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# Bostrychia edisondepaulae (Rhodophyta, Rhodomelaceae), a new freshwater species from Brazil

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# *Bostrychia edisondepaulae* (Rhodophyta, Rhodomelaceae), a new freshwater species from Brazil

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## Abstract

Samples tentatively identified as *Bostrychia moritziana* were collected in freshwater habitats from two locations in the States of São Paulo (Cananéia) and Rio de Janeiro (Trindade), southeastern Brazil. Culture observations for more than 20 years, combined with analyses of DNA sequences of the mitochondrial (COI-5P) and the plastid (*rbc*L) genes, supported the proposal of a new species of *Bostrychia*. The new species is morphologically closely related to *B. moritziana* and *B. radicans,* but it is phylogenetically distinguished by DNA sequence data. Reproduction in culture was by abortive tetrasporangia. Phylogenetic analyses showed

that the Brazilian samples formed a clade that was sister to *B. moritziana* sequences in the *rbc*L tree. Likewise, in the COI tree, these samples were a clade but nested among three *B. moritziana* sequences. The morphological similarity of the new species with *B. moritziana* and *B. radicans*, which have been regarded as species complexes, is intriguing. The sequence divergences found in this study (> 6% for *rbc*L and COI-5P) suggested that the new species is distant enough to be recognized as a distinct species in comparison to the closest species *B. moritziana.*

**Keywords:** cryptic species, culture, morphology, phylogeny

# Introduction

Most records of *Bostrychia* in Brazil are from mangrove habitats. The mangrove macroalgal community typically shows low species diversity, growing on pneumatophores, plantlets, roots and in the lower part of mangrove trees, mainly *Rhizophora mangle* Linnaeus, *Avicennia schaueriana* Stapf and Leechmann and *Laguncularia racemosa* (Linnaeus) Gaertner (Cordeiro-Marino *et al*., 1992). A recent update of Brazilian mangrove macroalgae listed 22 species, of which 14 are Rhodophyta and eight Chlorophyta (Yokoya *et al*., 2023). The most speciose genus growing on mangrove trees is *Bostrychia* Montagne, with seven species widely distributed on the Brazilian coast: *B. calliptera* (Montagne) Montagne, *B. kelanensis* Grunow, *B. montagnei* Harvey, *B. moritziana* (Sonder ex Kützing) J.Agardh, *B. pilulifera* Montagne, *B. radicans* (Montagne) Montagne, and *B. tenella* (J.V.Lamouroux) J.Agardh (Menezes *et al*., 2015; Flora e Funga do Brasil, 2020).

*Bostrychia* species tolerate wide fluctuations of salinity and higher water turbidity, being found attached to rocks in the highest and shaded parts in the intertidal zones to even more distant points of the estuary where there is practically no influence from the sea (Eston *et al*., 1992). According to Necchi & Vis (2021), species of *Bostrychia* have been reported in freshwater habitats primarily in tropical and sub-tropical regions and less often in temperate regions of Australasia, Asia, North and South America. Seven species of *Bostrychia* were recognized occurring in strictly freshwater habitats (Necchi & Vis, 2021): *B. flagellifera* Post, *B. hamana-tokidae* Post, *B. kelanensis* Post, *B. moritziana* (Sonder ex Kützing) J.Agardh, *B. radicans* (Montagne) Montagne, *B. scorpioides* (Hudson) Montagne and *B tenella* (J.V. Lamouroux). Some species have been reported as relatively geographically widespread (e.g. *B. moritziana* and *B. radicans*), while others are more geographically restricted (*B.*

*kelanensis, B. scorpioides* and *B. tenella*). However, it does not seem to have a geographic trend of occurrence for individual species in freshwater. Two species commonly reported in freshwaters (*B. moritziana* and *B. radicans*) are currently interpreted as a species complex (Zuccarello & West, 2003). There are only two records of *Bostrychia* in freshwaters in Brazil (Kumano & Necchi, 1987; Branco & Necchi, 1996). *Bostrychia moritziana* (as *B. radicans* f. *moniliformis* Post) was described from Ceará State, northeastern Brazil (Kumano & Necchi, 1987), whereas Branco & Necchi (1996) reported the occurrence of *B. moritziana* from São Paulo State, southeastern Brazil.

In February 1998, samples tentatively identified as *B. moritziana* (as *B. radicans* f. *moniliformis*), based on the monosiphonic last-order branches, were collected on Cardoso Island, in Cananéia, south coast of the state of São Paulo, and sent to Prof John West for culture studies at the University of Melbourne, Australia. Later, in 2001, other samples of *B. moritziana,* growing far from the influence of the sea, were collected at Trindade, Rio de Janeiro, by the now deceased Professor Dr. Edison de Paula and also sent to J. West. Since then, both samples have been kept under controlled conditions of temperature, light and salinity. The results of monitoring these samples for more than 20 years, combined with analyses of DNA sequences of the mitochondrial barcode region of the gene encoding cytochrome *c* enzyme (COI-5P) and the plastid large subunit gene encoding the Rubisco enzyme (*rbc*L), supported the proposal of a new species of *Bostrychia*, here presented.

# Materials and Methods

**Samples and culture conditions**

The present study was based on specimens kept in the algal culture laboratory at the Univ. Melbourne for more than 20 years since its collection on the south coast of Rio de Janeiro and south of São Paulo, Brazil. Details of the collection sites are described below.

Specimens previously identified as *Bostrychia moritziana* were collected by Edison de Paula on March 26, 2001, in a shaded stream, without tidal influence (salinity 1 psu), about 200 meters from the sea, in Trindade, State of Rio de Janeiro (23º21.100'S and 44º43.500'W). Samples in two 50 mL tubes were sent by airmail, which arrived on April 25, 2001, at the University of Melbourne, Australia.

Samples identified with culture codes as JAW3825 and JAW3826 were also originated from material collected by M.T. Fujii at the Sítio Grande River (SG2), Ilha do Cardoso State Park, in Cananéia, State of São Paulo (25°03.000' S and 047°55.000’ W) located about 270 km

south from São Paulo. SG2 mangrove is a soft-bottom shaded mangrove due to the high tree canopy that reduces light penetration to 10-30% of the incident irradiance (Oliveira, 1984). Salinity of the local water varied from 0 to 30 psu when measured every two months from December 1987 to December 1988 (Eston *et al*., 1992).

The cultures were started by cutting two apical tips (1mm), agar drag, and wash in 50x70 mm Pyrex culture dishes with 5 psu PEAT culture medium. Methods for isolation and maintenance of cultures are the same as described in West & Zuccarello (1999) and West (2005). Isolates were kept under the following culture conditions: temperature 18-20 ºC, irradiance of 2–4 μmol photons $m^{-2}$ $s^{-1}$ supplied by lighting of cool white LED. The long-term maintenance of these isolates is part of the work conducted by John West, with the support of University of Melbourne, to maintain a private culture collection of macro and microalgae (primarily red algae) that currently comprises about 2,500 isolates, including numerous strains of *Bostrychia* (West, 2005).

**Morphological analyses**

Morphological observations and measurements were made on material maintained under controlled culture conditions for over 20 years, as described above. These specimens represent clones of the population collected in Trindade, Rio de Janeiro. Measurements of cell length and diameter were taken from the surface view, as well as the thallus diameter in fully developed regions. At least ten measurements were taken of each element, and the measurement is given in length by width. Photomicrographs and measurements were taken with digital camera (Canon G3, Japan) using bright field optical on a Zeiss Axioscope microscope (Göttingen, Germany).

**DNA sequencing and phylogenetic analyses**

Material from seven culture isolates was prepared for DNA sequencing using silica desiccant (Table 1). The material was pulverized by grinding in liquid nitrogen using a mortar and pestle. DNA was extracted using the Nucleospin II Plant Mini kit. The protocol was modified with a longer incubation period of 40 minutes.

Two sets of primers were used to amplify, in two fragments, the *rbc*L gene and *rbc*L-S spacer region, which will be referred to as the *rbc*L throughout the rest of the paper. The first fragment utilized the F*rbc*L Start primer (5'-ATGTCTAACTCTGTAGAAG-3') paired with either the R753 primer (5'-GCTCTTTCATACATATCTTCC-3') or R753a (5'-GCTCTTTCRTACATATCYTC-3') (Freshwater & Rueness, 1994; Cassano *et al*., 2009).

The second fragment was generated with the F753 primer (5'-GGAAGATATGTATGAAAGAGC-3') and R*rbc*SStart primer (5'-GTTCTTGTGTTAATCTCAC-3') (Freshwater & Rueness, 1994). The PCR reaction mix consisted of 35.3 µL $dH_2O$, 5 µL 10x buffer, 1 µL dNTP, 1.5 µL $MgCl_2$, 0.25 µL Invitrogen Taq, 2.5 µL of each amplification primer, and 2 µL of extracted DNA. If a fragment was not produced, then the PuReTaq™ Ready-To-Go™ PCR Bead was used, consisting of 19 µL $dH_2O$, 2 µL of each primer and 2 µL DNA. The thermocycler settings were as follows: 94 °C for 4 min, then 35 cycles of 94 °C for 1 min, 40 °C for 1 min, 72 °C for 1:30 min, and a final hold at 72 °C for 10 min. For PCR of the COI-5P, the primer pair GWSFn (5'-TCAACAAATCAYAAAGATATYGG-3') and GWSRn (5'-GGRTGTCCRAARAAYCARAA-3') was used (Le Gall & Saunders, 2010). The PuReTaq™ Ready-To-Go™ PCR Bead was used, and the thermocycler settings were as follows: 94 °C for 5 min, then 35 cycles of 94 °C for 30 sec, 45 °C for 1 min, 72 °C for 2 min, and a final hold at 72 °C for 10 min. All PCR products were purified using the PureLink™ Quick PCR Purification kit and sequenced by Eton Bioscience Inc. Sequence data were visualized and checked using Geneious prime 2023.2.1 (https://www.geneious.com). All newly generated sequence data are available from GenBank (Benson *et al.,* 2017; Table 1) and were combined with closely related sequences from GenBank (Table S1). For the *rbc*L, seven new sequences and 12 from GenBank were aligned and likewise six new sequences and two GenBank sequences were aligned for the COI-5P. Phylogenetic analyses were conducted for each gene separately. Maximum likelihood (ML) using RAxML (Stamatakis, 2014) and Bayesian Inference (BI) analysis using MrBayes v.3.2.6 (Huelsenbeck & Ronquist, 2001) were run as plug-ins in Geneious Prime. For the ML and BI analyses, a GTR+G model was employed. Support values were determined using 1,000 ML bootstrap (bs) replicates and BI posterior probabilities (pp), 100,000 generations sampling every 200 generations.

# Result

### Phylogenetic analyses

New sequence data of the *rbc*L was generated for all seven culture isolates, but only six yielded new COI-5P data. When the two fragments of the *rbc*L were combined for the individual isolates, the resulting sequences ranged from 1521 to 1550 base pairs (bp) due to length differences in the *rbc*L-S spacer region. The COI-5P length was 661 bp. For the

phylogenetic analyses of the *rbc*L, the seven newly generated sequences were combined with eight sequences of *B. radicans*, and one each of *B. moritziana, B. radicosa* (Itono) J.A.West, G.C.Zuccarello & M.H.Hommersand, *B. tangatensis* Post and *B. simpliciuscula* Harvey ex J.Agardh. The *rbc*L sequences from GenBank, along with the new sequences, varied from 1163 bp to 1550 bp, and the final alignment was trimmed to 1528 bp. For the COI-5P, the six newly generated sequences were combined with one sequence each of *B. moritziana* and *B. simpliciuscula,* and the final alignment was trimmed to 661bp.

The phylogenetic analyses using ML and BI of the *rbc*L data showed similar tree topology, such that only the ML tree with support values is shown (Fig. 1). *Bostrychia radicans* was split into two clades with one newly sequenced culture isolate in each. Two new sequences were in a clade with previously sequenced *B. moritziana*. Three of the isolates formed a well-supported clade separate from all others and sister to *B. moritziana* (Fig. 1). These three cultures only differed from each other by 0.1% (uncorrected p-distance) but from *B. moritziana* by 8.2–8.3%.

The phylogenetic analyses using ML and BI of the COI-5P data showed similar tree topology, such that only the ML tree with support values is shown (Fig. 2). Among the eight sequences, *B. radicans* was sister to *B. simpliciuscula*. The three culture isolates formed a clade that was nested among the three *B. moritziana* sequences. The three isolates only differed from each other by 0-0.3% but from all three *B. moritziana* sequences by 6.0-6.5%.

**Morphology under culture conditions**

After approximately two months in culture, the isolates' growth reached 2.5 mm and 4.5 mm, and both were contaminated by unicell green. They were washed and transferred after 90 days, replicate 1 was 2.8 mm and 2 was 6 mm. After 105 days, replicate 1 was not growing and terminated, while replicate 2 was 16 mm with compound monosiphonous laterals. After six months, replicate 2 had robust branching and numerous tetrasporangial stichidia, spores released, but no viable sporelings, and after eight and a half months, it was still robust without sporelings. With one year of growth in culture, the isolate was still robust with no sporelings and large 3-4 cm thalli were produced with many tetrasporangia and free spores, but still no sporelings. After 20 months, it was still contaminated with green unicells, and short tips were agar dragged and washed. After 25 months, it was still contaminated, and was again agar dragged and washed. Thalli were robust with normal branches and tetrasporangia, many free spores, but still no sporelings. With 33 months, the isolate was clean and robust. After 39 months in culture, growth was vigorous, attached to glass, with no reproduction, and

after 42 months, thalli were robust, attached with abortive tetrasporangia, and still no sporelings. These same conditions were observed until this isolate reached seven and a half years in culture. From this date on, the conditions can be summarized as follows: good growth, not attached, free floating, normal branching, abortive tetrasporangia, no sporelings, occasionally contamination with green unicells. Thereafter, the isolate became non-reproductive, and this condition persisted until the most recent observations (after 23 years of isolation in culture) with transfer intervals at 5-9 months after 07 May 2013 and after 11 months of the latest transfer on 05 July 2023.

It is not possible to distinguish this new species from the closely related species, *B. radicans* and *B. moritziana*, based on morphology, except for the thinner and more delicate thallus, which may be due to the long time kept in culture. Although *B. edisondepaulae* is morphologically similar to *B. radicans* and *B. moritziana*, the genetic divergences (as uncorrected p-distances) among them are distinct: 6.7-9.9% for *rbc*L and 6.0-6.5% for COI-5P and justifies the present proposal for a new species.

**Taxonomic treatment**

***Bostrychia edisondepaulae*** J.A.West, Necchi, M.L.Vis & M.T.Fujii sp. nov. (Figs. 3A-F)

**Type:** BRAZIL: State of Rio de Janeiro, Trindade, Paraty municipality, about 60 km from the border of Ubatuba, in the north of São Paulo state, at a shaded stream, 200 m from sea, with no tidal influence, salinity 1 psu, 23º21.100'S, 44º43.500'W. BHO A-1929, dried material of culture strain JAW4146, collected 2 March 2001 by Edison de Paula (Holotype); Culture strains JAW3825 (BHO A-1928) and JAW3826 (BHO A-1987) collected 25 February 1998 in the Sitio Grande River (SG2), Ilha do Cardoso State Park, Cananéia, São Paulo state, Brazil (Paratypes).

**Diagnosis:** thallus ecorticate, attached to the substrate by a cladohapteron; main axes composed of two whorls of pericentral cells and two tier cells. The species is morphologically similar to *Bostrychia moritziana* and *B. radicans*, but phylogenetically distinct based on COI 5P and *rbc*L sequence data, which provide the only reliable means of differentiation

**Description:** Thallus ecorticate, main axes 25 mm long and up to 140 µm in diameter (Figs. 3A-B); main axes, 110-140 µm in diameter, formed by an axial cell that produces four pericentral cells with 72-82 µm in length, 45-52 µm in diameter. Each of the pericentral cells divides transversely, resulting in two-tier cells per axial cell (Fig. 3C); apices of determinate branches monosiphonous, straight (Figs. 3A-B). Branching dichotomous or

pseudodichotomous, alternate, with up to three orders of branches (Figs. 3A-B), with the first lateral transformed into a cladohapteron to attach to the substrates. The well-developed culture specimens show a delicate and disorganized appearance. Tetrasporangial stichidia slender, elongate-ellipsoidal or fusiform (Figs. 3D-E); tetrasporangia never produced viable tetraspores. Galls developed on stichidia (Figs. 3D, F). Specimens have been kept in the algal culture laboratory at Melbourne University since March 2001. No further specimens have been collected.

**GenBank Accession Numbers**: *rbc*L PZ445390; COI-5P PZ445397.
**Etymology:** epithet named in honor of Edison José de Paula, collector of the holotype, and for his enormous contribution to Brazilian phycology until his premature death at age of 52 in 2003.

## Discussion

Among the seven species of *Bostrychia* reported in freshwaters (Necchi & Vis, 2021), only *B. moritziana* has been observed in freshwater habitats in Brazil (Kumano & Necchi, 1987; Branco & Necchi, 1996). Due to the proximity of the sites where the two populations were collected in Cardoso Island, it is reasonable to assume that the previous record from São Paulo State by Branco & Necchi (1996) could be attributed to the new species here described. The taxonomic diversity of the species of *Bostrychia* is still poorly known in freshwaters in Brazil, since upstream segments of river estuaries have been scarcely sampled.
Species of *Bostrychia* reported from freshwater typically reproduce only by tetrasporangial stichidia (Necchi & Vis, 2021). The only report of sexual reproduction in freshwaters is by D'Lacoste & Ganesan (1987) from a population of *B. moritziana* from Venezuela with carpogonia and cystocarps. We found different reproductive patterns of freshwater populations of *Bostrychia* in culture. The *B. moritziana* isolates 3149 and 3150 from Venezuela, collected several km upstream in turbulent flow, have reproduced sexually in culture from 1992 to 2024 (Karsten *et al*., 1993; West unpublished data). On the other hand, the two isolates of *B. edisondepaulae* sp. nov., although also collected in freshwater habitats, only produced abortive tetrasporangial stichidia during the entire period under culture conditions. Galls were observed in the majority of culture isolates *B. moritziana/B. radicans* and were more frequent in male than female or tetrasporophytic plants (West et al., 2013). Furthermore, the authors reported that had never found galls on any *Bostrychia*

species in the field. In the present study galls were observed on tetrasporangial stichidia of tetrasporophytic plants.

Cryptic diversity has been reported in several species of *Bostrychia*. Saengkaew *et al*. (2016) employed RuBisCo spacer sequences to observe patterns of genetic diversity of *Bostrychia* species along the coasts of the Andaman Sea (Indian Ocean) and Gulf of Thailand (Pacific Ocean) in Thailand. Their results showed that, of the eight morphospecies of *Bostrychia* recognized on both coasts of Thailand, four (*B. binderi, B. calliptera, B. tenella* and *B. moritziana*) consisted of multiple cryptic species. In addition, several new haplotypes were discovered for *B. binderi, B. tenella* and the *B. moritziana/B. radicans* species complex. Genetic analysis using the plastid *rbc*L spacer from samples from the Andaman Sea and the Gulf of Thailand confirmed the occurrence of the two cryptic *B. tenella* species along both coasts (Bulan *et al*., 2022). Genetic data (partial COI sequences) revealed the occurrence of five cryptic *B. intricata* species along the coast of southeastern Australia (Muangmai *et al.,* 2022). The morphological and molecular similarity of the new species with *B. moritziana* and *B. radicans*, which has been regarded as a species complex (Zuccarello & West, 2003; Saengkaew *et al*., 2016), is intriguing. However, based strictly on the molecular data reported here, *B. edisondepaulae* does not correspond to any of the seven lineages previously identified within the *B. moritziana/B. radicans* complex (Zuccarello & West, 2003), but instead represents a distinct and previously unrecognized lineage.

The sequence divergences observed in this study (> 6% for *rbc*L and COI-5P) suggested that the new species is distant enough from the closest species *B. moritziana* to be recognized as a distinct species. It is strongly recommended that the current information based on only two populations from the Brazilian coast should be expanded to clearly demonstrate the range of genetic variation within this new species and to compare it with the species complex of the two closest species (*B. moritziana* and *B. radicans*).

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## Authors' Contributions

All authors contributed to the study conceptualization and all took part in manuscript writing. JAW conducted all culture experiments and observations and provided the samples for

sequence generation and photomicrograph; MLV sequenced the samples and performed the phylogenetic analyses; MTF prepared the species description, the figure plate and the initial draft of the manuscript; ONJ contributed to the phylogenetic analyses, morphological description and the writing the initial draft. All authors read and approved the final manuscript.

## Conflict of Interest

The authors declare that there are no competing interests involved in the manuscript concept and content.

## Data Availability Statement

All data supporting the findings of this study are included in the article.

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## Table and Figure Captions

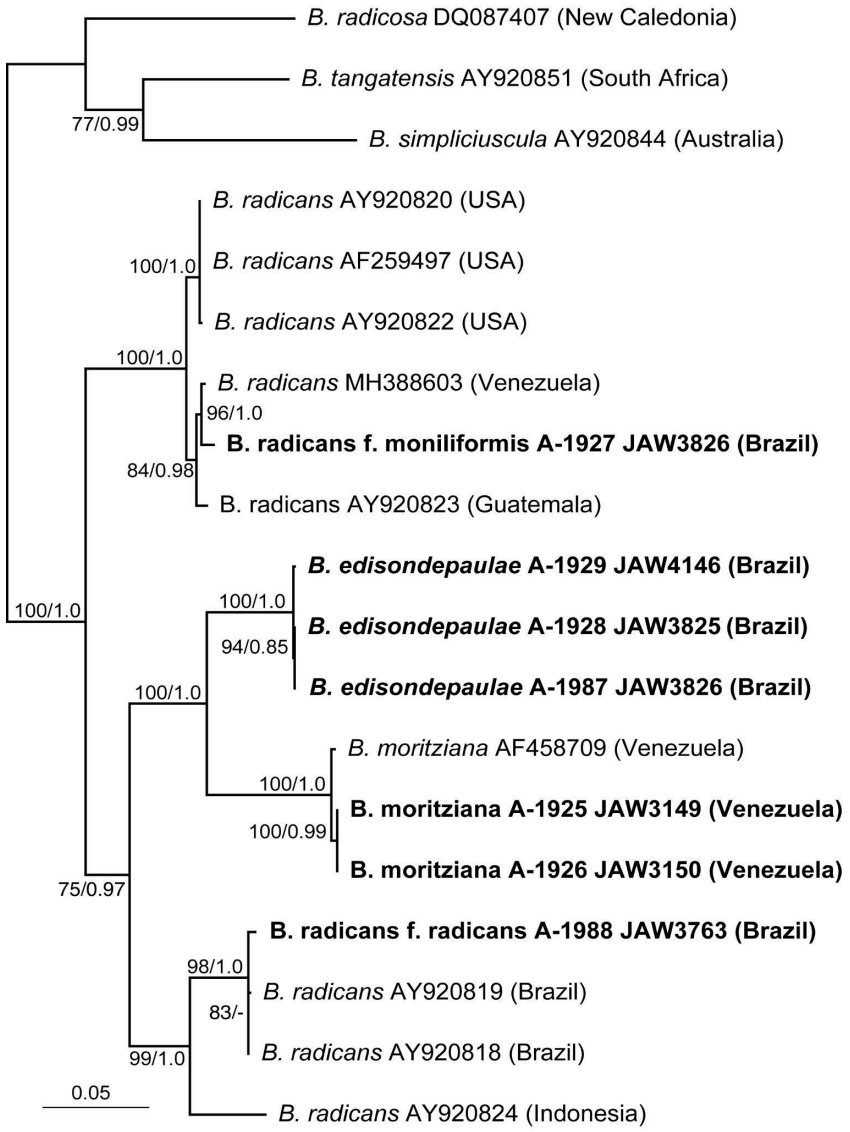


**Figure 1.** Maximum likelihood phylogenetic tree based on *rbc*L sequences showing the relationship of *Bostrychia edisondepaulae* sp. nov with other *Bostrychia* species. Information on the sequences is listed in Table S1. Scale represents substitutions per site and support values correspond to ML bootstrap and BI posterior probability. Newly generated sequences are in boldface.

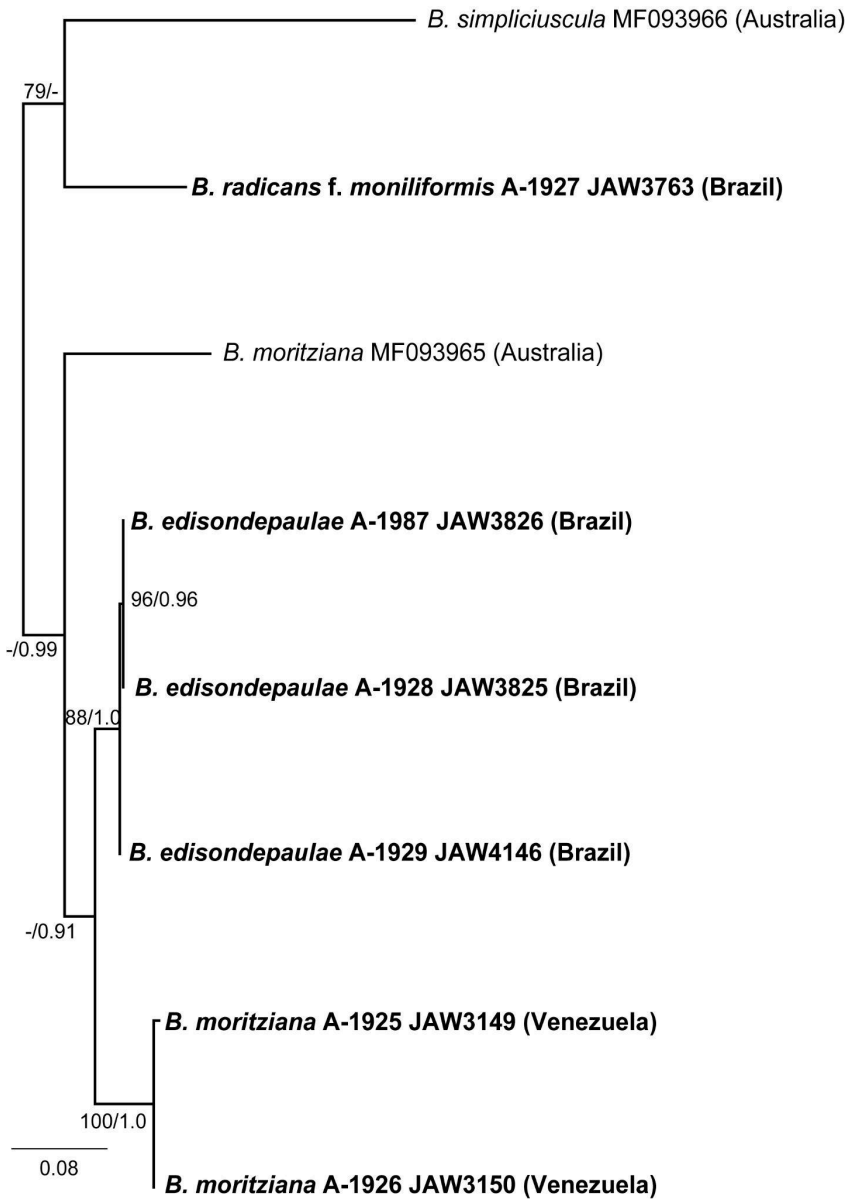


**Figure 2.** Maximum likelihood phylogenetic tree based on COI-5P sequences showing the relationship of *Bostrychia edisondepaulae* sp. nov with other *Bostrychia* species. Information on the sequences is listed in Table S1. Scale represents substitutions per site and support values correspond to ML bootstrap and BI posterior probability. Newly generated sequences are in boldface.

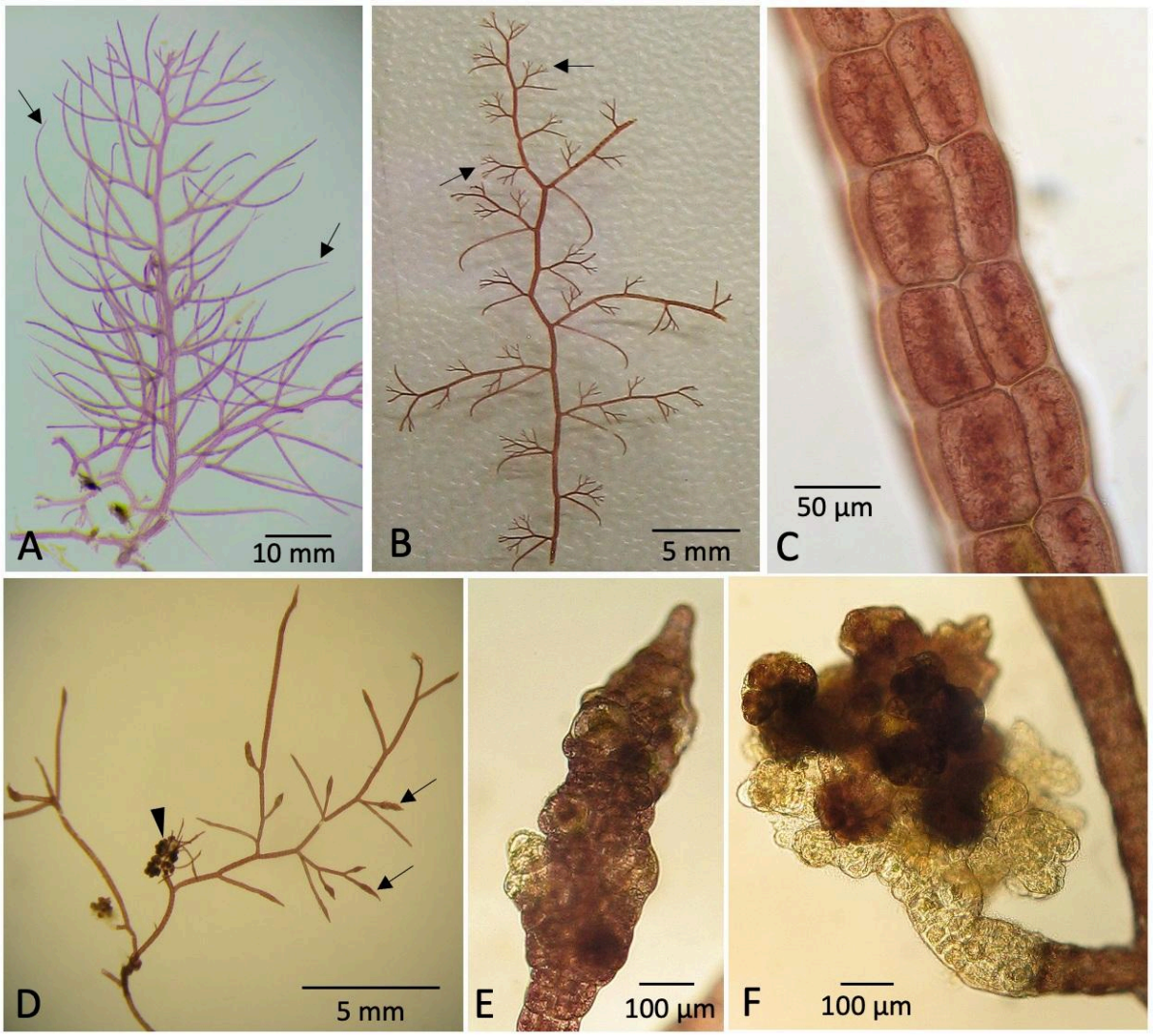


**Figure 3.** Morphological features of *Bostrychia edisondepaulae* sp. nov. **A.** Trindade strain JAW4146 (holotype), note monosiphonous ultimate branches (arrows). **B.** Ilha do Cardoso strain JAW3826, note monosiphonous ultimate branches (arrows). **C.** Detail of the lateral branch with four periaxial cells (three cells in the plane of vision). **D.** Specimen with abortive tetrasporangial stichidia (arrows) and galls developed on it (arrow head). **E.** Detail of an abortive tetrasporangial stichidium developed in culture. **F.** Detail of the tetrasporangial stichidium galls.

**Table 1.** Information on *Bostrychia* culture isolates used in this study.

| Taxon | Collection information (Location, collector, date, habitat) | Culture Number | Herbarium Voucher | GenBank Number *rbc*L, COI-5P |
|---|---|---|---|---|
| *B. edisondepaulae* | Brazil, Rio de Janeiro State, Trindade, E. J. Paula, E., 26 March 2001, freshwater | JAW4146 | BHO A-1929 | PZ445390, PZ445397 |
| *B. edisondepaulae* | Brazil, São Paulo State, Ilha do Cardoso, M.T. Fujii, 15 February 1998, freshwater | JAW3825 | BHO A-1928 | PZ445387, PZ445396 |
| *B. edisondepaulae* | Brazil, São Paulo State, Ilha do Cardoso, M.T. Fujii, 15 February 1998, freshwater | JAW3826 | BHO A-1987 | PZ445391, PZ445398 |
| *B. moritziana* | Venezuela, Rio Guire, 11 September 1991, freshwater | JAW3149 | BHO A-1925 | PZ445386, PZ445393 |
| *B. moritziana* | Venezuela, Rio Guire, 10 September 1991, freshwater | JAW3150 | BHO A-1926 | PZ445387, PZ445394 |
| *B. radicans* f. *moniliforme* | Brazil, Santa Catarina, N. S. Yokoya and M.T. Fujii, 10 October 1997, marine | JAW3762 | BHO A-1927 | PZ445388, PZ445395 |
| *B. radicans* f. *radicans* | Brazil, Santa Catarina, N.S. Yokoya, 5 October 1997, freshwater with little salt water influence | JAW3763 | BHO A-1988 | PZ445392 |

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