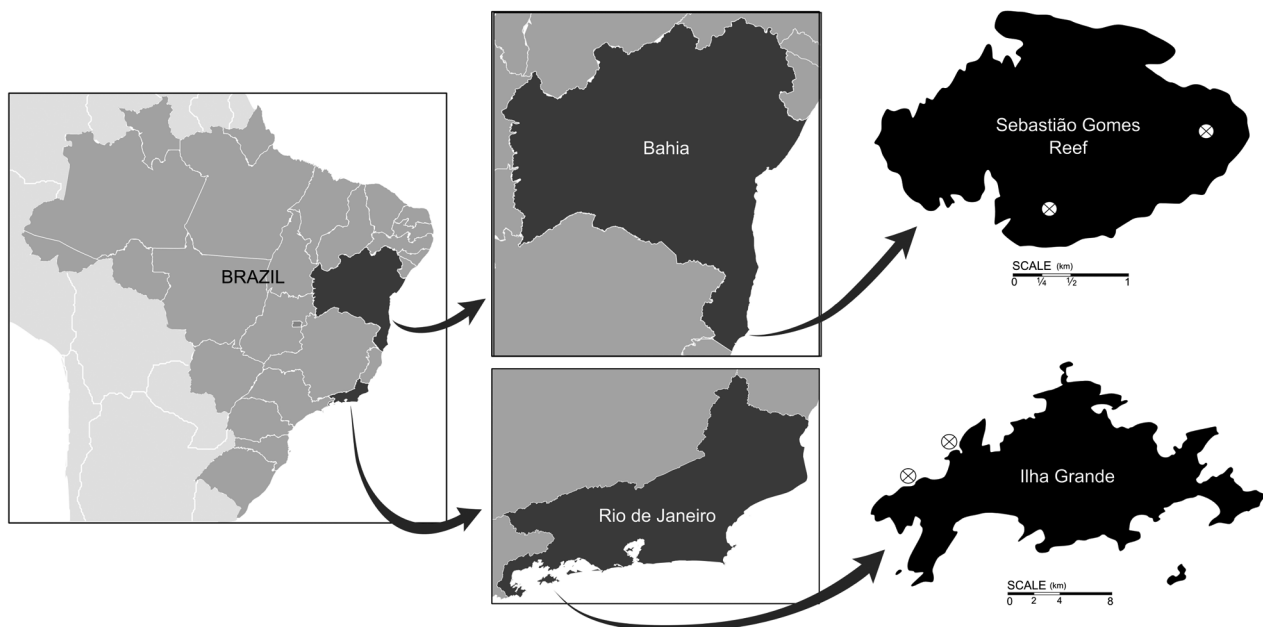


FIGURE 1. Sampling stations for *Paulsilvella* and its hosts in Brazil. Crossed white circles indicate collecting spots. Sebastião Gomes is a biogenic reef which is totally submerged during high tidal levels, while Ilha Grande is an island with subtidal rocky shores.

DNA extraction used a Chelex protocol (Goff & Moon 1993) and PCR was applied to obtain plastid (*psbA*, *rbcL* and UPA) and nuclear (SSU rDNA) markers. Primers were combined such as follows: for *psbA*, amplification used *psbAF1* × *psbAR2*, while sequencing used these and also 500F (Yoon *et al.* 2002) and the newly designed 550R (TTRTGTTTCRGCTGRAATAC); for *rbcL*, amplification used F57 × 897cR (CGTGAATATGTWGARTTACCDGC), 577F or 753F × *rbcS*-start and sequencing used these and 1150aR (Freshwater & Rueness 1994); for SSU rDNA, amplification used 18S5' × 1055R and 1055F × 18S3' and sequencing used these and also 530F and 536R (Milstein & Oliveira 2005); for UPA both amplifying and sequencing used p23SrV f1 × p23SrV r1 (Presting 2006). PCR cycles follow Saunders & Moore (2013; *psbA* and *rbcL*), Presting (2006, UPA) and Milstein & Oliveira (2005; SSU rDNA). PCR fragments were purified using illustra™ GTX™ PCR DNA and Gel Band Purification Kit (GE Healthcare, Buckinghamshire, UK) following the manufacturer's recommendations for direct sequencing. Sequencing reactions were performed with BigDye™ Terminator v3.1 Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and samples were loaded in an ABI 3730 DNA Genetic Analyzer (Applied Biosystems).

Consensus sequences were built by hand using BioEdit (Hall 1999), and chromatograms were checked to confirm the validity of ambiguous nucleotides. Datasets used for phylogeny inferences are: one with *psbA* and SSU rDNA combined, and another with *rbcL*. Additional individual analyses for *psbA* and SSU rDNA were also performed (see supplementary information). Matrices were aligned using Clustal W in BioEdit. Ambiguous regions on the nSSU matrices were removed to avoid inappropriate inferred relationships. Additional sequences were obtained in GenBank (Benson *et al.* 2013) to enrich the analysis. These sequences from GenBank were chosen concerning to the closest subfamilies to the Lithophylloideae (based on previous molecular data, *op. cit.*) and the groups where *Paulsilvella*'s hosts belong. For the *psbA* and SSU rDNA combined analysis we have selected only sequences which have used the same samples to amplify both molecular markers from the

Lithophylloideae, Corallinoideae, Mastophoroideae (including representatives from all subdivisions, as indicated in Kato *et al.* 2011) and Metagoniolithoideae subfamilies (Table 1). *RbcL* is represented by a much more limited number Corallinophycidae sequences available in GenBank, so that this dataset have failed to contain any Metagoniolithoideae or “Mastophoroideae” representatives (Table 2). Outgroups were *Mesophyllum engelhartii* (Foslie) W.H. Adey 1970: 23(JQ896243) for *psbA*, *M. engelhartii* (JQ896270) for SSU rDNA and *Mesophyllum vancouveriense* (HQ322337) for *rbcL*.

TABLE 1. List of new (in bold, including SPF herbarium number) and additional sequences for *psbA* and SSU rDNA datasets. Previously published sequences are referenced as follows: Bailey & Chapman 1998⁽¹⁾, Bailey 1999⁽²⁾, Harvey *et al.* 2003⁽³⁾, Vidal *et al.* 2003⁽⁴⁾, Bailey *et al.* 2004⁽⁵⁾, Broom *et al.* 2008⁽⁶⁾, Aguirre *et al.* 2010⁽⁷⁾, Bittner *et al.* 2011⁽⁸⁾, Kato *et al.* 2011⁽⁹⁾ and Hernandez-Kantun⁽¹⁰⁾ submitted.

Taxa	<i>psbA</i>	SSU rDNA
	GenBank accession no.	GenBank accession no.
CORALLINALES		
Corallinaceae		
Corallinoideae		
<i>Arthrocardia</i> sp.	EF628246 ^{6,9,10}	EF628230 ^{6,8,9,10}
<i>Cheilosporum sagittatum</i>	DQ167881 ^{6,9,10}	EF628226 ^{6,8,9,10}
<i>Corallina officinalis</i>	DQ168010 ^{6,9,10}	EF628232 ^{6,8,9,10}
<i>Haliptilon roseum</i>	EF628245 ^{6,9}	EF628229 ^{6,8,9}
<i>Jania</i> sp.	DQ167886 ^{6,9} / DQ167885 ^{6,9,10}	EF628227 ^{6,9,10} / EF628225 ^{6,9,10}
<i>Jania rubens</i> SPF57696 (host)	KM044019	KM044029
Lithophylloideae		
<i>Amphiroa</i> sp. SPF57697 (host)	KM044020	KM044030
<i>Amphiroa fragilissima</i>	GQ917498 ⁸	U60744 ^{1,2,3,4,6,7,8,10}
<i>Amphiroa fragilissima</i> SPF57694 (host)	KM044017	KM044027
<i>Amphiroa rigida</i>	JQ896250 ¹⁰	JQ896277 ¹⁰
<i>Amphiroa</i> sp.	GQ917472 ⁸	GQ917416 ^{8,10}
<i>Amphiroa</i> sp.	GQ917435 ⁸	GQ917380 ^{8,10}
<i>Amphiroa</i> sp.	GQ917491 ⁸	GQ917428 ⁸
<i>Lithophyllum</i> cf. <i>bamleri</i>	GQ917462 ^{8,10}	GQ917406 ^{8,10}
<i>Lithophyllum</i> cf. <i>bamleri</i>	GQ917473 ^{8,10}	GQ917417 ^{8,10}
<i>Lithophyllum byssoides</i>	JQ896251 ¹⁰	JQ896278 ¹⁰
<i>Lithophyllum dentatum</i>	JQ896255 ¹⁰	JQ896282 ¹⁰
<i>Lithophyllum dentatum</i>	JQ896237 ¹⁰	JQ896264 ¹⁰
<i>Lithophyllum incrustans</i>	JQ896236 ¹⁰	JQ896263 ¹⁰
<i>Lithophyllum kotschyannum</i>	AB576031 ^{9,10}	AB576011 ^{9,10}
<i>Lithophyllum kotschyannum</i>	AB576030 ^{9,10}	AB576010 ^{9,10}
<i>Lithophyllum kotschyannum</i>	AB576029 ⁹	AB576008 ⁹
<i>Lithophyllum margaritae</i>	Q896235 ¹⁰	JQ896262 ¹⁰
<i>Lithophyllum margaritae</i>	JQ896253 ¹⁰	JQ896280 ¹⁰
<i>Lithophyllum pustulatum</i>	DQ167872 ^{6,10}	EF628240 ^{6,8,10}
<i>Lithophyllum</i> cf. <i>pygmaeum</i>	GQ917459 ^{8,10}	GQ917403 ^{8,10}
<i>Lithophyllum stictaeforme</i>	DQ167970 ^{6,9,10}	EF628241 ^{6,8,9,10}
<i>Lithophyllum</i> sp.	JQ896239 ¹⁰	JQ896266 ¹⁰
<i>Lithophyllum</i> sp.	GQ917452 ⁸	GQ917397 ⁸

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TABLE 1 (continued)

Taxa	<i>psbA</i>	SSU rDNA
	GenBank accession no.	GenBank accession no.
<i>Lithophyllum</i> sp.	GQ917470 ⁸	GQ917413 ⁸
<i>Lithophyllum</i> sp.	GQ917474 ^{8,10}	GQ917418 ^{8,10}
<i>Paulsilvella huveorum</i> SPF57695	KM044018	KM044028
<i>Paulsilvella huveorum</i> SPF57698	KM044021	KM044031
<i>Titanoderma</i> sp.	GQ917477 ^{8,10}	GQ917421 ^{8,10}
Mastophoroideae		
<i>Hydrolithon</i> cf. <i>boergesenii</i>	GQ917447 ^{8,10}	GQ917378 ^{8,10}
<i>Hydrolithon onkodes</i>	GQ917483 ⁸	GQ917373 ⁸
<i>Hydrolithon reinboldii</i>	GQ917485 ^{8,10}	GQ917376 ^{8,10}
<i>Metagoniolithon radiatum</i>	GQ917496 ^{8,10}	GQ917432 ^{8,10}
<i>Metagoniolithon stelliferum</i>	GQ917497 ^{8,10}	GQ917433 ^{8,10}
<i>Pneophyllum conicum</i>	AB576040 ^{9,10}	AB576023 ^{9,10}
<i>Pneophyllum conicum</i>	GQ917471 ^{8,10}	GQ917414 ^{8,10}
<i>Spongites yendoii</i>	DQ16790 ^{6,9}	EF628234 ^{6,8,9}
Hapalidiaceae		
Melobesioideae		
<i>Mesophyllum engelhartii</i>	JQ896243 ¹⁰	JQ896270 ¹⁰

Sequence evolution models were determined in ModelGenerator (Keane *et al.* 2006) for each marker separately under the Akaike Information Criterion. The following parameters were obtained for *psbA*: GTR +I+G with gamma distribution = 1.716, proportion of invariable sites = 0.631, base frequencies A = 0.222, C = 0.222, G = 0.206, T = 0.350 and rate among sites [A-C] = 1.103, [A-G] = 17.562, [A-T] = 6.045, [C-G] = 0.619, [C-T] = 27.952, [G-T] = 1.000; for SSU: GTR +I+G with gamma distribution = 0.556, proportion of invariable sites = 0.617, base frequencies A = 0.253, C = 0.207, G = 0.285, T = 0.256 and rate among sites [A-C] = 0.814, [A-G] = 2.403, [A-T] = 1.586, [C-G] = 0.386, [C-T] = 2.926, [G-T] = 1.0000; and for *rbcL*: GTR +I+G with gamma distribution = 1.9122, proportion of invariable sites = 0.5827, base frequencies A = 0.3277, C = 0.1381, G = 0.2072, T = 0.3270 and rate among sites [A-C] = 3.5389, [A-G] = 9.5372, [A-T] = 5.0634, [C-G] = 2.0067, [C-T] = 37.8126, [G-T] = 1.0000. Neighbor joining (NJ) reconstructions were performed in MEGA 6.06 (Tamura *et al.* 2013). Maximum likelihood (ML) analyses were inferred with PhyML algorithm (Guindon & Gascuel 2003) in Topali 2.5 (Milne *et al.* 2004). Bootstrap tests were performed for 500 (ML) or 2000 (NJ) replicates.

For the Bayesian analysis(BI), two independent runs of four chains of the Markov Chain Monte Carlo were performed using MrBayes (Huelsenbeck & Ronquist 2001); one tree was sampled every 100 generations for 4,000,000 generations starting with a random tree. The first 400,000 generations were discarded as burnin after visually checking of the plateau in excel dispersion graphic and a 50% consensus tree was computed with the remaining data.

The *psbA* + SSU rDNA combined analysis was performed after inference on the best evolution model for each marker separately with MrModeltest 2.2.(Nylander 2004) and checking their mutual inconsistency using the Incongruence-Length Difference Test (ILD) on PAUP. Since both sequences requested the same evolution model (GTR+G+I; nst=6; rates=gamma) and the ILD pointed favorable to combining the data (P value = 0.02), we have followed on analyzing this final matrix without the need of prior partitioning and independent optimized parameters.

TABLE 2. List of new (in bold, including SPF herbarium number) and sequences obtained from GenBank included in *rbcL* dataset. Previously published sequences are referenced as follows: Gavio *et al.* 2005⁽¹⁾, Gabrielson *et al.* 2011⁽²⁾, Martone *et al.* 2012⁽³⁾, Freshwater *et al.* 2013⁽⁴⁾, Scott *et al.* 2013⁽⁵⁾.

Taxa	<i>rbcL</i>
	GenBank accession no.
CORALLINALES	
Corallinaceae	
Corallinoideae	
<i>Alatocladia yessoensis</i>	HQ322277 ⁽²⁾
<i>Arthrocardia corymbosa</i>	JN701475 ⁽³⁾
<i>Bossiella orbigniana</i>	HQ322279 ⁽²⁾
<i>Calliarthron cheilosporioides</i>	HQ322284 ⁽²⁾
<i>Calliarthron tuberosum</i>	HQ322316 ⁽²⁾
<i>Chiharaea bodegensis</i>	HQ322332 ⁽²⁾
<i>Chiharaea silvae</i>	JN701473 ⁽³⁾
<i>Corallina officinalis</i>	KC134323 ⁽⁵⁾
<i>Corallina pilulifera</i>	DQ787558
<i>Corallina pinnatifolia</i>	HQ322333 ⁽²⁾
<i>Corallina vancouveriensis</i>	HQ322334 ⁽²⁾
<i>Jania natalensis</i>	EU349111
<i>Jania rubens</i> SPF57696 (host)	KMO44024
<i>Jania sagittata</i>	KC134331 ⁽⁵⁾
<i>Serraticardia macmillanii</i>	HQ322338 ⁽²⁾
<i>Yamadaea melobesioides</i>	JN701477 ⁽³⁾
Lithophylloideae	
<i>Amphiroa</i> sp SPF57697 (host)	KM044025
<i>Amphiroa fragilissima</i>	U04039 ⁽⁴⁾
<i>Amphiroa fragilissima</i> SPF57694 (host)	KM044022
<i>Amphiroa zonata</i>	JN701462 ⁽³⁾
<i>Lithophyllum grumosum</i>	JX393106 / JX393121
<i>Lithophyllum impressum</i>	HQ322335 ⁽²⁾ / JX393128
<i>Lithothrix aspergillum</i>	HQ322336 ⁽²⁾
<i>Paulsilvella huveorum</i> SPF57695	KM044023
<i>Paulsilvella huveorum</i> SPF57698	KM044026
<i>Pseudolithophyllum muricatum</i>	AY294373 ⁽¹⁾
Hapalidiaceae	
Melobesioideae	
<i>Clathromorphum reclinatum</i>	KC134324 ⁽⁵⁾
<i>Mastophoropsis canaliculata</i>	KC134335 ⁽⁵⁾
<i>Mesophyllum vancouveriense</i>	KC134326 ⁽⁵⁾ / HQ322337 ⁽²⁾
<i>Synarthrophyton patena</i>	KC134328 ⁽⁵⁾

Results

Molecular analysis

Sequences were obtained for two specimens of *Paulsilvella huveorum* for each plastid marker (*psbA*, *rbcL*, and

UPA); for nuclear SSU rDNA one full sequence and a partial one were obtained; identity among the two samples were 100% for UPA, *psbA* and SSU rDNA (for the overlapping region) and 99% for *rbcL* (1 divergent nucleotide). Sequences for each marker were also obtained for the host species *Jania rubens* (Linn.) J.V. Lamouroux (1816: 272), *Amphiroa* sp. and *A. fragilissima*. The combined *psbA*+SSU matrix included 43 sequences with 2,028 nucleotides (Fig. 2), while the *rbcL* dataset used 34 sequences, 1,273 nucleotides (Fig. 3).

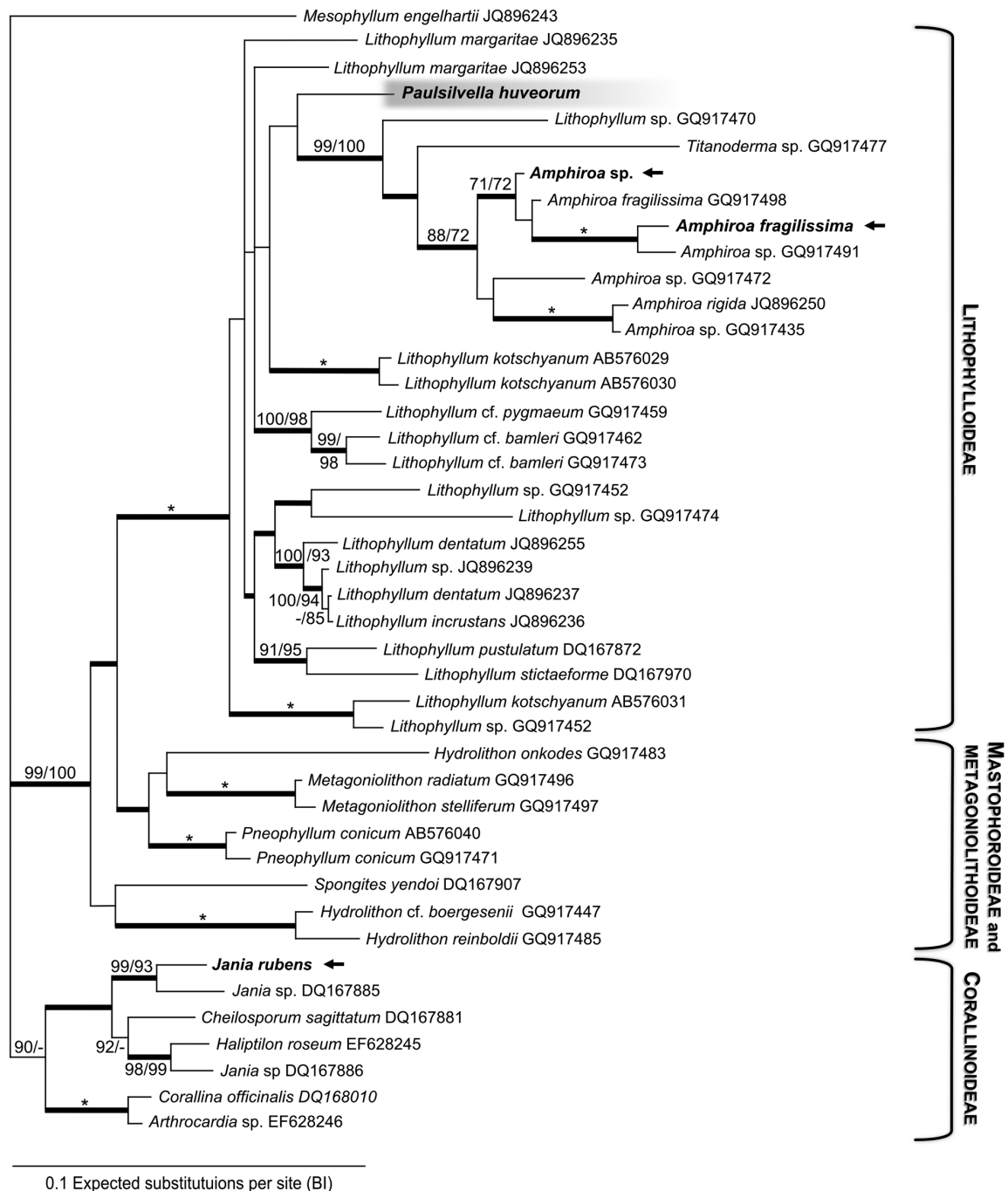


FIGURE 2. Molecular positioning of *Pausilvella* on the Lithophylloideae based on a combined *psbA* and SSU rDNA dataset. In the Bayesian inferred filogram posterior probabilities (when > 95 %) are shown as thicker branches. Bootstrap supports for NJ/ML (2,000/500 replicates) are shown at the nodes when higher than 70. Asterisks indicate 100 % bootstrap support for both NJ and ML. Sequences generated in this work are in bold; *Pausilvella* hosts included on this analysis are indicated by an arrow.

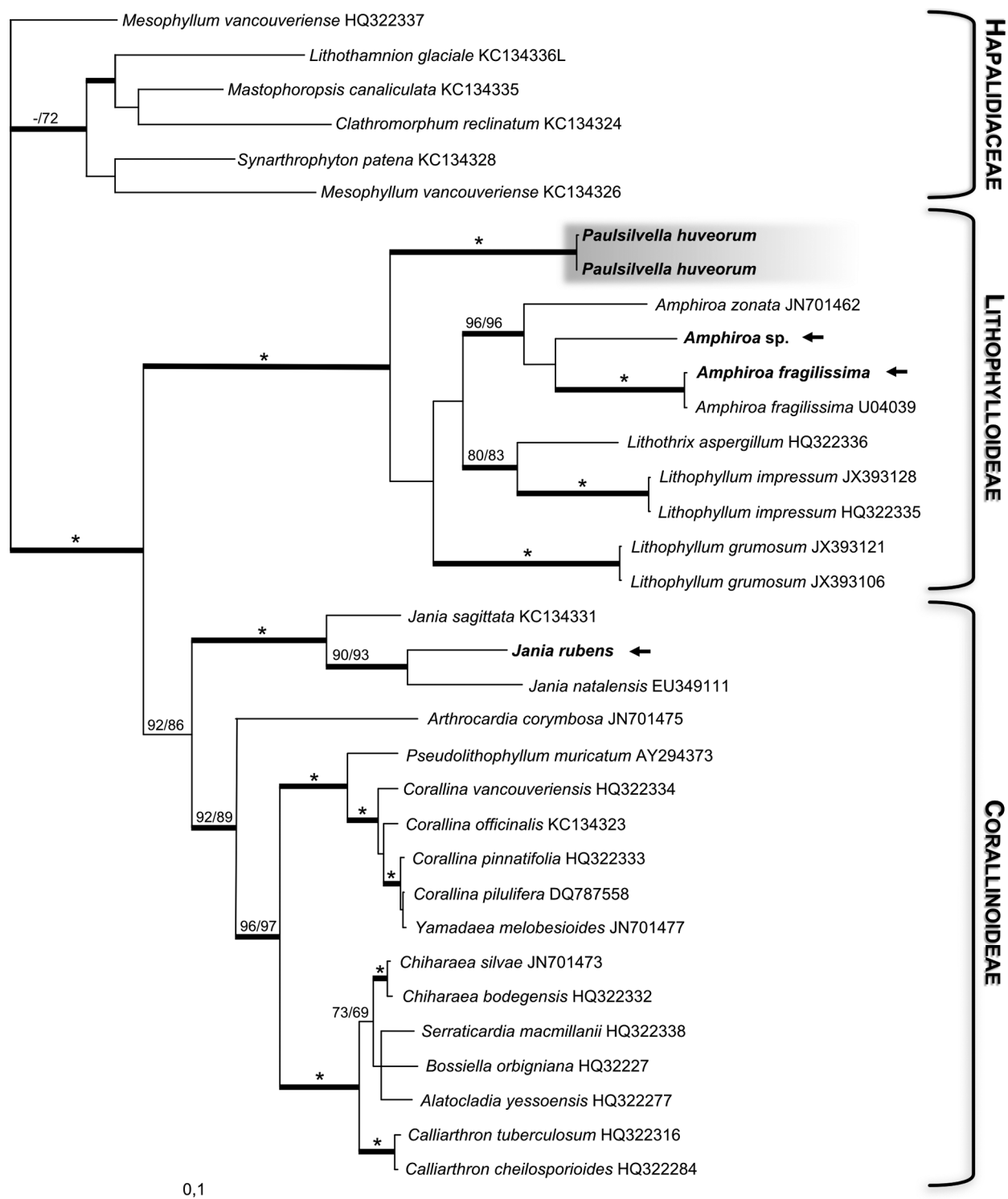


FIGURE 3. Molecular positioning of *Pausilvella* on the Lithophylloideae using *rbcL*. In the Bayesian inferred filogram posterior probabilities (when > 95 %) are shown as thicker branches. Bootstrap supports for NJ/ML (2,000/500 replicates) are shown at the nodes when higher than 70. Asterisks indicate 100 % bootstrap support for both NJ and ML. Sequences generated in this work are in bold; *Pausilvella* hosts included on this analysis are indicated by an arrow.

No phylogeny was performed for UPA, due to the low number of identified Corallinales sequences in the data banks; nonetheless, *P. huveorum* UPA sequences (KM044013 and KM044016) are consistent with the results obtained for the other markers, since these differ from host sequences (KM044012, KM044014 and KM044015) and are most similar to other Lithophylloideae from Genbank using Blast tool (Altschul *et al.* 1990).

The Lithophylloideae, including *P. huveorum*, formed a monophyletic grouping with high support on all analyses performed (NJ/ML/BI as follows: 100/100/100 for *psbA*+SSU, Fig. 2, and 100/100/100 for *rbcL*, Fig. 3).

The use of the combined *psbA*+SSU dataset (Fig. 2) increased resolution at basal nodes, when compared to analyzing *psbA* and SSU rDNA separately (data not shown). In these combined analyses *P. huveorum* is nested within the Lithophylloideae, but its grouping to a specific internal branch is poorly supported. For the *rbcL* analyses (Fig. 3) *P. huveorum* is positioned at the base of the available Lithophylloideae sequences, but this placement is probably an artifact due to the small number of *rbcL* sequences available. *Amphiroa* species formed a monophyletic clade with high support in all analysis (NJ/ML/BI as follows: 88/100/100 for *psbA*+SSU and 96/96/100 for *rbcL*). The genus *Lithophyllum* is paraphyletic in all phylogenies. As there are few samples in the databases for which sequences were obtained for both *psbA*+SSU markers, individual analyses for those markers were produced using a higher number of available sequences from Genbank, which are presented as a supplementary material 1 and 2. In these datasets more representatives for the Lithophylloideae and for the “Mastophoroideae” were included, and also representatives for the Hapalidiaceae, Sporolithales and Rhodogorgonales in order to eliminate the possibility of biased relationships driven by the absence of representatives of more distantly related clades on the analysis. Details are shown on supplementary material section, but it is relevant to mention that: i. *P. huveorum* remains nested within the Lithophylloideae; ii. despite including more sequences, the basal relationships get little support and inner relationships of the Lithophylloideae are not resolved.

The host species *Jania rubens*, *Amphiroa* sp. and *A. fragilissima*, in both phylogenetic analyses indicate their close relation to other species in their respective genera—emphasizing the lack of cross contamination with the epiphyte *P. huveorum* during sequences production. For *A. fragilissima* there were available Genbank sequences for *rbcL* and SSU rDNA, the former from Key Largo, Florida, USA—close to the type location, in Jamaica; this would be the first molecular evidence confirming the morphological identification of this taxon for the Brazilian coast.

Morphological analysis

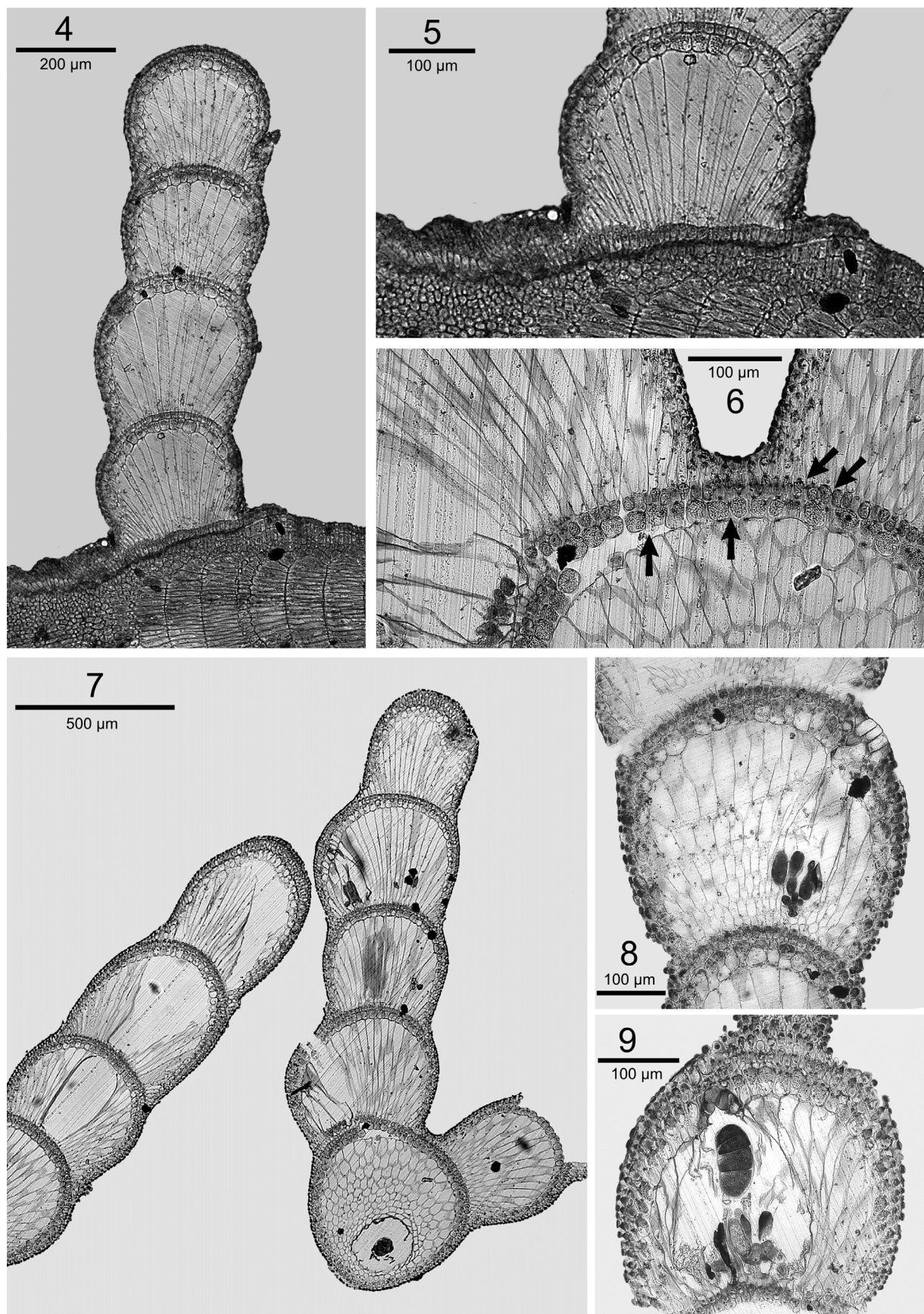
Paulsilvella huveorum Woelkerling, Sartoni & Boddi 2002: 358 (Figs. 4–20; Table 3)

Holotype:—F.T. Sartoni 11/010a (Woelkerling, Sartoni & Boddi 2002: 362). Notes: The holotype preparation includes male, female, carposporangial and tetrasporangial individuals. Isotype LTB 18056.

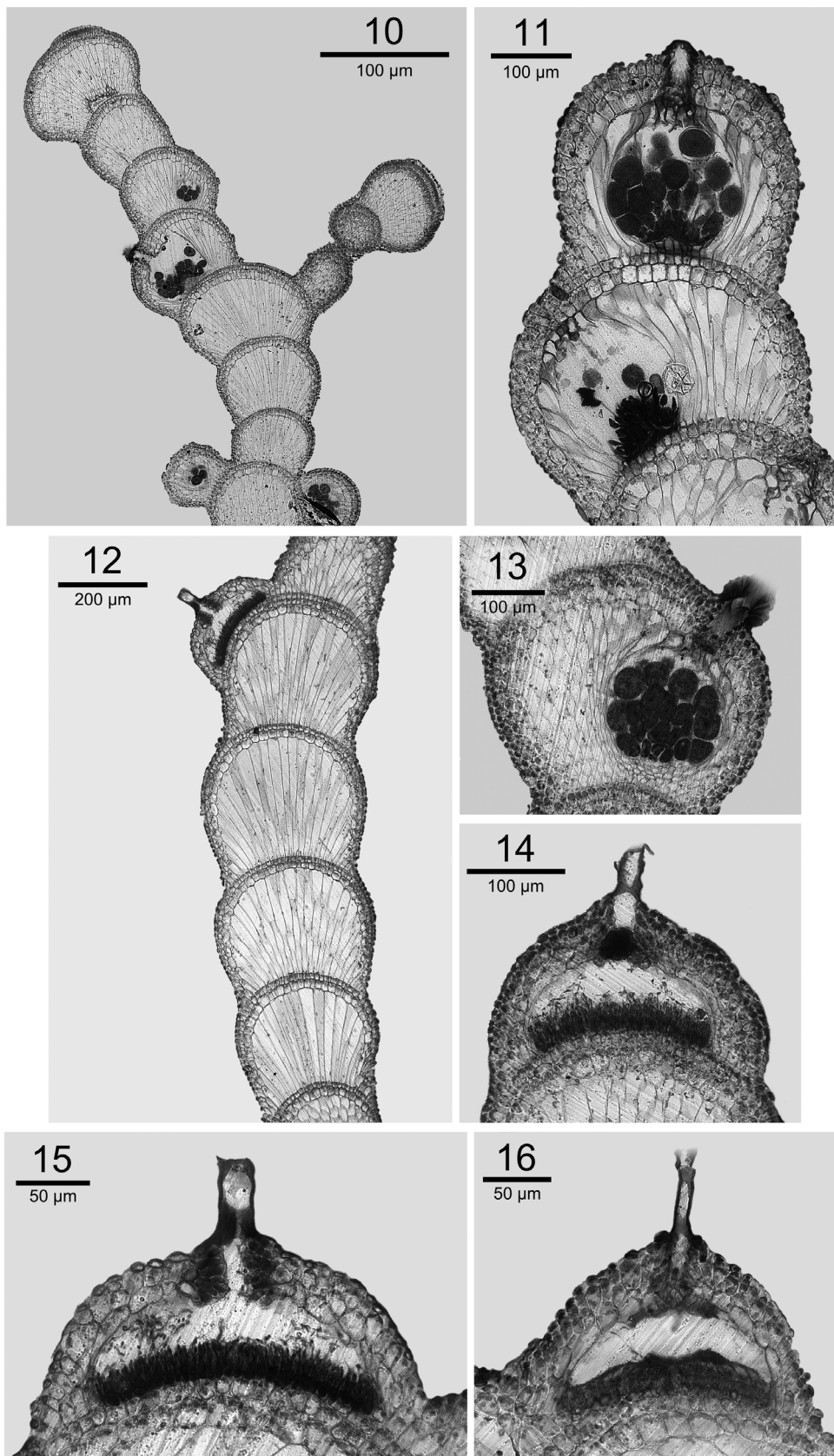
Type locality:—Gandersha, Somalia (Woelkerling, Sartoni & Boddi 2002: 362). Holotype: G. Sartoni; 27 January 1982; on *A. fragilissima* growing on the inshore side of the reef.

Description:—Plants nongeniculate, entirely attached ventrally to living substrate by cell adhesion; discrete encrusting base allied to monomeric non-articulated branches (Fig. 4). Encrusting portions, 21(33)56 mm across; protuberances ascending and becoming dichotomous branched. Branches cylindrical, up to 5 mm long and 0.5 mm in diameter, simple or irregularly branched. Plants pseudoparenchymatous; internal organization dimerous in crustose portions (Fig. 5) and monomeric/radial in protuberances. Crustose portions mostly consisting of one palisade cell layer followed by one compressed epithelial layer. Branches arise where a second palisade layer emerges and elongates to the columnar portion of the first monomeric segment, which will also bring forward two layers of smaller cells. The second layer of smaller cells gives rise to the next monomeric segment. Basal palisade cells 16(17)20 µm length and 9(11)13 µm diameter; columnar cells 110(231)310 µm long, 7(10)13 µm diameter proximally and 22(29)51 µm diameter distally; first tier of shorter cells 20(27)35 µm length and 15(27)35 µm diameter; second tier of shorter cells 10(15)21 µm length and 10(12)15 µm diameter. Cells of adjacent filaments linked by secondary pit-connections (Fig. 6), what is more evident on the palisade basal layer or among cells from the first and second smaller layers on protuberant branches.

Tetrasporangial conceptacles (Figs. 7–9) uniporate, flush and confined to a branch sector; conceptacle roofs 3(4) cells thick; pore canals surrounded by elongated block cells. Conceptacle chambers 83(144)201 µm diameter and 169(199)230 µm tall, usually without a central columella; tetrasporangia initials originated from cells from the second tier of shorter cells from the previous monomeric sector; each mature sporangium presents 71(93)109 µm long and 20(35)49 µm in diameter, containing four zonately arranged tetraspores.



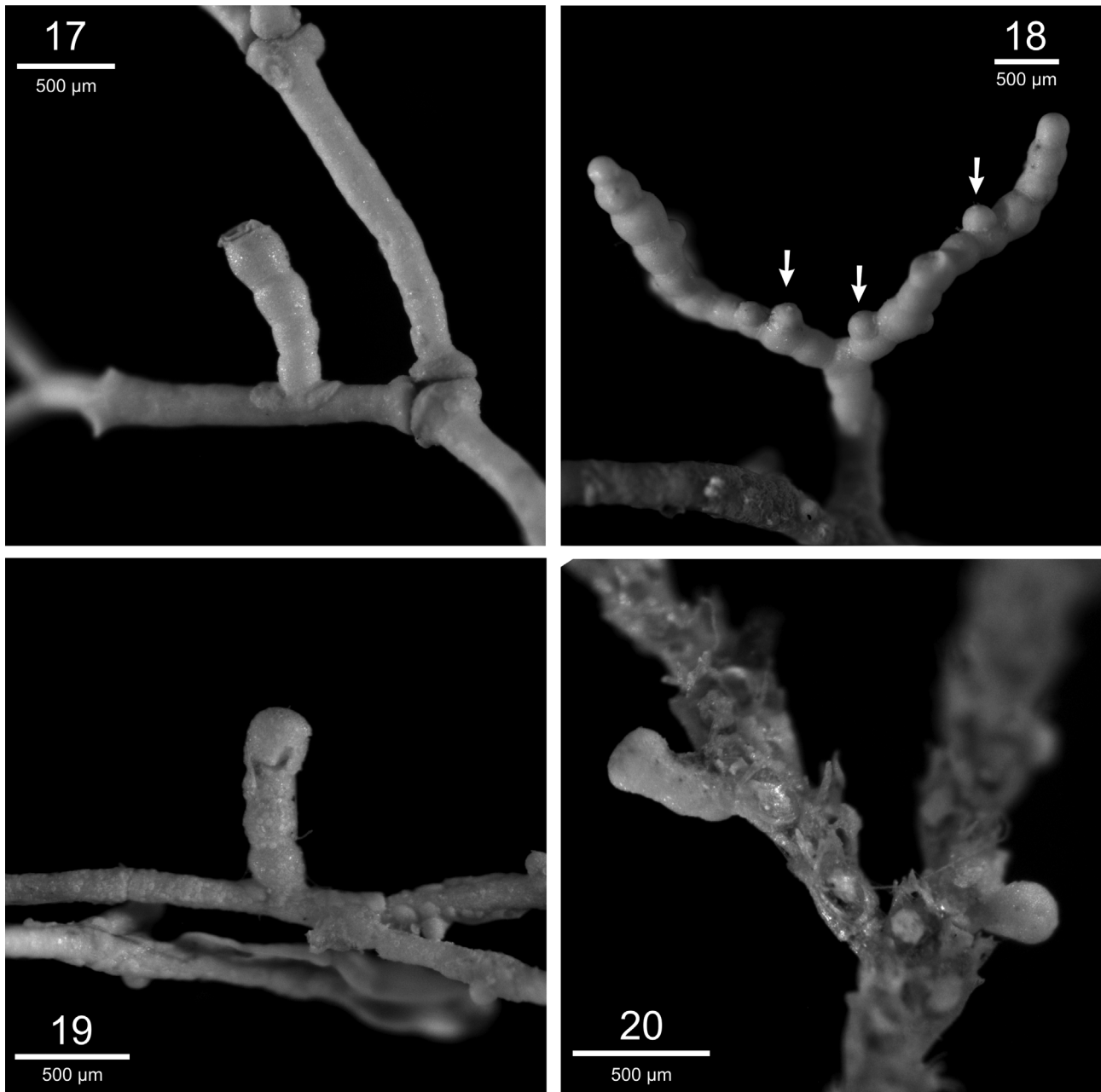
FIGURES 4-9. *Paulsilvella*'s vegetative and tetrasporophytic details. **4.** Erect axis growing on *Amphiroa fragilissima*. **5.** Detail on basal cell arrangement, on which is evident a dimerous growing pattern. **6.** Secondary pit connections are particularly evident on the first and second tiers of shorter cells (arrows). **7.** General habit of two tetrasporophytic thalli. **8.** Tetrasporangial conceptacle; block cells line the pore of canal. **9.** Tetrasporangial conceptacle; detail on a mature tetrasporangium and formation of block cells which would line the pore canal at final stages of development.



FIGURES 10–16. *Paulsilvella*'s carposporophytic and male reproductive details. **10.** General habit of a reproductive carposporophytic thalli. Observe both protruding (below) and flushed (above) positioning of conceptacles. **11.** Carposporangial conceptacles on the two final sectors of a thalli. **12.** General habit of a reproductive male thalli. Observe the protruding positioning of conceptacle. **13.** Mature carposporangial conceptacle on the moment of mucilage liberation. **14.** Male conceptacle with spout and Initial stage of mucilage liberation. Observe the simple arrangement of spermatia. **15.** Mature male conceptacle with moving mucilage on spout canal. **16.** Male conceptacle with final spout and initial degeneration of chamber and spermatia.

Gametangial plants dioecious. Mature carposporangial conceptacles (Figs. 10, 11 and 13) flush to a branch sector or protruding from it; conceptacle roofs 3(4) cells thick. Conceptacle chambers 134(161)190 μm in diameter and 137(194)234 μm tall; supporting cells of carpogonial branches originate from cells from the second tier of shorter cells from the previous monomerous sector; carposporophytes developing within female conceptacles after presumed karyogamy, each comprising a central more or less flattened fusion cell and several celled gonimoblast filaments arising from the whole surface of the fusion cell and bearing terminal carposporangia; carposporangia liberation is presumed by mucilage extravasation (Fig. 13).

Male conceptacles (Figs. 12 and 14–16) always protruding from branches surface; conceptacle chambers 130(168)206 μm in diameter and 52(61)73 μm tall, disposing simple spermatangia which spread all over the floor; spermatangia liberation seem to involve mucilage extravasation (Figs. 14–16) and to be guided by a spout.



FIGURES 17–20. *Paulsilvella*'s observed habit details viewed on stereomicroscope. **17.** Growing on *Amphiroa fragilissima*. **18.** Growing on *Amphiroa beauvoisii* arrows indicate protruding conceptacles; **19.** Growing on *Jania rubens*. **20.** Growing on a bryozoan.

Ecological observations:—In the original description the host plants were collected from tide pools, lower intertidal and upper subtidal levels. In our study collections were obtained both at tidal pools at reef plateau (at

Sebastião Gomes reef) and attached to subtidal rocks at 5–7 m depth (at Ilha Grande). It is also important to stress that *Paulsilvella* has shown to thrive on distant latitudinal spots (from tropical, Bahia, to subtropical areas, Rio de Janeiro), which implies a broader ecological range considering light intensity, water movement and temperature.

TABLE 3. Comparative morphological measurements (µm) of *Paulsilvella* species.

		<i>P. huveorum</i> (Western Atlantic) this work	<i>P. huveorum</i> (Western Indic) Woelkerling <i>et al.</i> 2002	<i>P. antiqua</i> (Western Indic) Woelkerling <i>et al.</i> 2002
Encrusting phase				
	palisade cell height	16(17)20	18 to 2 (?)	(no data)
	palisade cell diameter	9(11)13	6 to 13	(no data)
Protuberant branches				
	diameter of branches	303(389)532	165–1450	270–501
	length of branch sectors	212(283)354	112–525	116–331
	columnar cell length	110(231)310	203–318	(33–)136–268
	columnar cell diameter (distal)	22(29)51	15–40	17–35
	columnar cell diameter (proximal)	7(10)13	6–15	8–17
	1 st tier of shorter cells - cell length	20(27)35	12–21	18–45
	1 st tier of shorter cells - cell diameter	15(27)35	9–27	16–31
	2 nd tier of shorter cells - cell length	10(15)21	9–21	15–30
	2 nd tier of shorter cells - cell diameter	10(12)15	7–15	15–17
Conceptacles				
male	conceptacle position	lateral, protruding	lateral, protruding	(no data)
	conceptacle chamber - height	52(61)73	62–124	(no data)
	conceptacle chamber - diameter	130(168)206	165–350	(no data)
	conceptacle roof - number of cells	3(4)	3–4	(no data)
carposporangial	conceptacle position	flush or protruding	flush	protruding? ^{*2}
	conceptacle chamber - height	134(161)190	124–268	245–250
	conceptacle chamber - diameter	137(194)234	185–268	180–185
	conceptacle roof - number of cells	3(4)	3–4	–
tetrasporangial	conceptacle position	flush	flush	protruding? ^{*2}
	conceptacle chamber - height	169(199)230	124–247	(no data)
	conceptacle chamber - diameter	83(144)201	165–247	(no data)
	conceptacle chamber - number of cells	3(4)	3(4) (* ¹)	(no data)
	tetrasporangia length	71(93)109	60–80	(no data)
	tetrasporangia diameter	20(35)49	(no data)	(no data)

*¹ obtained from photo 28 from Woelkerling *et al.* 2002 (p. 368).

*² there is an uncertainty if the only conceptacle found could be tetrasporangial or carposporangial, since it was empty (Woelkerling *et al.* 2002, p. 370)

New hosts are now referred for the genera: *A. beauvoisii*, *Jania cubensis* ex Kützinger (1849: 709) and non-identified hydrozoa and bryozoans. Apart from that, the previously registered host *A. fragilissima* was observed as the most common substrate.

Geographical distribution:—This is the first time *Paulsilvella* is registered out of East Africa and registered on a subtropical area (for Ilha Grande Island; Fig. 1).

Examined material:—BRAZIL, Bahia: Sebastião Gomes reef (morphological approach). SPF 567001, *A. beauvoisii* J.V. Lamouroux (1816: 299) turf (*J. cubensis* also present), 17°54'38,88"S 39°06'44,28"W; SPF 567003,

25 January 2008, B.N. Torrano-Silva & E.C. Oliveira; *A. beauvoisii* turf, 17°54'38,88"S 39°06'44,28"W, 25 January 2008, B.N. Torrano-Silva & E.C. Oliveira; SPF 56997, *A. fragilissima* turf, 17°55'22,62"S 39°07'54,48"W, 17 October 2008, B.N. Torrano-Silva & C.E. Amancio; SPF 56998, *A. fragilissima* turf, 17°54'38,88"S 39°06'44,28"W, 15 October 2008, B.N. Torrano-Silva & C.E. Amancio.

BRAZIL, Rio de Janeiro: Ilha Grande Island (both morphological and molecular approach). SPF 57694, *A. fragilissima* turf, 23°06'54.8" S 044°17'36.0" W, 8 September 2013, B.N. Torrano-Silva & F. Nauer; SPF 57695, *P. huveorum* on various hosts, 23°06'54.8" S 044°17'36.0" W, 8 September 2013, B.N. Torrano-Silva & F. Nauer; SPF 57696, *J. rubens*, 23°06'54.8" S 044°17'36.0" W, 8 September 2013, B.N. Torrano-Silva & F. Nauer; SPF 57697, *Amphiroa* sp. turf, 23°06'54.8" S 044°17'36.0" W, 8 September 2013, B.N. Torrano-Silva & F. Nauer; SPF 57698, *P. huveorum* on various hosts, 23°06'57.3"S 044°16'12.8"W, 8 September 2013, B.N. Torrano-Silva & F. Nauer.

Discussion

The observed morphology and anatomy from Brazilian and Eastern Africa *Paulsilvella* living specimens confirm these are the same morphospecies. In spite of that, some reproductive features contradict the Woelkerling *et al.* (2002) view of species delimitation for this genus. The existence of both flushed and protruding carposporangial conceptacles (even on the same thalli) unify the characteristics which originally separated *P. huveorum* and *P. antiqua* (Woelkerling *et al.* 2002), but we decided not propose the synonym because more data about this genera is need to solve this controversy. Apart from that, there is no certainty if the only conceptacle found for *P. antiqua* was carposporangial or tetrasporangial, as it was empty and the roof features are similar in both phases (Woelkerling *et al.* 2002, 370: 35).

In the molecular analysis, we have found *Paulsilvella* included either at the base (*rbcL*) or nested (*psbA* + SSU rDNA) within the sequences of other Lithophylloideae representants, confirming its original placing in the subfamily based on the morphological features (Woelkerling *et al.* 2002). Also *Paulsilvella* and *Lithothrix* were not monophyletic (supplementary material 1 and 2), confirming the transfer of the fossil species *P. antiqua* from the genus *Lithothrix* (Woelkerling *et al.* 2002) to *Paulsilvella*. The basal placing of *Paulsilvella*, in the *rbcL* phylogeny (or a polytomy if we consider only the Bootstrap supported branches) is probably a consequence of the limited set of species included in the *rbcL* analysis.

A closer relationship between *Titanoderma* and *Amphiroa* based on *psbA* + SSU rDNA sequences is once more shown, following the SSU rDNA inferences from Bailey (1999), Broom *et al.* (2008) and Bittner *et al.* (2011). Unfortunately there were no available *psbA* and SSU rDNA sequences from the same specimen of *Lithothrix*, but this genera closer relationship to crustose genera (rather than to *Amphiroa*) is shown on *psbA* and SSU more replete analyses (supplementary materials 1 and 2). At the same time, *Lithothrix* closer relation to *Lithophyllum* samples at the *rbcL* dataset (prior to *Amphiroa* clade) could be artificial, once there were no available *rbcL* sequences for *Titanoderma*. For the encrusting genus *Titanoderma*, there are only SSU rDNA sequences available and the three species analysed are more closely related to articulated genera, *Amphiroa* and *Lithothrix*, rather than to other encrusting forms of *Lithophyllum* (Fig. 2), also according to previous published data (op. cit.).

The addition of *P. huveorum* sequences to the present molecular inferences also didn't resolve the positioning of *Lithophyllum* within the Lithophylloideae, which is already being suggested as a non-monophyletic genus (Hernandez-Kantun *et al.* submitted). The present phylogenetic analyses indicate that the three growing forms found within the Lithophylloideae (encrusting, geniculate and monomerous) are not distributed as a continuous pattern. Morphological changes from the believed encrusting ancestor of the Lithophylloideae may have been driven independently, or following an initial shift event, which could have then evolved by different pathways to the geniculate and monomerous forms, including reversal steps on *Titanoderma*. These two possible evolutionary pathways are now reworked after their initial proposal by Bailey 1999 but; even after the inclusion of *Paulsilvella*'s molecular data, the geniculate and monomerous forms origins remain a mystery and may be elucidated with future additions on concatenated anatomical and molecular data from *Amphiroa*, *Lithophyllum* and *Titanoderma*, and also the missing genera, *Ezo* and *Tenarea*, to the Lithophylloideae phylogeny inferences.

Unfortunately, until this moment, nothing can be said regarding molecular similarities from Western Africa and Western Atlantic specimens, since this is the first time *Paulsilvella* DNA is isolated and there are no sequences from outside Brazil to compare. Additional collections are needed to verify phyletic distances. The new

geographical record for *Paulsilvella* suggests a relationship between Indo Pacific and Atlantic by the old Tethys Sea. Detecting this species on Brazilian coast also reinforces the hypothesis on the Indo-Pacific origin of the Brazilian marine macroflora composition (Horta *et al.* 2001).

It is important to note that, although *Paulsilvella* is uncommonly seen on field, it does not mean that it is indeed a rare species—or even that it has not been collected many times previously. Their thallus' inconspicuous size for human eyes may have led to the present existence of *P. huveorum* individuals attached to their hosts, which have already been deposited on herbaria. Some of their hosts are worldwide distributed: as an example, *A. fragilissima*, the commonest host, grows on Europe, Western and Eastern Africa, Indian Ocean Islands, South-west and Eastern Asia, Australia, New Zealand, the Pacific Islands, North, Central and South America, including the Caribbean islands (Guiry 2014). Another fact to register is the possibility that *P. huveorum* grows on a much wider range of hosts, including other species of coralline geniculate algae, bryozoans and hydrozoans. As *Paulsilvella* has not been spotted growing on crustose coralline algae until this moment, apparently this epiphyte prefers the turf micro-environment, avoiding washable surfaces. Adding the present data to the previous notes from Woelkerling *et al.* (2002), we can list a variable set of marine environments on which *P. huveorum* was found, including rocky shores, reef barriers, mangroves and seagrass meadows. Following this reasoning, and considering the worldwide distribution of geniculate algae, it is very reasonable to consider that *Paulsilvella* could be soon registered for a wider range of distribution, on condition that careful collections are taken and meticulous turf scavenging (for new and old collections) is worked out.

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