Halotia gen. nov., a phylogenetically and physiologically coherent cyanobacterial genus isolated from marine coastal environments

Diego Bonaldo Genuário,1 Marcelo Gomes Marçal Vieira Vaz,1 Guilherme Scotta Hentschke,2,3 Célia Leite Sant’Anna2 and Marli Fátima Fiore1

1Center for Nuclear Energy in Agriculture, University of São Paulo, Avenida Centenário 303, 13400-970 Piracicaba, SP, Brazil
2Institute of Botany, Nucleus of Phycology, Avenida Miguel Estêfano 3687, 04301-012 São Paulo, SP, Brazil
3Universidade Luterana do Brasil, Rua Martinho Lutero 301, 96501-595 Cachoeira do Sul, RS, Brazil

Nostoc is a common and well-studied genus of cyanobacteria and, according to molecular phylogeny, is a polyphyletic group. Therefore, revisions of this genus are urged in an attempt to clarify its taxonomy. Novel strains isolated from underexplored environments and assigned morphologically to the genus Nostoc are not genetically related to the ‘true Nostoc’ group. In this study, four strains isolated from biofilms collected in Antarctica and five strains originated from Brazilian mangroves were evaluated. Despite their morphological similarities to other morphotypes of Nostoc, these nine strains differed from other morphotypes in ecological, physiological and genetic aspects. Based on the phylogeny of the 16S rRNA gene, the Antarctic sequences were grouped together with the sequences of the Brazilian mangrove isolates and Nostoc sp. Mollenhauer 1 : 1-067 in a well-supported cluster (74% bootstrap value, maximum-likelihood). This novel cluster was separated phylogenetically from the ‘true Nostoc’ clade and from the clades of the morphologically similar genera Mojavia and Desmonostoc. The 16S rRNA gene sequences generated in this study exhibited 96% similarity to sequences from the nostocacean genera mentioned above. Physiologically, these nine strains showed the capacity to grow in a salinity range of 1–10% NaCl, indicating their tolerance of saline conditions. These results provide support for the description of a new genus, named Halotia gen. nov., which is related morphologically to the genera Nostoc, Mojavia and Desmonostoc. Within this new genus, three novel species were recognized and described based on morphology and internal transcribed spacer secondary structures: Halotia branconii sp. nov., Halotia longispora sp. nov. and Halotia wernerae sp. nov., under the provisions of the International Code of Nomenclature for Algae, Fungi and Plants.

The genus Nostoc is a cosmopolitan, nitrogen-fixing group of cyanobacteria with approximately 300 described species (Komárek, 2013). Species of the genus Nostoc can occur in aquatic, terrestrial and aerial ecosystems and in symbioses with fungi, mosses, liverworts, ferns and vascular plants (Komárek, 2013). Strains assigned to this genus have been documented in marine coastal environments ranging from tropical mangroves to Antarctica (Komárek et al., 2014; Silva et al., 2014). In Brazilian mangroves, six novel morphotypes of the genus Nostoc were isolated and characterized phylogenetically by Silva et al. (2014). In Antarctica, members of this genus are the most common heterocytous cyanobacteria in aerophytic and aquatic ecosystems (Fritsch, 1912; Wharton et al., 1983; Parker & Wharton, 1985; Simmons et al., 1993; Fumanti et al., 1997; Novis & Smissen, 2006; Taton et al., 2006a, b, 2008; Komárek et al., 2014). The seashore strains of Nostoc investigated in this

Abbreviations: ITS, internal transcribed spacer; ML, maximum-likelihood; NJ, neighbour-joining.

The GenBank/EMBL/DDBJ accession numbers for the 16S–23S rRNA genes and nifH sequences of the strains reported in this study are KC695852–KC695854, KC695857, KC695877 and KJ843310–KJ843313 (16S–23S rRNA genes) and KJ830938–KJ830946 (nifH).

Four supplementary figures are available with the online Supplementary Material.
study are directly and indirectly affected by tidal variation in the mesolittoral zone and by sea spray and wind in the supralittoral zone (Lewis, 1961), resulting in salted habitats. In general, the ocean salinity is approximately 3.5%, which is tolerated or even required by some organisms. According to Ventosa & Aralá (2002), organisms that live at this salt concentration are classified as slightly halophilic organisms, among which several cyanobacterial species are included. Many cyanobacterial species are able to grow at salinities ranging from 0 to 9.9% (Thajuddin & Subramanian, 1992; Oren, 2000, 2008; Moisand et al., 2002; Hagemann, 2011), and some genera and species have been described as inhabiting saline habitats (Golubic, 1980; Chatuchawan et al., 2012; Dadheech et al., 2012, 2013).

Morphologically, the genus Nostoc is characterized by uniseriate, isopolar and non-branched filaments, and its vegetative cells can differentiate into akinetes, heterocytes and hormogonia (Komárek & Anagnostidis, 1989). The akinetes are formed apoheterocyclicly, while heterocyte development occurs in terminal and intercalary positions (Komárek & Anagnostidis, 1989). The production of mucilaginous colonies and the complex life cycle are other traits common to all species of the genus (Lazaroff & Vishniac, 1961; Lazaroff, 1966; Mateo et al., 2011; Hrouzek et al., 2013). Despite its well-established morphological traits, according to the current taxonomy, the genus Nostoc is genetically heterogeneous, and phylogenetic analysis based on 16S rRNA gene sequences has revealed that several genotypes fall outside the ‘true Nostoc’ cluster (Hrouzek et al., 2005; Rajaniemi et al., 2005; Papaeftihimiou et al., 2008; Lukešová et al., 2009; Genuário et al., 2010; Hrouzek et al., 2013; Silva et al., 2014). Hence, many studies have recommended a revision of this genus, taking into account mostly phylogenetic data. As a result, some Nostoc-related morphotypes have been placed in the new genera Mojavia and Desmonostoc (Reháková et al., 2007; Hrouzek et al., 2013). Likewise, the strains of Nostoc investigated in this study, which were isolated from saline environments in Brazilian mangroves and maritime Antarctica, did not fall inside the ‘true Nostoc’ cluster. Therefore, a polyphasic approach was used that considered morphological traits, phylogenies of the 16S rRNA gene and nifH gene sequences, the growth response to saline culture conditions and internal transcribed spacer (ITS) secondary structures. Additionally, the presence of genes involved in cyanotoxin and protease inhibitor biosynthesis was evaluated.

Environmental samples collected in Antarctica (Almirantar Bay, King George Island, South Shetlands) were used for cyanobacterial isolation. Biofilm samples growing on rocks and bones were collected close to the Baranowski glacier (62°11’54”S 58°26’53”W) and on the seashore of Punta Plaza (62°05’28”S 58°24’22”W), respectively. Subsamples of these materials were spread onto BG-11, BG-11G (BG-11 without a nitrogen source) (Allen, 1968) and 3NP (Taton et al., 2006a) agarose media (1.2%, w/v) containing cycloheximide (70 mg l⁻¹) (Rippka, 1988). Monospecific cultures were obtained by successive streaking onto fresh solid medium followed by optical microscope observations. In all of the isolation steps, the cultures were grown with a 14:10 h light/dark cycle under white fluorescent irradiance (40 µmol photons µm⁻² s⁻¹) at 24±1°C. Morphological observations were conducted using a Zeiss Axioskop 40 optical light microscope equipped with an AxioVision LE 4.6 digital imaging system (Carl Zeiss). Morphological characterization was performed according to the reference literature (Bornet & Flahault, 1886; Komárek & Anagnostidis, 1989; Komárek, 2013). Five strains that were previously identified as members of Nostoc and were isolated from Brazilian mangroves (Silva et al., 2014) were included in this study. All nine cyanobacterial strains are maintained at the Culture Collection of the Center for Nuclear Energy in Agriculture (CENA/USP), Piracicaba, Brazil, under the conditions described above.

Total genomic DNA was extracted using a modified CTAB method adapted for cyanobacteria (Fiore et al., 2000). The 16S rRNA gene and the 16S–23S ITS (from position 27 of the 16S rRNA gene to position 30 of the 23S rRNA gene of Escherichia coli) were amplified by PCR using primers 27F and 23S30R (Taton et al., 2003). In addition, a fragment of the cyanobacterial nifH gene was amplified by PCR using the primer set designed by Olson et al. (1998). PCR amplification, cloning and sequencing of the two targets (16S–23S rRNA and nifH) were performed as described by Genuário et al. (2013). The sequenced 16S–23S rRNA and nifH gene fragments were assembled into two separated contigs using the software Phred/Phrap/Consed (Philip Green, University of Washington, Seattle, USA), and only bases with >20 quality were considered (Ewing et al., 1998; Ewing & Green, 1998; Gordon et al., 1998).

The nucleotide sequences obtained in this study and the reference sequences retrieved from GenBank were aligned using CLUSTAL W, trimmed (16S rRNA gene matrix with a 1465-bp length and nifH matrix with a 328-bp length) and used to reconstruct phylogenetic trees. 16S rRNA gene phylogenetic trees were reconstructed using the neighbour-joining (NJ), maximum-likelihood (ML) and Bayesian methods, while the nifH phylogenetic tree was reconstructed using ML only. The NJ and ML trees were reconstructed using the MEGA program package, version 5 (Tamura et al., 2011), using Kimura’s two-parameter model of sequence evolution. The robustness of the phylogenetic trees was estimated via bootstrap analysis using 1000 replications. Bayesian inference was conducted using MrBayes 3.2 (Ronquist & Huelsenbeck, 2003) applying two separate runs with four chains each and 100 000 000 Markov chain Monte Carlo generations. The tree was viewed in FigTree 1.3.1 (http://tree.bio.ed.ac.uk/software/figtree). The general time reversible evolutionary model of substitution with gamma distribution and with an estimate of proportion of invariable sites was selected as the fittest for the alignment by jmodelTest 2.1.1 (Darriba et al., 2012). Given that the NJ, ML and Bayesian methods resulted in nearly identical topologies, only the ML tree is presented, with indications of bootstrap values (NJ and ML) and Bayesian probabilities.
The ITS sequences and the D1–D1′, box B, V2 and V3 secondary structures were aligned and folded using CLUSTAL W from MEGA package (Tamura et al., 2011) and MFold (Zuker, 2003), respectively, following the default settings.

Three microcystin synthetase genes (mcyD, mcyE and mcyG), two intergenic spacer regions for protease inhibitors [aeruginosin (aerA/aerB) and cyanopeptolin (mcnC/mcnE)] and one saxitoxin synthetase gene (sxtI) were investigated in all of the strains. The PCR amplifications were performed as described by Genuário et al. (2013).

The nine strains were tested for their capacity to grow under different NaCl concentrations. Initially, each strain was grown for 15 days in 250 ml glass flasks containing 50 ml BG-110 medium under the conditions described above. Next, the biomass was resuspended and homogenized by syringe flow and used as the pre-inoculum (1% of biomass, v/v) for the assays. The experiments were conducted in 15 ml glass flasks containing 5 ml liquid BG-110 with NaCl added. The following NaCl concentrations were tested: 1, 3, 5 and 10 %, which are equivalent to 0.171, 0.513, 0.855 and 1.711 M NaCl, respectively. As a control, the strains were grown in BG-110 medium without NaCl. The effect of NaCl on the strains was analysed by determination of dry weight (the salt mass was discounted) and microscopic morphological inspection after 21 days of cultivation. All of the experiments were conducted in duplicate. For the dry weight determination, the flasks were dried for 7 days or until they reached a constant mass (Fiore et al., 2000). The dry weight data were analysed, and the values were compared using the Kruskal–Wallis non-parametric test, with a significance level of 5 %.

Nearly complete 16S rRNA gene sequences (1413 or 1414 bp) were obtained for all of the studied strains. A comparative analysis among the novel 16S rRNA gene sequences and related sequences retrieved from GenBank revealed Nostoc sp. Mollenhauer 1:1-067 (≤97.9 % similarity) and Nodularia sp. Lukesova 1/91 (≤96.9 % similarity) as the highest scoring hits. In the 16S rRNA gene phylogenetic reconstruction, the new Antarctic and Brazilian sequences were grouped together with the sequence of Nostoc sp. Mollenhauer 1:1-067 in a highly stable cluster (74 % bootstrap value, ML) and were related to species of Nodularia (including Nodularia sp. Lukesova 1/91), Cyanospira rippkae and species of Anabaenopsis (Fig. 1). The cluster representing Halotia gen. nov. was subdivided into four subclusters (C-Ia, C-Ib, C-Ic and C-Id), indicating the existence of four different species. Within subclusters C-Ia, C-Ic and C-Id, the sequences shared ≥99.7, ≥99.7 and ≥99.6 % 16S rRNA gene sequence identity, respectively. The sequence of Nostoc sp. Mollenhauer 1:1-067 (subcluster C-Ib) shared ≥96.3 % identity with the sequences of the other subclusters.

The lengths of the ITS of the nine strains varied from 591 to 641 bp (Table 2). Both tRNAs (Ile and Ala) and the regions

<table>
<thead>
<tr>
<th>Strain</th>
<th>Medium</th>
<th>Origin</th>
<th>Date of collection</th>
<th>Geographical coordinates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Halotia branconii</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CENA186</td>
<td>MN0</td>
<td>Soil, mangrove, Cardoso Island, Brazil</td>
<td>December 2006</td>
<td>25° 05’ 02” S 47° 57’ 42” W</td>
</tr>
<tr>
<td>CENA390</td>
<td>3NP</td>
<td>Whale bones on the seashore of Punta Plaza, Admiraity Bay, Antarctica</td>
<td>January 2009</td>
<td>62° 05’ 28” S 58° 24’ 22” W</td>
</tr>
<tr>
<td>CENA392</td>
<td>BG-110</td>
<td>As above</td>
<td>As above</td>
<td>As above</td>
</tr>
<tr>
<td>Halotia wernerae</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CENA1358</td>
<td>ASN-III</td>
<td>Soil, mangrove, Cardoso Island, Brazil</td>
<td>February 2006</td>
<td>25° 05’ 02” S 47° 57’ 42” W</td>
</tr>
<tr>
<td>CENA1359</td>
<td>ASN-III</td>
<td>As above</td>
<td>As above</td>
<td>As above</td>
</tr>
<tr>
<td>CENA1660</td>
<td>ASN-III</td>
<td>As above</td>
<td>As above</td>
<td>As above</td>
</tr>
<tr>
<td>CENA391</td>
<td>BG-11</td>
<td>Whale bones on the seashore of Punta Plaza, Admiraity Bay, Antarctica</td>
<td>January 2009</td>
<td>62° 05’ 28” S 58° 24’ 22” W</td>
</tr>
<tr>
<td>Halotia longispora</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CENA184</td>
<td>MN0</td>
<td>Brackish water, mangrove, Cardoso Island, Brazil</td>
<td>February 2006</td>
<td>25° 05’ 02” S 47° 57’ 42” W</td>
</tr>
<tr>
<td>CENA420</td>
<td>BG-11</td>
<td>Rock, close to Baranowski glacier, Antarctica</td>
<td>January 2009</td>
<td>62° 11’ 54” S 58° 26’ 53” W</td>
</tr>
</tbody>
</table>
D1–D1’, D2, box B, box A, D4, V2 and V3 were identified, and the secondary structures of the D1–D1’, box B, V2 and V3 regions were aligned, folded and compared (Fig. 2 and Fig. S1, available in the online Supplementary Material).

The functional nifH gene that encodes the Fe protein component of nitrogenase was partially sequenced (325 bp) for all of the strains. Comparisons of these nifH sequences with sequences available from GenBank showed the highest
identities with sequences of cultured cyanobacterial strains and uncultured cyanobacterial clones retrieved from nature. In the phylogenetic tree, the nifH sequences of the novel strains were dispersed in three clades (Fig. 3). The first clade was formed by five nifH sequences of our novel strains. The closest related sequence, from uncultured bacterial clone I-5.33, originated from intertidal microbial mats in the Netherlands (Severin et al., 2012). The second cluster, consisting of three other nifH sequences from our strains, has a sister cluster formed by the sequences of Desmonostoc muscorum UTEX 1933 and Nodularia harveyana CCAP 1452/1. The last nifH sequence from our novel strains was loosely affiliated with two sequences of cultured cyanobacterial strains (Myxosarcina sp. ATCC 29377 and Xenococcus sp. ATCC 29373) and two uncultured organisms (20107A04 and 20141A06) from a coral reef lagoon (Hewson et al., 2007).

Regarding the genes encoding the production of cyanotoxins and protease inhibitors (Table 3), at least two genes of microcystin (mcy) biosynthesis were amplified by PCR from all of the strains isolated from Brazilian mangroves. No mcy gene amplification was observed for the Antarctic strains. The xtl gene was amplified by PCR from three strains from the mangroves. Aeruginosin and cyanopeptolin intergenic regions were amplified by PCR from three strains, two from the mangroves and one from Antarctica.

The nine strains were able to grow at different NaCl concentrations (1, 3, 5 and 10 %), and statistically significant differences were observed in biomass production among the treatments (Kruskal–Wallis rank sum test: Kruskal–Wallis $\chi^2=22.6287$, d.f.=4, $P=0.0001502$). Additionally, the treatments were compared using the Mann–Whitney non-parametric test, which indicated that biomass production was higher in BG-11 $0^\circ$ alone and with 1 % NaCl (Table 4). Despite the differences in biomass production, all strains grew at all the tested NaCl concentrations, showing higher biomass production at salinities around seawater concentration (until 3 % NaCl).

The morphological, ecological and molecular data generated in this study, as discussed further, allowed the proposal of the new genus Halotia gen. nov., with the description of three novel species, Halotia branconii sp. nov., Halotia longispora sp. nov. and Halotia wernerae sp. nov. These descriptions were prepared considering all of the isolated strains. These descriptions are given under the provisions of the International Code of Nomenclature for Algae, Fungi and Plants.

### Diagnois for Halotia D. B. Genuário et al. gen. nov.

**Etymology:** Halotia (Ha.lo’ti.a. Gr. hals, halos salt. N.L. fem. n. Halotia referring to the salinity of the sampling sites, Brazilian mangroves and maritime Antarctica).

In nature, members of Halotia are found on soil from tropical mangroves and on bones and rocks in maritime regions of Antarctica. In nature, thalli are microscopic and, in liquid media, they grow as a gelatinous biomass on the bottom of the test tube or attached to the tube walls. Colonies are irregularly spherical and are sometimes enclosed by firm mucilage and are always enclosed by diffusent mucilage. Isodiametric motile hormogonia are present, and the terminal heterocytes develop first, followed by the intercalary ones. Members of Halotia have trichomes with spherical or sub-spherical cells (2.0–6.8 µm long, 2.3–4.6 µm wide) and heterocytes, which are generally straight at first and then become condensed and entangled in older colonies. Terminal heterocytes are dominant and conical, while intercalary heterocytes are spherical. The cells occasionally divide into two planes, forming pluriserrate trichomes. Akinetes are apoheterocytic, subspherical or elongated (2.7–7.5 µm long, 3.0–6.4 µm wide) and always germinate into two vegetative cells.

**Type species:** Halotia branconii.

### Diagnosis of Halotia branconii D. B. Genuário et al. sp. nov. (Figs 4 and S2)

Thallus composed mainly of colonies of trichomes enclosed by a firm and outer diffusent mucilage layer. Trichomes sometimes free-living and enclosed by diffusent sheaths;

---

### Table 2. Lengths of the 16S–23S ITS regions in the Halotia strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Complete ITS (nt)</th>
<th>D1–D1’</th>
<th>D2</th>
<th>D3</th>
<th>tRNAIle</th>
<th>V2</th>
<th>tRNAAla</th>
<th>Box B</th>
<th>Box A</th>
<th>D4</th>
<th>V3</th>
<th>D5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Halotia branconii</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CENA186</td>
<td>583</td>
<td>65</td>
<td>14</td>
<td>3</td>
<td>73</td>
<td>76</td>
<td>73</td>
<td>39</td>
<td>12</td>
<td>7</td>
<td>44</td>
<td>11</td>
</tr>
<tr>
<td>CENA390</td>
<td>583</td>
<td>65</td>
<td>14</td>
<td>3</td>
<td>73</td>
<td>76</td>
<td>73</td>
<td>39</td>
<td>12</td>
<td>7</td>
<td>44</td>
<td>11</td>
</tr>
<tr>
<td>CENA392</td>
<td>583</td>
<td>65</td>
<td>14</td>
<td>3</td>
<td>73</td>
<td>76</td>
<td>73</td>
<td>39</td>
<td>12</td>
<td>7</td>
<td>44</td>
<td>11</td>
</tr>
<tr>
<td>Halotia wernerae</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CENA158</td>
<td>546</td>
<td>65</td>
<td>14</td>
<td>3</td>
<td>73</td>
<td>68</td>
<td>73</td>
<td>45</td>
<td>12</td>
<td>7</td>
<td>36</td>
<td>13</td>
</tr>
<tr>
<td>CENA159</td>
<td>546</td>
<td>65</td>
<td>14</td>
<td>3</td>
<td>73</td>
<td>68</td>
<td>73</td>
<td>45</td>
<td>12</td>
<td>7</td>
<td>36</td>
<td>13</td>
</tr>
<tr>
<td>CENA160</td>
<td>546</td>
<td>65</td>
<td>14</td>
<td>3</td>
<td>73</td>
<td>68</td>
<td>73</td>
<td>45</td>
<td>12</td>
<td>7</td>
<td>36</td>
<td>13</td>
</tr>
<tr>
<td>CENA391</td>
<td>546</td>
<td>65</td>
<td>14</td>
<td>3</td>
<td>73</td>
<td>68</td>
<td>73</td>
<td>45</td>
<td>12</td>
<td>7</td>
<td>36</td>
<td>13</td>
</tr>
<tr>
<td>Halotia longispora</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CENA184</td>
<td>534</td>
<td>66</td>
<td>14</td>
<td>3</td>
<td>73</td>
<td>76</td>
<td>73</td>
<td>37</td>
<td>12</td>
<td>7</td>
<td>36</td>
<td>13</td>
</tr>
<tr>
<td>CENA420</td>
<td>533</td>
<td>65</td>
<td>14</td>
<td>3</td>
<td>73</td>
<td>76</td>
<td>73</td>
<td>37</td>
<td>12</td>
<td>7</td>
<td>36</td>
<td>13</td>
</tr>
</tbody>
</table>
Fig. 2. Secondary structures of the 16S–23S ITS sequences from the strains of *Halotia* gen. nov.
straight at first (hormogonia) and then become densely entangled and condensed forming colonies enclosed by firm mucilage. Akinetes spherical or subspherical (2.7–4.3 \( \mu m \) long, 3.4–5.6 \( \mu m \) wide) and slightly larger than the vegetative cells (2.0–3.3 \( \mu m \) long, 3.7–4.6 \( \mu m \) wide). Cell content dull green to brownish; mucilage hyaline or yellowish.

Holotype: ANTARCTICA, Punta Plaza, Admiralty Bay, 20/01/2009, Diego B. Genuário (SP 428496) (herbarium preparation of cultured material CENA392), Herbarium of São Paulo State, São Paulo, Brazil.

Reference strain: CENA392

Myxosarcina sp. (U73133)

Nodularia sphaerocarpa UP16a (EU381376)

Nodularia spumigena FL2f (AY493375)

Anabaenopsis sp. NRE1 (EU381375)

Klebsiella pneumoniae (U01740)

Fig. 3. ML phylogenetic tree based on partial nifH gene sequences. Strains of Halotia gen. nov. are shown in bold. A bootstrap test involving 1000 resamplings was performed, and bootstrap values greater than 50% are displayed at the relevant nodes. Bar, 0.1 substitutions per nucleotide position.
Table 3. PCR screening for genes related to microcystin, saxitoxin, aeruginosin and cyanopeptolin biosynthesis

<table>
<thead>
<tr>
<th>Strain</th>
<th>mcyD</th>
<th>mcyE</th>
<th>mcyG</th>
<th>aer</th>
<th>mcn</th>
<th>sxtI</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Halotia branconii</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CENA186</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CENA390</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CENA392</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Halotia wernerae</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CENA158</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>CENA159</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CENA160</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CENA391</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Halotia longispora</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CENA184</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CENA420</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

The genes and intergenic regions screened for are involved in biosynthesis of microcystin (mcyD, mcyE, mcyG), aeruginosin (aer), cyanopeptolin (mcn) and saxitoxin (sxtI).

Type location: Grows on bones in Punta Plaza, Admiralty Bay, Antarctica.

Studied material: BRAZIL, São Paulo State, Cardoso Island, 06/12/2006, Diego B. Genuário (SP 428497); ANTARCTICA, Punta Plaza, Admiralty Bay, 20/01/2009, Diego B. Genuário (SP 428498).

Habitat: Grows on bones in Antarctica and mangrove soils in Brazil.

Etymology: *Halotia branconii* (bran.co‘ni.i. N.L. gen. n. branconii of Branco; named for L. H. Z. Branco, a Brazilian taxonomist of cyanobacteria).

**Diagnosis of Halotia longispora** D. B. Genuário *et al.* sp. nov. (Figs 5 and S3)

Thallus composed of colonies of loosely entangled trichomes that are mainly enclosed by diffluent mucilage and sometimes by firm mucilage. Trichomes sometimes enclosed only by diffluent mucilage; straight at first (hormogonia) and then become lightly condensed and entangled when older, originating colonies are enclosed by firm mucilage. Akinetes elongated, granulated, bright green and twisted before being released (3.0–7.5 µm long, 3.0–6.1 µm wide). Cells subspherical or slightly elongated and bright green to brownish (3.6–5.8 µm long, 2.9–3.1 µm wide). Sheaths hyaline or yellowish.

Holotype: ANTARCTICA, close to the Baranowski glacier, 19/01/2009, Diego B. Genuário (SP 428499) (herbarium preparation of cultured material CENA420), Herbarium of São Paulo State, São Paulo, Brazil.

Reference strain: CENA420

Type location: Grows on rocks close to the Baranowski glacier, Antarctica.

Studied material: BRAZIL, São Paulo State, Cardoso Island, 17/04/2006, Diego B. Genuário (SP 428499)

Habitat: Mangrove soils in Brazil.

Etymology: *Halotia longispora* (lon.gi.spo’ra. L. adj. longus long; Gr. n. spora a seed and, in biology, a spore; N.L. n.

Table 4. Treatments and median values of biomass production

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Median dry weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BG-110</td>
<td>0.00649†</td>
</tr>
<tr>
<td>1% NaCl</td>
<td>0.00564‡</td>
</tr>
<tr>
<td>3% NaCl</td>
<td>0.00331§</td>
</tr>
<tr>
<td>5% NaCl</td>
<td>0.00029†</td>
</tr>
<tr>
<td>10% NaCl</td>
<td>0.00004†</td>
</tr>
</tbody>
</table>

Medians followed by the same symbol in the column do not differ by non-parametric Mann–Whitney test.
longispora the long spore, referring to the elongated shape of the akinetes).

**Diagnosis of Halotia wernerae D. B. Genuário et al. sp. nov. (Figs 6 and S4)**

Thallus composed of trichomes enclosed by a diffuent mucilage layer. Trichomes are straight at first (hormogonia) and then can be parallel, slightly condensed or intensely coiled. Mature cells subspherical and brownish or dull green (3.3–6.8 μm long, 2.3–3.4 μm wide). Akinetes subspherical, the same size as or slightly larger than vegetative cells (4.9–7.0 μm long, 3.0–6.4 μm wide) and granulated. Sheaths hyaline.

Holotype: BRAZIL, São Paulo State, Cardoso Island, 15/02/2006, Diego B. Genuário (SP 428501) (herbarium preparation of cultured material CENA158), Herbarium of São Paulo State, São Paulo, Brazil.

Reference strain: CENA158

Type location: Grows in mangrove soil in Cardoso Island, São Paulo State, Brazil.

Studied material: BRAZIL, São Paulo State, Cardoso Island, 15/02/2006, Diego B. Genuário (SP 428502); (SP 428503); ANTARCTICA, Punta Plaza, Admiralty Bay, 20/01/2009, Diego B. Genuário (SP 428504).

Habitat: Grows in mangrove soil in Brazil and on bones in Antarctica.

Etymology: *Halotia wernerae* (wer’ner ae. N.L. gen. n. wernerae of Werner; named for V. R. Werner, a Brazilian taxonomist of cyanobacteria).
The nine studied strains are morphologically indistinguishable from species of *Nostoc*, *Desmonostoc* and *Mojavia*; however, genetic, physiological and ecological results support the description of the novel genus *Halotia*. The phylogenetic tree based on the 16S rRNA gene sequences of several nostocacean genera resulted in the formation of seven clusters (C-I to C-VII) containing sequences of *Nostoc* morphotypes (Fig. 1). The novel *Halotia* sequences were placed in cluster C-I, supported by bootstrap values of 75 and 97 % (ML and NJ, respectively) and by a probability of 0.83 according to Bayesian analysis. The sequence of *Nostoc* sp. Mollenhauer 1:1-067 was the only one retrieved from GenBank that was positioned inside cluster C-I. This strain was isolated from an association with the lichenized fungus *Peltigera didactyla* from an unknown location; however, its 16S rRNA gene sequence was more closely related to sequences of members of the genus *Nodularia* than to sequences of other symbiotic members of the genus *Nostoc* (O’Brien et al., 2005). Likewise, clusters C-I and C-II were both more closely related to sequences of the genera *Nodularia*, *Anaebaenopsis* and *Cyanothrix* (Fig. 1). Among the *Nostoc* morphotype clusters, clades C-IV, C-VI and C-VII were established phylogenetically. In our phylogenetic tree, cluster C-IV consisted of sequences of *Mojavia pulchra* JTC2-VF2 (Reháková et al., 2007) and *Nostoc verrucosum* KU005 (Sakamoto et al., 2011), without bootstrap support (Fig. 1). The monospecific genus *Mojavia*, morphologically related to *Nostoc*, was described as having a subaerobic habitat in sandy desert soil of Cadiz Valley, Joshua Tree National Park, San Bernardino County, CA, USA (Reháková et al., 2007). Cluster C-VI was formed by sequences of typical members of the genus *Nostoc*, represented by the species *Nostoc punctiforme*, *N. commune*, *N. calcicola* and *N. edaphicum* (Svenning et al., 2005; Reháková et al., 2007). Cluster C-VII contained sequences of the recently described genus *Desmonostoc*, also related morphologically to *Nostoc* (Hrouzek et al., 2013). This genus has as its type species *Desmonostoc muscorum*, which was isolated from meadows and arable and abandoned soils ranging from pH 5.3 to 7.1; the reference sequence is NIVA-CYA818. The remaining clusters were spread throughout the phylogenetic tree and require further research.

Based on the phylogenetic tree clusters formed, a subset of the 16S rRNA gene sequences was compared. The 16S rRNA gene sequences of members of *Halotia* gen. nov. showed high identity with those of *Cyanothrix rippkae* PCC9501 (96.8 %), *Nostoc edaphicum* X (95.3 %) (‘typical *Nostoc*’ cluster) and *Nostoc* sp. PCC8112 (95.8 %) (phylogenetically the closest cluster). These values are close to the cut-off point for genus definition (95 %) (Stackebrandt & Goebel, 1994; Ludwig et al., 1998), which alone would not justify the separation of the *Halotia* cluster from the cluster mentioned above. However, this strict identity percentage (95 %) cannot be considered the unique diacritical feature to separate genera, since some exceptions have been applied for separation of cyanobacterial genera in the literature with broader ranges of identities for the nostocacean order (up to 99 %) (Berrendero et al., 2011; Flechtner et al., 2002). Likewise, the phylogenetic relationships within the clusters summarize all these data supporting the separation of our clade in a new genus.

A comparison among the 16S rRNA gene sequences within the *Halotia* cluster (C-I) revealed identities ≥96.3 %. This value indicates the existence of multiple species when considering an identity of 97.5 % as the cut-off point for bacterial species delimitation (Stackebrandt & Goebel, 1994; Ludwig et al., 1998). Furthermore, according to the phylogenetic tree (Fig. 1), cluster C-I contained four subclusters (C-Ia, C-Ib, C-Ic and C-Id), each representing a different species. Within each of subclusters C-Ia, C-Ic and C-Id, the sequences shared ≥99.7, ≥99.7 and ≥99.6 % identities, respectively. The sequence of *Nostoc* sp. Mollenhauer 1:1-067 (subcluster C-Ib) shares ≥96.3 % identity with the novel species.

The existence of three *Halotia* species is also supported by the 16S–23S ITS sequences. These sequences were very stable in terms of length and secondary structures (Table 2 and Figs 2 and S1). Reháková et al. (2007) analysed the 16S–23S ITS sequences of *Mojavia* and typical *Nostoc* strains and found box B and V3 regions of 40–55 nt and 32–110 nt, respectively. These variations seemed to be narrower in the box B and V3 regions of the *Halotia* strains. Furthermore, the tRNAs (Ala and Ile) and the D1–D1 helices for the *Halotia* strains, suggesting that they are specific for this novel genus (Table 2 and Fig. S1). Among the *Halotia* strains, the tRNAs (Ala and Ile) and D1–D1 had the same patterns of secondary structure (data not shown), whereas the box B, V2 and V3 regions exhibited four, three and two different patterns, respectively (Fig. 2). At first glance, the conserved pattern of the secondary structures of the D1–D1 helices for the *Halotia* strains matched that of *Mojavia* and those from members of *Nostoc sensu stricto* (Reháková et al., 2007). However, a detailed analysis revealed two and three additional nucleotides between the terminal and subterminal loops in *Mojavia* and *Nostoc sensu stricto*, respectively (Reháková et al., 2007). In addition, in two cases, the D1–D1 helices of members of *Halotia* exposed two unpaired nucleotides on the 5’ side of the helix of their basal unilateral bulges in comparison to no unpaired nucleotides and one unpaired nucleotide, respectively, for *Mojavia* and *Nostoc sensu stricto*. According to these minor variations, this structure separates *Halotia* from the other genera mentioned above. Moreover, the box B and V3 secondary structures showed wide differences in comparison with those published for *Mojavia* and *Nostoc sensu stricto* (Reháková et al., 2007). Consequently, D1–D1’, box B and V3 secondary structures separate *Halotia* from the other genera and also support the description of a new genus.

The separation of the strains of *Halotia* into three species is supported by the formation of three internal clusters within cluster I in the phylogenetic tree, by the three length
patterns of the 16S–23S ITS rRNA (complete ITS, box B, V2, V3), by the V2 secondary structure and by the diacritical morphological traits observed.

The characterization of members of the new genus *Halotia* regarding their potential for nitrogen fixation revealed the presence of *nifH* in all strains. The topology of the phylogenetic tree based on the novel *nifH* nucleotide sequences (Fig. 3) was not correlated with the topology obtained based on the 16S rRNA gene sequences (Fig. 1). Although some studies have demonstrated congruence between *nifH* gene and 16S rRNA gene phylogenies (Zehr & Capone, 1996; Zehr et al., 1997, 2003), phylogenetic analysis of the *nifH* gene reflects its own evolution and not the evolutionary relationships of the organisms. Its phylogeny can only distinguish broad taxonomic groups within the domains *Bacteria* and *Archaea*, and not at lower taxonomic levels, because of variations in amino acid and nucleotide sequences (Ben-Porath & Zehr, 1994). Our current knowledge has been revised considering the mechanisms involved in the distribution and maintenance of this functional gene. The gene cluster involved in nitrogen fixation is not distributed universally among the phylum *Cyanobacteria*, and the processes of its dispersion and maintenance are unknown (Young, 1992; Bolhuis et al., 2010), reflecting, at first glance, the disjunctive distribution of the *Halotia* species in the *nifH* phylogenetic tree. Swingley et al. (2008), studying conserved families of proteins in cyanobacteria, stressed that nitrogen-fixing ability in cyanobacteria is a paraphyletic feature, since it occurs in multiple and dispersed points in their phylogenetic tree. These authors suggested that the ability to fix nitrogen appeared independently three times in this group of microorganisms. Also, they mention that the loss of genes and horizontal gene transfer or a combination of these two processes can explain the dispersion of genes related to nitrogen fixation in the phylum *Cyanobacteria* (Swingley et al., 2008). Taking account of the patchy distribution and the mechanisms mentioned above (horizontal gene transfer and loss of genes), incongruence between the *nifH* and 16S rRNA gene phylogenies would be expected.

The presence of functional genes involved in the biosynthesis of toxins (microcystin and saxitoxin) and protease inhibitors (cyanopeptolin and aeruginosin) was investigated as being indicative of the potential for metabolite production (Table 3). Genes involved in the biosynthesis of toxins were detected more frequently in the Brazilian mangrove strains than in the Antarctic strains, whereas protease-biosynthetic genes occurred in strains isolated from both environments. The PCR amplification of microcystin and saxitoxin genes corroborates and complements a previous study that showed the presence of *mcyA* and *sxtI* in strains CENA158, CENA159 and CENA160 (Silva et al., 2014). Although *mcy* and *sxt* genes were detected, production of these cyanotoxins must be confirmed by chemical analyses.

Tolerance of high salinities is not a general trait in cyanobacteria, but some strains can tolerate or even require highly saline environments (Apte et al., 1997; Oren, 2000; Moisander et al., 2002; Andreote et al., 2014). Several studies have focused on the delimitation between organisms that are able to survive and those that can grow under highly saline conditions. Studies conducted by Hoff & Frémy (1933) and Flannery (1956) recognized different limits of salt concentration to determine the halophilic or halotolerant features of an organism. According to Hoff & Frémy (1933), true halophiles must be able to grow in 3 M NaCl (17.55 %) or above, whereas Flannery (1956) reported 2 % NaCl to be the limit between obligate and facultative halophiles. Otherwise, organisms that live in oceans, which present an almost constant 3.5 % salinity, are classified as slightly halophilic organisms (Ventosa & Arahal, 2002). Therefore, according to Hoff & Frémy (1933), our nine strains can be classified as halotolerant, as they grow in salt concentrations below 17.55 %. In addition, considering the classification of Flannery (1956) and Ventosa & Arahal (2002), the *Halotia* strains can be recognized as slightly halophilic, as their biomass production (growth) was reduced by increasing the salt concentration. In bacteriology, saline requirements can be used to describe novel species or genera (Garcia-Pichel et al., 1998). For cyanobacteria groups, in addition to the present study, physiological studies of responses to salinity have also been conducted to describe *Oxynema* and *Acaryochloris marina* as a new genus and species, respectively (Miyashita et al., 2003; Chatchawan et al., 2012). Despite the importance of physiological characterization, many novel cyanobacterial taxa have been described based only on the chemical and environmental conditions of their sampling site.

Morphologically, the species of the genera *Mojavia*, *Desmonostoc* and *Halotia* are very similar to the species of the true genus *Nostoc*, even considering their life cycle. Thus, morphological characteristics are insufficient to separate groups within the genus *Nostoc sensu lato*; hence, the search for diacritical morphological features is worthless. The wide genetic diversity of *Nostoc* is shown by the non-monophyletic status of this genus, which was demonstrated in both this and previous studies (Hrouzek et al., 2005, 2013; Rajaniemi et al., 2005; Papaefthimiou et al., 2008; Silva et al., 2014). The monophyly of the *Halotia* cluster and its phylogenetic separation from the well-defined genera *Mojavia*, *Nostoc* and *Desmonostoc* indicate that strains of the genus *Halotia* share a common ancestor and belong to a novel genus. Furthermore, the description of three novel species is well supported considering morphological, phylogenetic (16S rRNA gene) and ITS secondary structure data.

Tropical Brazilian mangroves and marine Antarctic ecosystems shelter a genetic lineage of nostocacean strains that is presented as a novel genus, *Halotia*, with three novel species. The lack of information regarding the origin of strain Mollenhauer 1:1-067 hampers better definition of the distribution and habitat of this emerging genus. Currently, we can assert that the distribution and habitat of *Halotia* includes maritime areas from Brazilian mangroves.
and Antarctic maritime regions, and the physiological traits of *Haløtia* include a tolerance of high salinity.

**Acknowledgements**

This study was supported by grants from the State of São Paulo Research Foundation (FAPESP) (2004/13910-6) and the Brazilian National Research Council (CNPq) (520194/2006-3, 490570/2010-0). D. B. G. and M. G. M. V. V. were supported by FAPESP graduate scholarships 2010/00321-3 and 2010/18732-0, respectively. M. F. F. would like to thank CNPq for a research fellowship (306607/2012-3). G. S. H. would also like to thank CNPq for a graduate scholarship (158353-2010/2). We are grateful to Dr Aharon Oren for help with the scientific names and etymology and MSc Danillo O. Alvarenga for conducting Bayesian analysis.

**References**


