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First record of *Nocturama* (Batrachospermales, Rhodophyta) in South America, with the description of a new species *N. novamundensis*

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Abstract

Specimens from southeastern and southern Brazil previously identified as *Sheathia arcuata (= Batrachospermum arcuatum)* are shown to be members of the recently described genus *Nocturama,* previously known only from Australia and New Zealand. Morphological and molecular evidence support recognizing the Brazilian specimens as a new species, described here as *Nocturama novamundensis, sp. nov.* Comparison of DNA sequences of the plastid-encoded ribulose-1,5-bisphosphate-carboxylase–oxygenase large subunit (*rbc*L) and the nuclear small subunit ribosomal DNA (SSU rDNA) markers showed *Nocturama* as a well supported clade. The sequence divergences between the new and the type species were high (95-98bp, 7.4–7.6%) for *rbc*L and 19bp, 1.1% for SSU), and those within each species were extremely low (0-1 bp, 0-0.1%). The new species can be distinguished from *N. antipodites* in having curved primary fascicles composed of non-‘audouinelloid’ cells (compared to straight primary fascicles with audouinelloid—cylindrical—cells) and in being always dioecious (only rarely is *N. antipodites* dioecious).

Key words: biogeography, Brazil, freshwater red algae, *rbc*L gene, molecular systematics, morphology, rDNA SSU marker

Introduction

The genus *Nocturama* was recently established by Entwisle *et al*. (2016) to accommodate a single species from Australia and New Zealand formerly known as *Batrachospermum antipodites* (Entwisle 1995). The species was always considered morphologically distinctive but Entwisle & Foard (1997) allocated it to an informal cluster of Australian and New Zealand species within *Batrachospermum* called the ‘*Batrachospermum antipodites* Group’, distinguished only by ‘carpogonia subtended by a relatively short filament of modified cells’. Other taxa included in this cluster—*B. antiquum* Entwisle & Foard, *B. discorum* Entwisle & Foard, *B. kraftii* Entwisle & Foard and *B. ranuliferum* Entwisle & Foard—are now considered part of the expanded genus *Nothocladus* (Entwisle *et al.* 2016).

As part of an effort to monograph all members of Batrachospermales from Brazil using morphological and molecular evidence, records of the genus *Sheathia* were reviewed. Only one species had been recorded from Brazil, *Sheathia arcuata* (Kylin) Salomaki & Vis—as *Batrachospermum boryanum* Sirodot by Necchi (1990) and *Batrachospermum arcuatum* Kylin by Necchi *et al.* (1999) and Branco *et al*. (2014). However, a comparison of DNA sequences of the plastid-encoded ribulose-1,5-bisphosphatecarboxylase–oxygenase large subunit (*rbc*L) and the nuclear small subunit ribosomal DNA (SSU rDNA) markers showed specimens attributed to *S. arcuata* were more closely related to the genus *Nocturama* than to *Sheathia*. Thus, the aim of this study was to compare the Brazilian specimens more closely with the Australian type species of *Nocturama*, *N. antipodites*, and to make any necessary taxonomic changes.

Material and methods

All specimens previously reported from Brazil (Necchi 1990, Necchi *et al*. 1999, Branco *et al*. 2014)—preserved in formaldehyde 4% and lodged at herbaria SJRP and SP (Thiers *et al*. 2016)—were examined. Some specimens more recently collected (Branco *et al*. 2014) were preserved in silica desiccant for DNA analysis.

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Morphological analyses followed the general procedures outlined in Necchi (1990) and Entwisle & Foard (1997). Where possible, at least 20 measurements or counts were taken for each character in each sample. Images are from a Leica DFC 320 digital camera using LAS capture and image analysis software coupled to a Leica DM5000 microscope (Leica Microsystems, Wetzlar, Germany). All characters referred to in previous studies of Batrachospermales, and particularly of *Nocturama* (Entwisle 1995, Entwisle & Foard 1997, Entwisle *et al*. 2016) were considered.

For DNA extraction, material was processed with a Precellys 24 tissue homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France), followed by DNA extraction using 'DNeasy plant mini kit' (Qiagen GmbH, Hilden, Germany) and 'NucleoSpin plant II mini kit' (Macherey-Nagel, Düren, Germany), according to the manufacturers' protocols. A 1282bp fragment of the plastid-encoded ribulose-1,5-bisphosphatecarboxylase–oxygenase large subunit gene (*rbc*L) was polymerase chain reaction (PCR) amplified using primers and cycles described by Vis *et al*. (1998) and Stewart & Vis (2007). The full sequence of SSU rDNA (1765 bp) was obtained by PCR using the primers and cycles described by Vis *et al.* (1998) and Milstein & Oliveira (2005). Two different amplification systems were used for PCR reactions for all markers as follows: (1) puReTaq Ready-to-go PCR beads (GE HealthCare Life Sciences, Buckinghamshire, UK); (2) Top Taq Master Mix (Qiagen). We used the PCR products of the system that worked the best (determined by the brightest band in the gel). The resulting PCR products of the two markers were purified using the QIAquick PCR (Qiagen) or NucleoSpin Gel and PCR Clean-up (Macherey-Nagel), according to manufacturers' protocols. Sequencing reactions were run using the ABI PRISM Big Dye v3.1 Terminator Cycle Sequencing Ready Reaction kit and the ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems, Foster City, California, USA). Sequence alignments were assembled in Geneious 7 (Kearse *et al*. 2012).

For phylogenetic analyses of the *rbc*L and SSU data, a GTR + I + G was determined as the best-fit model of sequence evolution by the Akaike Information Criterion using jModelTest 2.1.4 (Darriba *et al*. 2012). Separate analyses were conducted for the *rbc*L and the SSU rDNA marker data sets. A combined analysis with the two markers was not tried because the few sequences available for SSU rDNA of closely related taxa severely limited the quality of the analysis. Maximum likelihood (ML) topologies and bootstrap values from 10,000 replicates were inferred using the Randomized Accelerated Maximum Likelihood graphic user interface (RAxMLGUI version 1.2; Silvestro & Michalak 2011). Bayesian analysis (BA) was performed in MrBayes 3.2 (Ronquist & Huelsenbeck 2003) with three runs of five chains of Metropolis coupled Markov Chain Monte Carlo for 10 x10^6 generations. 500,000 chains were removed as burn-in prior to determining the posterior probabilities.

Results and discussion

Two sequences of *rbc*L and two of SSU rDNA were generated in this study (see below). Phylogenetic analyses of *rbc*L sequences resulted in the same tree topologies for BA and ML, showing high support for the genus *Nocturama* as a monophyletic group (Fig. 1). The *Nocturama* clade was sister to a large clade formed by species of *Sheathia*, *Nothocladus* sensu Entwisle *et al*. (2016) and *Setacea* sensu Rossignolo & Necchi (2016). Two groups with high support were formed within *Nocturama*, representing the type species (*N. antipodites*) and the Brazilian specimens. The sequence divergences between these two groups were high (95–98 bp, 7.4–7.6%), whereas within each clade were much lower (0–1 bp, 0–0.1%).

Data analysis of SSU sequences revealed *Nocturama* as a clade with medium (ML) to high support (BA) (Fig. 2). The *Nocturama* clade is sister to *Lemanea* and *Paralemanea* but we do not attribute this to a close relationship but rather the reduced dataset of SSU. On the basis of these data, two species are supported within *Nocturama*: the type species and the Brazilian specimens, the latter having identical sequences. The sequence divergences between these two species was high (19bp, 1.1%).

The molecular evidence supports continued recognition of *Nocturama* as a single genus, with the addition of another species for the Brazilian specimens. This proposed new taxon is described below.

Nocturama novamundensis Necchi & Entwisle, sp. nov.
Figures 3–13.

Holotype:—Brazil: Rio Grande do Sul, Parque Estadual Florestal do Turvo, Tigre River, 27°12'25"S, 53°50'02"W, 18.viii.2007, C.C.Z. Branco *et al.* (SJRP29741); Isotype MEL 2401959.

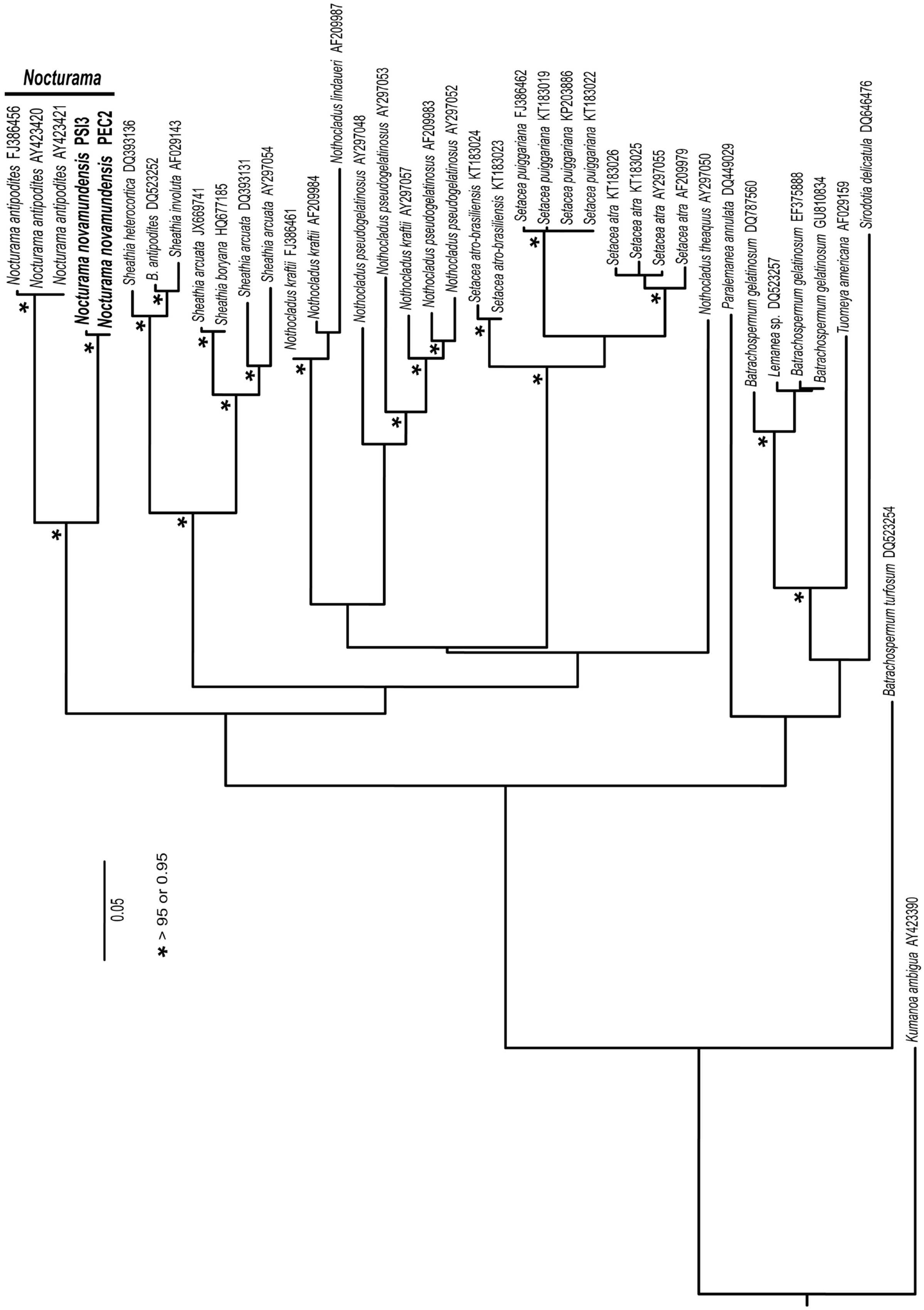


FIGURE 1. ML Phylogenetic tree based on *rbc*L sequences. The numbers associated with the nodes indicate the bootstrap values (BS) for maximum likelihood and posterior probability (PP) for Bayesian analysis; nodes without values indicate BS < 70% and PP <0.70. Newly generated sequences are in boldface. *Setacea* is alternatively treated as a section of the genus *Nothocladus* s. lat. (Entwisle *et al.* 2016).

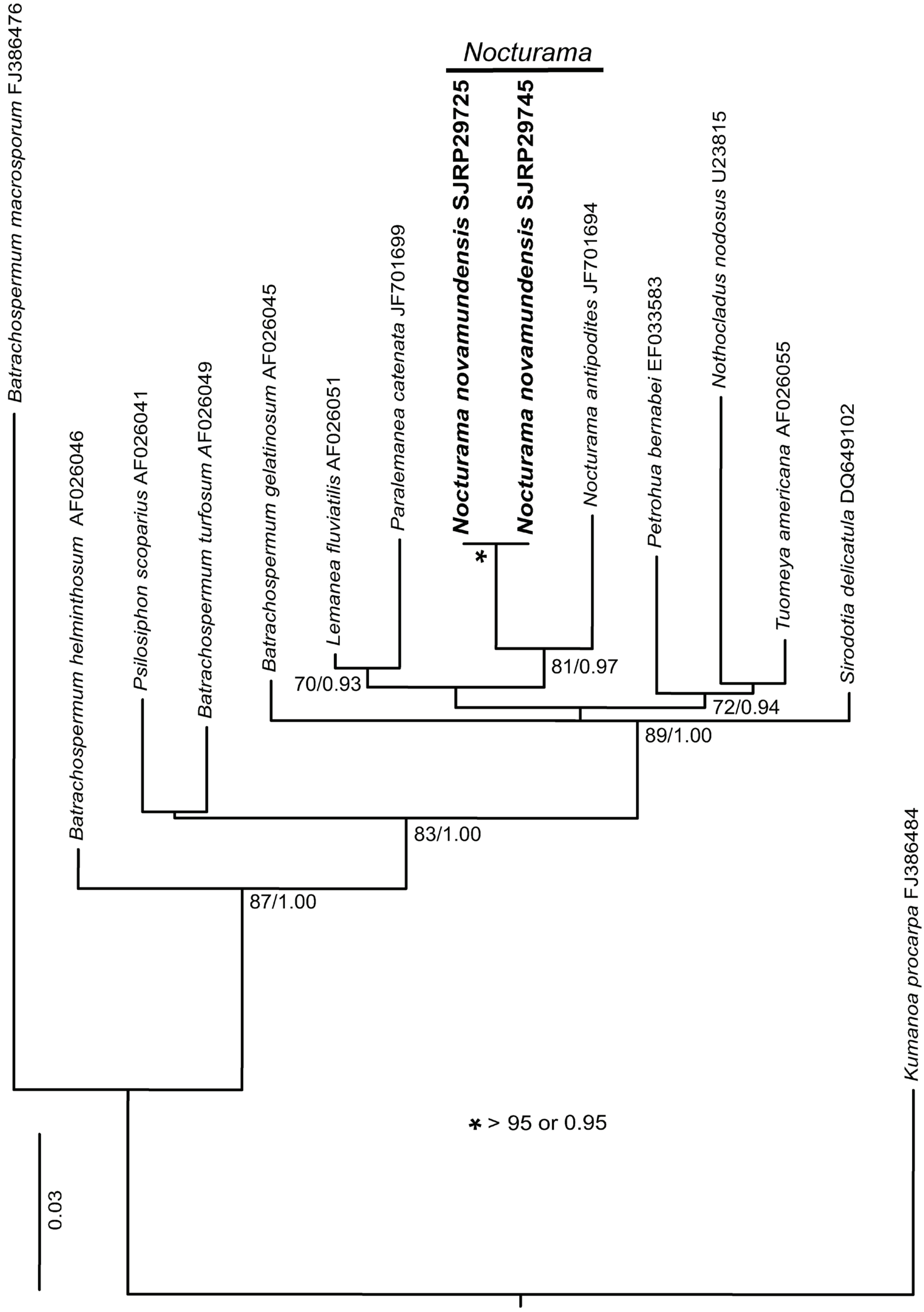
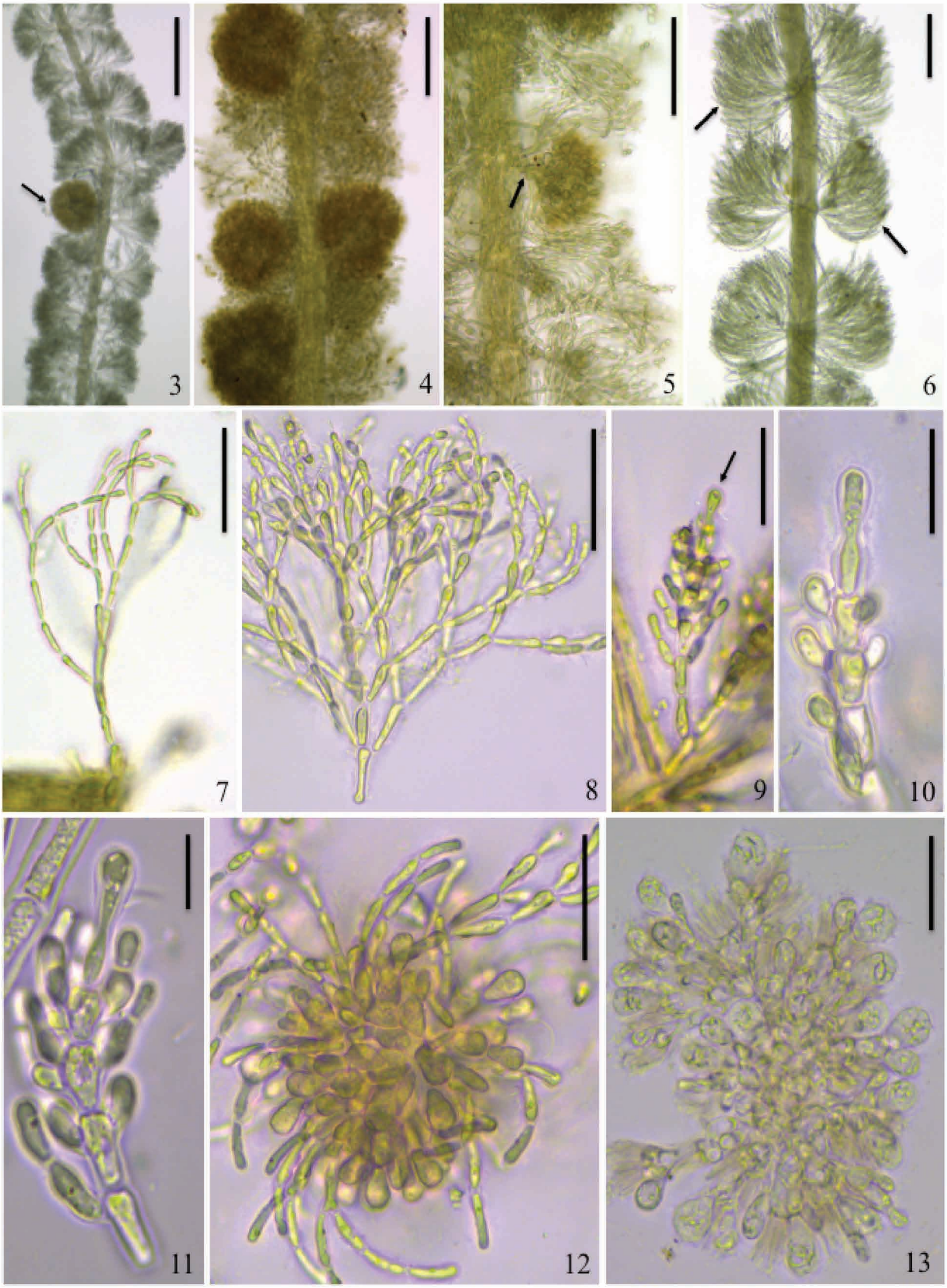


FIGURE 2. ML Phylogenetic tree based on SSU rDNAsequences. The numbers associated with the nodes indicate the bootstrap values (BS) for maximumlikelihood and posterior probability (PP) for Bayesian analysis; nodes without values indicate BS < 70% and PP <0.70. Newly generated sequences are in boldface.



FIGURES 3–13. *Nocturama novamundensis* Necchi & Entwisle sp. nov. 3–4. Whorls and carposporophytes (arrow); 5. Developing carposporophyte showing pedicellate (arrow); 6. Whorls showing curved primary fascicles (arrows); 7–8. Details of primary fascicles showing the curved disposition; 9. Entire carpogonial branch showing trichogyne (arrow); 10–11. Apical part of carpogonial branches showing young (10) and mature carpogonia; 12–13. Details of an intact (12) and a squashed carposporophyte. Scale bars: 250 µm for figures 3; 100 µm for figures 4–6; 50 µm for figures 7–8; 25 µm for figures 9, 12–13; 10 µm for figures 10–11.

Etymology:—*novamundensis*, from the Latin term for New World (*Mundus Novus*), the somewhat romantic and misleading term for the known distribution of this species, the ‘Americas’. The epithet is in harmony with that of the type species, *antipodites*, which is based on a colloquial term used for the inhabitants of Australia and New Zealand (being, it seems, on the ‘opposite side of the Earth’).

Material examined:—Paraná: Foz do Iguaçu, Iguaçu National Park, 25°09’38”S, 53°49’44”W, 02.v.2008, C.C.Z. Branco *et al.* (SJRP29760). Rio Grande do Sul: Parque Estadual Florestal do Turvo, Calisto River, 27°13’49”S, 53°54’92”W, 17.viii.2007, C.C.Z. Branco *et al.* (SJRP29739); Fábio River, 27°16’32”S, 54°00’56”W, 17.viii.2007, C.C.Z. Branco *et al.* (SJRP29735). Canela, Caracol State Park, Tiririca River, 29°18’59”S, 50°51’01”W, 02.vi.2008, C.C.Z. Branco *et al.* (SJRP29725). Santa Catarina: Blumenau, Serra do Itajaí National Park, Prata River, 27°02’17”S, 49°05’57”W, 28.v.2008, C.C.Z. Branco *et al.* (SJRP29745). São Paulo: Cananéia: Cardoso Island, Perequê River, 25°05’37”S, 47°55’45”W, 13.vi.1984, O. Yano & M.G.L. Wanderley (SP187226); between Ipanema and Cambriú beaches, 25°09’41”S, 47°55’48”W, 29.xi.1983, D.M. Vital (SP186936). Santo Antonio do Pinhal, Route SP-50, 2 km from town, 22°49’06”S, 45°44’58”W, 23.x.1996, O. Necchi Jr. & C.C.Z. Branco (SJRP23376). São Luiz do Paraitinga, Ponte Preta Farm, Chapéu Stream, 23°12’34”S, 45°20’10”W, 14.vii.1983, O. Yano (SP187153).

Geographic distribution:—the species occurs in four forested Brazilian biomes (Ab’Saber 2006): dense ombrophyllous forest (Atlantic rainforest), mixed ombrophyllous forest (subtropical rainforest with *Araucaria*), deciduous and semi-deciduous seasonal forests in southeastern and southern Brazil (Fig. 14).

FIGURE 14. Distribution map of *Nocturama novamundensis* Necchi & Entwisle sp. nov. in Brazil.

Environmental characteristics (n = 7): water temperature16.6 ± 3.4°C (mean ± standard-deviation); specific conductance 31 ± 8 μS cm^{-1}; turbidity 5 ±3 NTU ; pH 6.8 ± 0.5; dissolved oxygen 5,4 ± 1.2; current velocity 55 ± 25 cm s^{-1}; depth 19± 5 cm; shaded stream or river segments, with variable shading degrees: partly shaded (58% of collecting sites), shaded (28%) and heavily shaded (14%). These environmental data are summarized from Necchi *et al.* (1999) and Branco *et al*. (2014), in which this species is reported as *B. arcuatum*.

Description:—thalli dioecious, brownish, moderately mucilaginous, abundantly and irregularly branched, up to 7 cm long, 300–535 µm in diameter. Whorls spheric or barrel-shaped, separated or contiguous (Figures 3, 6). Internode 150–350 µm in length. Rhizoidal filaments composed of cylindrical cells only. Primary fascicles curved, composed of 8–15 cells; proximal cells cylindrical or ellipsoid, distal cells ellipsoid or obovoid (Figures 6–8). Secondary fascicles lacking, few and sparse in older thalli (Figures 3, 6). Spermatangia spherical or obovoid, terminal or sub-terminal on distal cells of primary fascicles, 3.5–6.0 µm in diameter. Carpogonial branches straight, 30–70 µm in length, on pericentral or proximal fascicle cells, composed of 3–10 cells, undifferentiated from fascicle cells; proximal cells cylindrical or ellipsoid, distal cells cylindrical or short-cylindrical; involucral filaments long (3–5 cells) in proximal portion, short (1–2 cells) in distal portion (Figures 9–11). Carpogonia symmetric, 13–25.5 µm in length; trichogyne club-shaped, sessile (Figures 10–11).

Carposporophytes pedicellate or indistinctly pedicellate, 1–2 per whorl, dense, spherical, 75–175 µm in diameter; carposporophyte inserted in the inner or outer part of the whorls; diameter to whorl radius ratio 0.5–1.0; gonimoblast filaments 3–5 cells (Figures 3–5, 12–13). Carposporangia obovoid, 10–18 µm in length, 7–10 µm in diameter (Figures 12–13).

DNA sequences:—*rbc*L-KX764639 (SJRP29725), KX764640 (SJRP29745); SSU-KX764641 (SJRP29725), KX764642 (SJRP29745)).

TABLE 1. Diagnostic taxonomic characters for the two species of *Nocturama*.

Characters	*N. antipodites*	*N. novamundensis*
Habitat	Heavily shaded streams	Partly to heavily shaded streams
Thallus colour	Red, red-brown or dark grey	Brownish
Thallus diameter (μm)	330–800	300–535
Primary fascicle cell number	8–13	8–15
Primary fascicle cell shape	Audouinelloid (cylindrical)	Cylindrical, ellipsoid or obovoid
Primary fascicle disposition	Straight	Curved
Secondary fascicles	Lacking (or rarely on very old thalli)	Lacking
Sexuality	Monoecious (rarely dioecious)	Dioecious
Spermatangia position	Primary fascicles (rarely on secondary fascicles in old thalli)	Primary fascicles
Carpogonial branch cell number	6–9	3–10
Carpogonial branch length (μm)	30–55	30–70
Carpogonial length (μm)	19–39	13–25.5
Trichogyne shape	Club-shaped	Club-shaped
Carposporophyte number	1–2	1–2
Carposporophyte shape	Spherical	Spherical
Carposporophyte diameter (μm)	70–200	75–175
Carposporangia shape	Obovoid	Obovoid
Carposporangia size (μm)	10–18 x 6–12	10–18 x 7–10
Carposporophyte diameter--to-whorl radius ratio	0.5–1.0	0.5–1.0
References	Entwisle & Kraft (1984, as *B. ectocarpum*) Entwisle (1995), Entwisle & Foard (2007)	Necchi (1990), Branco *et al.* (2014), as *B. arcuatum* This study

Diagnostic characters:—the species can be distinguished from *N. antipodites* in having curved rather than straight primary fascicles, composed of non-audouinelloid cells (Table 1). In addition, Brazilian populations of *N. novamundensis* are dioecious, whereas the Australian specimens of *N. antipodites* are monoecious, only rarely dioecious. Sequence divergences for both molecular markers analyzed corroborate the morphological characters, indicating a clear separation of the two species.

Circumscription of *Nocturama*:—with the addition of a new species, the circumscription of *Nocturama* remains the same except for the fascicle cells not always being audouinelloid (Entwisle *et al*. 2016). The Southern Hemisphere (ex-Gondwanan) distribution, red to brownish colour of the thallus, generally well-shaded habitat and the lack of secondary fascicles are useful if not exclusive diagnostic characters for the genus.

Within the Batrachospermales, *Nocturama* more closely resembles members of the genus *Sheathia* and section *Batrachospermum* of the genus *Batrachospermum* in lacking secondary fascicles and having long and undifferentiated carpogonial branches, short and usually club-shaped trichogynes, and pedicellate carposporophytes. It can be distinguished from both of those taxa by the presence of only one or two large carposporophytes per whorl (carposporophyte diameter-to-whorl-radius ratio ranging from 0.5 to 1.0). In contrast, members of *Sheathia* and section *Batrachospermum* typically have more numerous carposporophytes (two to six, exceptionally up to ten, per whorl) that are much smaller than whorls (carposporophyte diameter-to-whorl-radius ratio < 0.5) (Kumano 2002, Salomaki *et al*. 2014). This discovery highlights yet again the richness and distinctiveness of the Batrachospermales in the Southern Hemisphere, and that there are important discoveries still to be made even among existing collections.

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