INTERNATIONAL JOURNAL OF SYSTEMATIC AND EVOLUTIONARY MICROBIOLOGY

TAXONOMIC DESCRIPTION

Alvarenga *et al.*, *Int J Syst Evol Microbiol* 2017;67:3301–3309

DOI 10.1099/ijsem.0.002109

MICROBIOLOGY SOCIETY

Kryptousia macronema gen. nov., sp. nov. and *Kryptousia microlepis* sp. nov., nostocalean cyanobacteria isolated from phyllospheres

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Abstract

Tropical ecosystems worldwide host very diverse microbial communities, but are increasingly threatened by deforestation and climate change. Thus, characterization of biodiversity in these environments, and especially of microbial communities that show unique adaptations to their habitats, is a very urgent matter. Information about representatives of the phylum *Cyanobacteria* in tropical environments is scarce, even though they are fundamental primary producers that help other microbes to thrive in nutrient-depleted habitats, including phyllospheres. In order to increase our knowledge of cyanobacterial diversity, a study was conducted to characterize isolates from *Avicennia schaueriana* and *Merostachys neesii* leaves collected at a mangrove and an Atlantic forest reserve located at the littoral of São Paulo state, south-east Brazil. The morphological, ultrastructural, phylogenetic, molecular and ecological features of the strains led to the recognition of the new genus *Kryptousia*, comprising two new species, *Kryptousia macronema* gen. nov., sp. nov. and *Kryptousia microlepis* sp. nov., described here according to the International Code of Nomenclature for algae, fungi and plants. The new genus and species were classified in the nostocalean family *Tolypotrichaceae*. This finding advances knowledge on the microbial diversity of South American ecosystems and sheds further light on the systematics of cyanobacteria.

Tropical and subtropical ecosystems hold a number of niches colonized by biogeochemically relevant micro-organisms that are responsible for maintaining ecological dynamics and equilibrium. The habitat established by the phyllosphere, in particular, constitutes an important micro-ecological zone, which hosts microbial communities that remain mostly unknown despite their hyperdiversity and importance to host plants and nutrient cycling [1, 2]. Host plants and associated microbes are profoundly influenced by each other, which results in trees presenting characteristic microbiomes that undergo selection [3–5]. In tropical environments, phyllospheric bacteria may become major contributors to the dynamics of elements such as nitrogen [6].

Among the phyllosphere microbiota, cyanobacteria can be highlighted for their physiological capacities and the ecological roles they perform. Cyanobacteria are valuable primary producers that help to ease the nutrient depletion typical of leaf surfaces and favour the survival of other microbes, allowing for rich communities to thrive in spite of the harsh conditions of these habitats. In addition to producing organic compounds, epiphyllic cyanobacteria may provide fixed nitrogen [7, 8] and accumulated phosphorus [9], secrete plant-growth-regulating and signalling molecules [10, 11], counteract the negative effects of salt on plant development [12], and protect against insects [13, 14].

A large number of unknown cyanobacteria occurring on leaf surfaces from Atlantic forest [15] and mangroves [16] has been estimated in south-east Brazil. However, few epiphyllic cyanobacterial taxa have been isolated and characterized from these ecosystems. Though it is currently possible to evaluate microbial diversity by using culture-independent techniques, isolation is still crucial for characterizing unknown micro-organisms. We have recently described two phyllospheric cyanobacterial genera from a mangrove in south-east Brazil [17]. The present work aimed to characterize a new group of phyllospheric strains isolated from the same mangrove trees and another tree in an Atlantic forest reserve.

Samples were collected in two ecosystems located at the littoral region of the state of São Paulo, Brazil. *Avicennia schaueriana* Stapf and Leech. (Acanthaceae) leaves were

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Keywords: tropical environments; Atlantic forest; mangroves; *Tolypotrichaceae*.

The GenBank/EMBL/DDBJ accession numbers for the 16S–23S rRNA gene sequences of CENA329, CENA334, CENA336, CENA338^T, CENA343 and CENA354^T are KY508607-KY508612, respectively.

Two supplementary figures are available with the online Supplementary Material.

sampled in a mangrove forest at the Bertioga resort, north of the municipality of Guarujá (23° 53′ 50.40″ S, 46° 12′ 30.60″ W). *Merostachys neesii* Rupr. (Poaceae) leaves were sampled in the Santa Virgínia nucleus of the Serra do Mar state park, north of the municipality of Ubatuba (23° 20′ 36″ S, 45° 04′ 22″ W). The leaves collected were placed into sterile bags and stored at 4 °C until isolation, which happened within a week. Isolation was carried out by submerging leaves into 500 ml Erlenmeyer flasks containing 200 ml sterile BG-11 [18] or BG-11_0 [19] media and cycloheximide (Sigma-Aldrich) at a final concentration of 75 mg ml^{-1}. Inoculated flasks were kept at 25±1 °C under a fluorescent light of 40 µmol photons $m^{-2}s^{-1}$ with a photoperiod of 14 h light and 10 h dark. The inoculated flasks were constantly monitored for growth by using an Axiostar Plus light microscope (Carl Zeiss) and cyanobacterial colonies were transferred to solid BG-11/BG-11_0 for isolation by streaking.

After the strains were isolated, the cultures were photographed with an Olympus BX53 light microscope equipped with differential interference contrast (Olympus). For ultrastructural characterization, cells from a representative strain were fixed for 64 h at 4 °C with modified Karnovsky solution [20] and post-fixed for 1 h at room temperature with 1 % osmium tetroxide. Afterwards, the cells were pre-stained for 18 h at 4 °C with 2.5 % uranile acetate and dehydrated with increasing concentrations of acetone solutions (25, 50, 75, 90 and 100 %). Dehydrated cells were subjected to infiltration with Spurr resin for 24 h followed by submersion in resin for polymerization for 48 h at 70 °C [21]. Ultrathin sections of 600–1000 µm were cut from polymerized blocks with a Porter Blum MT-2 ultramicrotome (Sorvall Instruments) and transferred to 200 mesh copper grids covered with 5 % colodium. The sections were stained with uranile acetate for 20 min and lead citrate for 15 min. A Zeiss EM 900 transmission electron microscope (Carl Zeiss) was used for visualizing and photographing samples at 50 kV.

Genetic characterization began by collecting 3-week-old cultures for DNA extraction according to the method described by Fiore *et al.* [22]. Amplification of the 16S rRNA gene was performed by PCR in the Techne TC-412 termocycler (Bibby Scientific) using primers 27F1 and 23S30R under previously described conditions [23]. Amplicons were cloned into *Escherichia coli* DH5*α* cells after ligation to a pGEM-T vector (Promega). Positive colonies were selected by plating and blue-white screening and confirmed by PCR with the primers CYA359F, CYA781Ra and CYA781Rb [24]. A BigDye XTerminator kit (GE Healthcare) was used for amplifying plasmids with the vector primers M13F/M13R and the internal 16S rRNA gene primers 341–357F, 357–341R, 685–704F, 704–685R, 1099–1114F and 1114–1099R (modified from [25]). Sequencing was performed in an ABI PRISM 3500 Genetic Analyzer (Applied Biosystems).

The reads obtained were assembled by using the Phred/Phrap/Consed software package version 1.090518/020425 [26–28] and regions containing bases with qualities lower than Phred 20 were resequenced. Multiple sequence alignments were produced with Clustal Omega 1.2.1 [29] and jModelTest 2.1.10 [30] was used for selecting evolutionary models. Identification of conserved 16S–23S rRNA intergenic spacer (ITS) regions in the alignments was carried out according to Iteman *et al.* [31], and secondary structures were predicted with Mfold 3.6 [32] and visualized with PseudoViewer 3.0 [33]. MrBayes 3.2.2 [34] was used for Bayesian inference with two independent runs, four chains and 5 000 000 generations, and maximum likelihood analysis was performed with RaxML 7.2.8 [35] using a bootstrap resampling value of 1000. The phylogenetic tree was drawn with FigTree 1.4.2 (http://tree.bio.ed.ac.uk/software/figtree) and Inkscape 0.48.4 (www.inkscape.org).

Six filamentous strains were isolated from the samples, five from *Avicennia schaueriana* leaves and one from *Merostachys neesi*. All strains presented heterocytes, a typical feature of the cyanobacterial order *Nostocales*, suggesting that they can contribute to the phyllospheres from where they were isolated by fixing atmospheric nitrogen in addition to carbon dioxide; no akinetes were observed (Figs 1, 2). An ultrastructural evaluation also showed thylakoid arrangements similar to those commonly observed in the order *Nostocales*, with irregularly arranged membranes (Fig. 3). The presence of untapered or very slightly tapered, false-branched, heteropolar filaments guided the classification of these strains in the family *Tolypotrichaceae*, following the cyanobacterial taxonomic classification system proposed by Komárek *et al.* [36].

The isolated strains presented morphological features very similar to each other, including: frequent unilateral branching and occasional bilateral branching; sheaths open at the apex; hairless, cylindrical trichomes; granules and necridia; and hormogones, among others. Nonetheless, some differences in morphology could be observed among these strains, mainly regarding colony aspect and cell and filament length and diameter, leading to their separation into two groups (Table 1). Phylogenetically, 16S rRNA gene sequences obtained from these strains were grouped with other sequences from strains classified in the order *Nostocales*, and their separation into two close, but separate groups was confirmed with high statistical support in both Bayesian inference and maximum-likelihood analyses (Fig. 4). Additionally, secondary structures from conserved sections of their 16S–23S ITS regions supported the distinctions observed, most evidently in the D1–D1′ and V3 helices (Fig. 5). These observations indicated that these strains represent a single, coherent genus composed of two distinct species.

Morphological characteristics alone were not fully informative in regard to genus identification due to the overall visual similarity of the isolated strains to other Tolypotrichacean taxa. However, when combined, detailed morphological, phylogenetic, molecular and ecological data supported the classification of these strains into a new Tolypotrichacean genus composed of two novel species, one

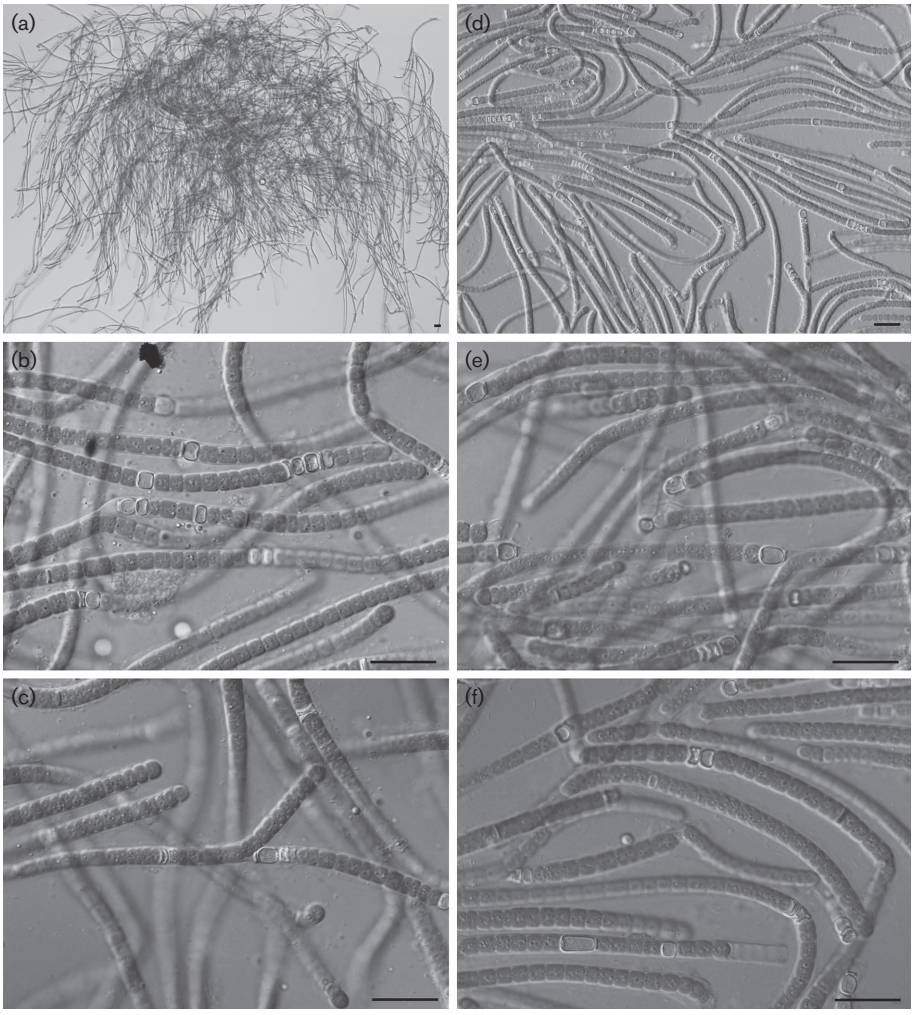

Fig. 1. Morphological aspects of *Kryptousia macronema* strains CENA336 (a–c) and CENA338^T (d–f). Scale bar, 20 µm. A colour version is available in the Supplementary Material as Fig. S1.

represented by strains CENA336 and CENA338^T and another by strains CENA329, CENA334, CENA343 and CENA354^T. Thus, these strains were respectively described as *Kryptousia macronema* gen. nov., sp. nov. and *Kryptousia microlepis* sp. nov. according to the International Code of Nomenclature for algae, fungi, and plants.

DESCRIPTION OF *KRYPTOUSIA* GEN. NOV.

Kryptousia (Krypt.ou′si.a. Gr. adj. *kryptos -ê -on* secret, hidden; Gr. fem. n. *ousía* the being, the essence of a thing; N.L. fem. n. *Kryptousia* a hidden essence; referring to the cryptic nature of its taxonomy, with few morphological differences when compared to related taxa).

Filaments interwoven or forming flake-like groups. Unilateral branches (Y type) common, with one or two single-pore heterocytes at the base, rarely without basal heterocytes. Bilateral branches (X type) occasionally present. Sheath thin, firm, colourless, homogeneous. Trichomes cylindrical, not or very little narrowed to the apex, without hairs. Cells shorter than broad, isodiametric or a little longer than broad. Cell content blue-green to yellowish blue-green. Granules and necridia present. Single-pored heterocytes isolated or in rows, basal, occasionally in the middle of the trichome (probably before trichome fragmentation and when in pairs also indicate a fragmentation spot). Reproduction by trichome fragmentation forming hormogones, akinetes not observed.

The type species is *Kryptousia macronema*.

DESCRIPTION OF *KRYPTOUSIA MACRONEMA* SP. NOV.

Kryptousia macronema (ma.cro.ne'ma. Gr. adj. *makros*, long; Gr. neut. n. *nema* thread; N.L. neut. n. *macronema* long thread; in reference to the length of filaments).

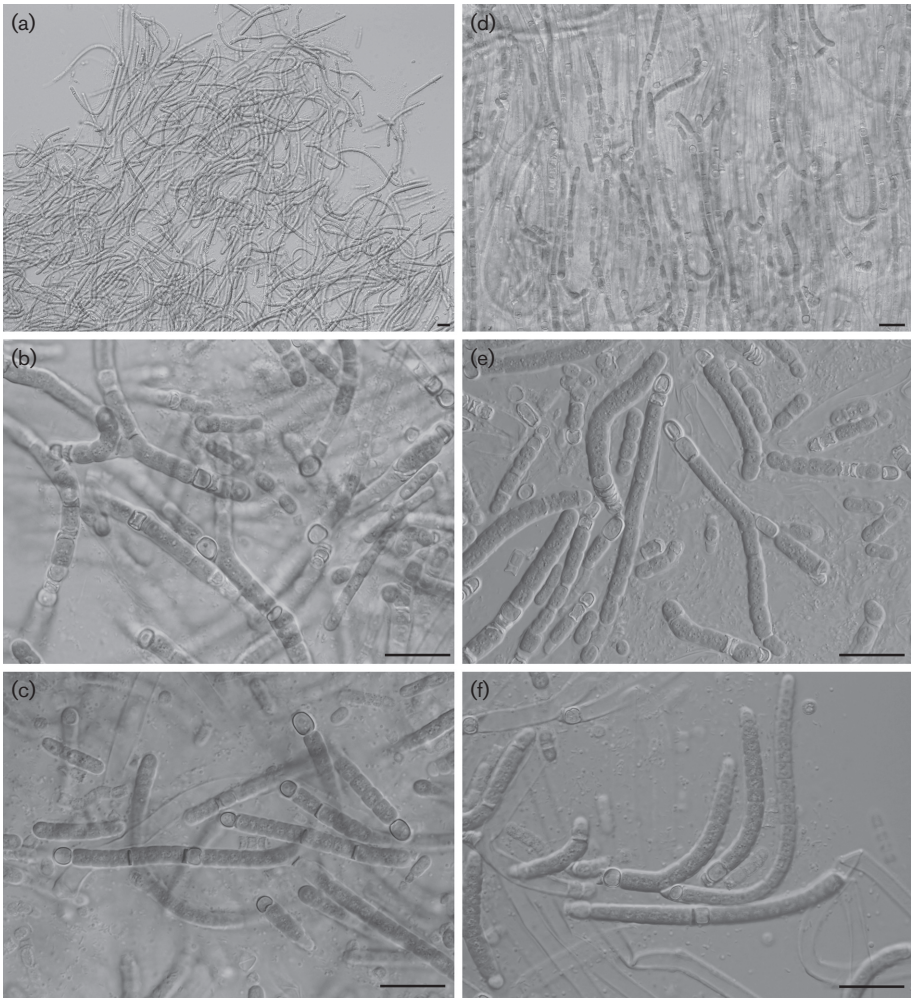

Fig. 2. *Kryptousia microlepis* micrographs illustrating its morphological features. (a) CENA329; (b) CENA334; (c, d) CENA343; (e, f) CENA354^T. Scale bar, 20 µm. A colour version is available in the Supplementary Material as Fig. S2.

Filaments interwoven, more or less oriented in parallel, forming more expanded masses (cultivation conditions), usually longer than 600 µm, (4.5−)5.0–6.7(−7.2) µm diameter (5.8 µm on average; *n*=122). Unilateral branches (Y type) common, with one or two single-pore heterocytes at the basis, rarely without basal heterocytes. Bilateral branches (X type) occasionally present. Sheath thin, firm, colourless, homogeneous, opened at the apex of the adult filament. Trichomes cylindrical, distinctly constricted at cross-walls, without hairs. Cells 1.9–7.4 (−8.0) µm long (4.4 µm on average; *n*=127), 3.9–6.3 µm diameter (5.0 µm on average; *n*=127), length:diameter ratio from 0.4 to 1.6 (−1.7) (0.9 in average; *n*=127). Cell content blue-green to yellowish blue-green, centroplasm and chromatoplasm not always distinguishable, granulated. Necridia present. Single-pored heterocytes isolated or in rows (up to four; usually with one or two apparently functional), basal, occasionally in the middle of the trichome (probably before trichome fragmentation and when in pairs also indicate a fragmentation spot), very variable in shape and size, conical, rounded, haemispherical, discoid, cylindrical or ellipsoid, 2.6–10.2 µm long, 3.5–7.4 µm diameter at the larger axis. Two-pored heterocytes occasionally observed in the middle of the trichome, isolated. Reproduction by trichome fragmentation, akinetes not observed.

Holotypus: SP469781, dried material deposited at the herbarium of the São Paulo Institute of Botany, São Paulo, Brazil.

The type strain is CENA338^T.

DESCRIPTION OF *KRYPTOUSIA MICROLEPIS* SP. NOV.

Kryptousia microlepis (mi.cro.le′pis. Gr. pref. *micro-* small; Gr. fem. n. *lepis* flake; N.L. fem. n. *microlepis* small flake; in reference to its flake-like arrangement of filaments).

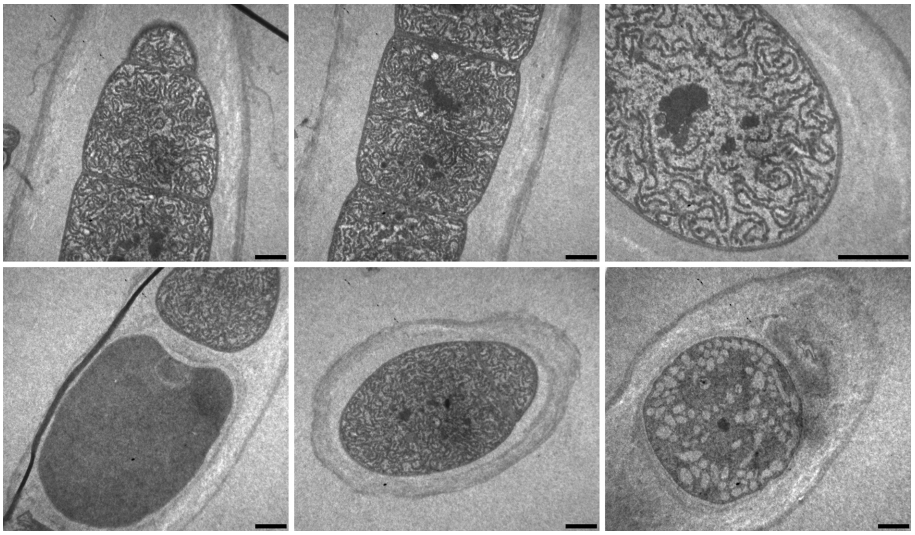

Fig. 3. Transmission electron micrographs of *Kryptousia macronema* CENA338^T filaments evidencing thylakoid arrangements typical of the order *Nostocales*. Scale bar, 1 µm.

Filaments forming flake-like groups (cultivation conditions), usually up to 400 µm long, (4.3−)4.5–7.8 (−9.8) µm diameter (6.1 µm in average; *n*=112). Unilateral branches (Y type) common, sometimes occurring repeatedly (up to three times) along a filament, with one or two single-pored heterocytes at the basis, rarely without basal heterocytes. Bilateral branches (X type) occasionally present. Sheath thin, firm, colourless, homogeneous, opened at the apex of the adult filament. Trichomes cylindrical, distinctly constricted at cross-walls, without hairs. Cells 2.6–9.2 (−11.4) µm long (5.4 µm on average; *n*=212), 3.1–6.4 µm diameter (4.7 µm on average; *n*=212), length:diameter ratio from 0.5 to 2.1 (−2.9) (1.2 on average; *n*=212). Cell content blue-green to yellowish blue-green, centroplasm and chromatoplasm usually distinguishable, granulated. Necridia present. Single-pored heterocytes isolated or in rows (up to four; usually with only one apparently functional), basal, occasionally in the middle of the trichome (probably before trichome fragmentation and when in pairs also indicate a fragmentation spot), very variable in shape and size, conical, rounded, hemispherical, discoid, cylindrical or ellipsoid, 2.6–8.6 (−10.2) µm long, 3.5–7.0 (−7.4) µm diameter at the larger axis. Two-pored heterocytes not observed. Reproduction by trichome fragmentation forming hormogones, akinetes not observed.

Table 1. Features commonly found in *Kryptousia* species as observed in the strains described in this work

Feature	*K. macronema*	*K. microlepis*
Form	Filamentous	Filamentous
Filament arrangement	Uniseriate	Uniseriate
Single false branching	Frequent	Frequent
Double false branching	Occasional	Occasional
Heterocytes	Present	Present
Akinetes	Absent	Absent
Sheaths	Open at the apex	Open at the apex
Hairs	Absent	Absent
Necridia	Present	Present
Reproduction	Hormogonia	Hormogonia
Colony	Interwoven filaments	Flake-like
Filament length	>600 µm	<400 µm
Cell length	4.4 µm	5.4 µm
Cell diameter	5.0 µm	4.7 µm
Length:diameter ratio	0.9	1.2

Holotypus: SP469782, dried material deposited at the herbarium of the São Paulo Institute of Botany, São Paulo, Brazil.

The type strain is CENA354^T.

Atlantic forests and mangroves are increasingly threatened by a wide range of processes, especially deforestation [37, 38] and climate change [39, 40]. Tropical and subtropical environments face a great and irreversible loss of biodiversity as a consequence of such combined threats [41–43]. Microbial diversity and activities may also be significantly impacted by anthropogenic processes, and their loss affects biogeochemical cycles and compromises ecosystem stability [44–46]. Therefore, although culture-independent methods are being extensively employed in phyllosphere microbiology [47, 48], the isolation of novel cyanobacteria from threatened ecosystems is still important because it also allows the preservation of genetic resources that might otherwise become unavailable.

The strains described in the present work were previously hypothesized as novel species belonging to the genus

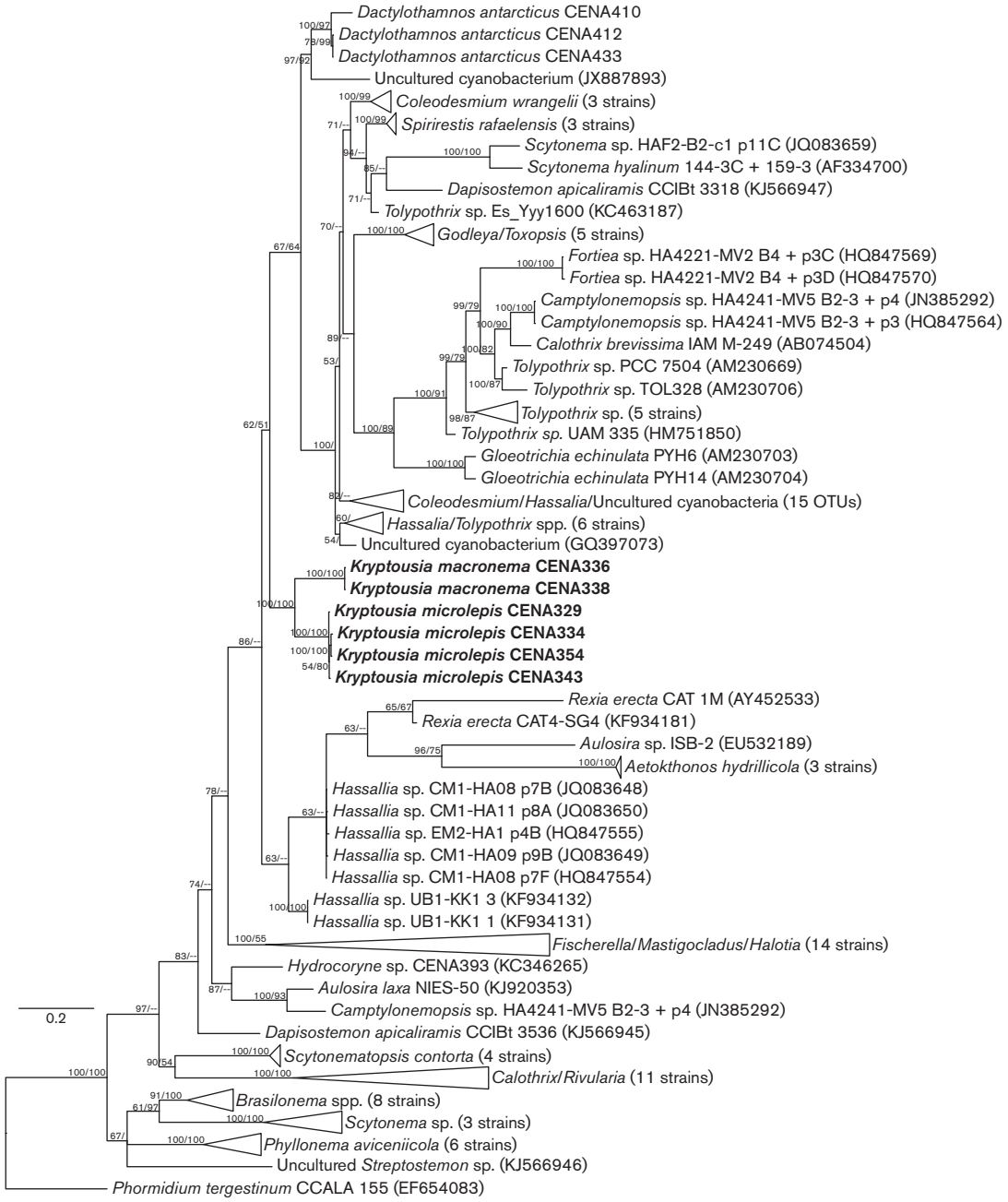

Fig. 4. Phylogenetic tree reconstructed by using the Bayesian inference method. Posterior probabilities are included in the nodes, followed by maximum-likelihood bootstrap values in branches present in both analyses.

Dactylothamnos, recently isolated from Antarctic soil [49]. That suspicion was mainly based on morphological similarities between the *Dactylothamnos* and *Kryptousia* strains, as the phylogenetic tree presented in the *Dactylothamnos* report included a reduced number of sequences and did not provide statistical reliability to the grouping of the Antarctic strain and the Brazilian isolates into a single clade. Consequently, no descriptions were provided for those hypothetical species. The present work renders robust phylogenetic and molecular evidence that, together with ecological considerations, can clearly distinguish the strains from distinct continents and habitats and indicate they constitute different genera.

Cryptic species are a common occurrence in cyanobacterial systematics [50–52]. Cryptic taxa in cyanobacteria may also be found in taxonomic levels higher than species, such as cryptic genera or cryptogenera, as exemplified by *Kryptousia* and a number of recently described cyanobacterial

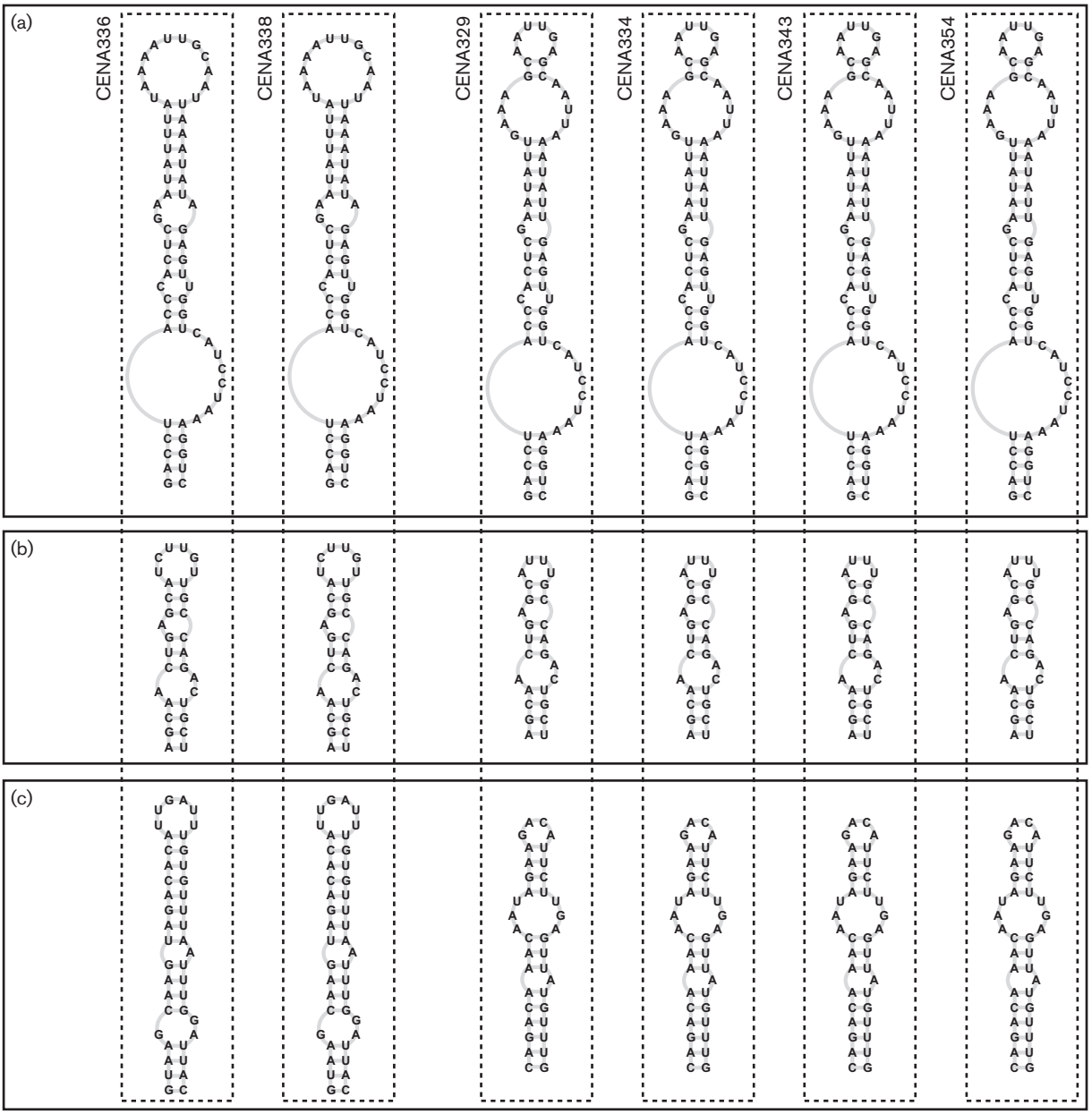

Fig. 5. Secondary structures of the D1–D1′ (a), box B (b), and V3 (c) helices from 16S–23S intergenic spacers of *K. macronema* and *K. microlepis* strains.

genera, including *Aliterella* [53], *Ammassolinea* [54], *Ancylothrix* [55], *Pinnochia* [56], *Potamolinea* [57] and *Roseonema* [58]. Similar morphology in different taxa frequently causes underestimation of biodiversity, but polyphasic taxonomy can be used successfully for sorting out these issues, especially when relying on robust molecular methods, which allow a more refined view of microbial evolution [51].

The cyanobacterial family *Tolypotrichaceae* has been recently proposed from genera previously classified in the family *Microchaetaceae*, which has also been split into the family *Godleyaceae*, differing mainly in regard to morphological, ecological and molecular data [59]. Like *Kryptousia*, Tolypotrichacean genera present as main features heteropolar, sheathed filaments, with frequent single false branching and sporadic double false branching, and reproduction by hormogonia [59]. Currently, this family is composed of the genera *Borzinema*, *Coleodesmium*, *Dactylothamnos*, *Hassalia*, *Rexia*, *Seguenzaea*, *Spirirestis*, *Streptostemon* and *Tolypothrix* [36].

In addition to *Dactylothamnos*, *Kryptousia* also resembles *Tolypothrix* among the Tolypotrichacean genera. However, *Tolypothrix* has been demonstrated to be polyphyletic [59],

a finding that exemplifies the limitations of morphology-based identification for these taxa. A polyphasic approach that includes molecular data appears to be essential for distinguishing *Kryptousia* from related Tolypotrichacean genera. Nonetheless, the current members of the family *Tolypotrichaceae* are found exclusively in terrestrial or freshwater environments, while *Kryptousia* has also been found in the saline leaf surfaces of *Avicennia*, indicating that ecological distinction might be possible.

Avicennia schaueriana is a mangrove tree species that is dominant in Brazil, the Caribbees and the Guianas, having been restricted to Atlantic America as a result of evolutionary processes [60, 61]. The distribution of *Merostachys neesii* is even more restricted, as it is endemic to the Brazilian Atlantic forest and was presumably extinct until 2008, when it was rediscovered [62]. Whether the restricted distribution of these trees is reflected on the biogeography of *Kryptousia* spp. remains to be verified. However, as observed in the phylogenetic tree presented here, 16S rRNA gene sequences from representative strains do not show close relationships to currently available sequences of strains either from South America or other continents, including uncultured cyanobacteria.

Avicennia schaueriana is known for the salt-secreting glands present in its leaves, which add further complexity to the harsh conditions usually observed in the phyllosphere [63]. This plant is also capable of producing a range of antimicrobial compounds [64]. Less is known about *Merostachys neesii* and its the leaf surface, but it most likely does not present such osmotically challenging conditions, suggesting that *K. microlepis* might have fewer restrictions regarding habitats exposed to salt, unlike *K. macronema*, which currently has only been found in mangroves.

Funding information
D.O. Alvarenga was supported by São Paulo Research Foundation (FAPESP) and National Council for Scientific and Technological Development (CNPq) graduate fellowships (grants #2008/52556-4 and #132494/2010-8, respectively). A.P.D. Andreote was supported by a FAPESP graduate fellowship (grant #2009/15402-1). M.F. Fiore thanks the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for a research fellowship (#310244/2015-3).

Acknowledgements
We wish to thank Professor Dr Neusa de Lima Nogueira and Mônica Lanzoni Rossi for helping with the preparation of samples for transmission electron microscopy, and Professor Dr Francisco André Ossamu Tanaka, Professor Dr Elliot Watanabe Kitajima and their team at NAP-MEPA/ESALQ-USP for permission to use the transmission electron microscope and assistance with the equipment. We are grateful to Dr Andrea Tucci for the deposit of reference samples at the São Paulo Institute of Botany. We would also like to thank the reviewers for their critical comments, which have greatly improved this manuscript.

Conflicts of interest
The authors declare that there are no conflicts of interest.

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