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TAXONOMIC DESCRIPTION

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Extending the ecological distribution of *Desmonostoc* genus: proposal of *Desmonostoc salinum* sp. nov., a novel *Cyanobacteria* from a saline–alkaline lake

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Abstract

Cyanobacteria is an ancient phylum of oxygenic photosynthetic microorganisms found in almost all environments of Earth. In recent years, the taxonomic placement of some cyanobacterial strains, including those belonging to the genus *Nostoc sensu lato*, have been reevaluated by means of a polyphasic approach. Thus, 16S rRNA gene phylogeny and 16S–23S internal transcribed spacer (ITS) secondary structures coupled with morphological, ecological and physiological data are considered powerful tools for a better taxonomic and systematics resolution, leading to the description of novel genera and species. Additionally, underexplored and harsh environments, such as saline–alkaline lakes, have received special attention given they can be a source of novel cyanobacterial taxa. Here, a filamentous heterocytous strain, Nostocaceae CCM-UFV059, isolated from Laguna Amarga, Chile, was characterized applying the polyphasic approach; its fatty acid profile and physiological responses to salt (NaCl) were also determined. Morphologically, this strain was related to morphotypes of the *Nostoc sensu lato* group, being phylogenetically placed into the typical cluster of the genus *Desmonostoc*. CCM-UFV059 showed identity of the 16S rRNA gene as well as 16S–23S secondary structures that did not match those from known described species of the genus *Desmonostoc*, as well as distinct ecological and physiological traits. Taken together, these data allowed the description of the first strain of a member of the genus *Desmonostoc* from a saline–alkaline lake, named *Desmonostoc salinum* sp. nov., under the provisions of the International Code of Nomenclature for algae, fungi and plants. This finding extends the ecological coverage of the genus *Desmonostoc*, contributing to a better understanding of cyanobacterial diversity and systematics.

Cyanobacteria is an ancient phylum of oxygenic photosynthetic microorganisms within the *Bacteria* domain. Members of this phylum present basic nutritional requirements (CO_2, H_2O and light mainly) and display great morphological/metabolic diversity, allowing them to grow and to disperse in a variety of environments, including extreme ones [1, 2]. Saline–alkaline lakes, usually called soda lakes, are an example of such harsh environments. These lakes normally contain amounts of sodium-carbonate/bicarbonate salts up to saturation, forming a buffer system which maintains high pH values (from 9.5 to 11.0) [3]. Saline–alkaline lakes are distributed worldwide, mainly in arid and semi-arid environments, as in the Rift Valley in East Africa [3], but are also found in tropical and sub-tropical regions, as in the Brazilian Pantanal wetlands [4–6] and the Chilean southern region [7]. Laguna Amarga (Torres del Paine National Park, Chile) is a soda lake located in Southern Patagonia [8], a semi-arid area 105 m above sea level, with a windy cold climate (mean annual air temperature of 6.6 °C) and evaporation rates normally greater than precipitation rates [7].

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Keywords: 16S rRNA gene phylogeny; ITS; *Nostoc sensu lato*; physiology; polyphasic approach; saline environments.

Abbreviations: BI, Bayesian inference; FAMEs, fatty acid methyl esters; GTR+G+I, evolutionary model of substitution with gamma distribution and with an estimate of proportion of invariable sites; ITS, internal transcribed spacer; ML, maximum-likelihood.

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Three supplementary tables and six supplementary figures are available with the online version of this article.

It seems clear that such extreme environments cause serious limitations to the development of life and it seems reasonable to assume that cyanobacteria strains isolated from such conditions can provide valuable information concerning taxonomy, systematics and ecological distribution.

The taxonomic classification of cyanobacteria was formerly based mainly on morphological characters [9–12]. However, during recent decades, the additional application of molecular data using 16S rRNA gene sequences, has greatly contributed to improving cyanobacterial taxonomy and systematics [13–17]. Ecological and ultrastructural features have also been suggested as fundamental characteristics allowing a more robust characterization [18–20]. Moreover, physiological and metabolic traits can also be powerful tools to enhance our understanding of cyanobacterial evolution, assisting in the systematics of this group [5, 21–24]. The combination of these different methods, known as the 'polyphasic approach', has demonstrated that many morphologically well-defined genera are indeed polyphyletic, resulting in the description of novel genera and species [15, 23, 25–28]. This is notably important for the *Nostoc sensu lato* group, which has been recently split into novel generic entities, *Mojavia* [29], *Desmonostoc* [30], *Halotia* [23] and *Aliinostoc* [28].

In this context, we proposed the hypothesis that saline–alkaline environments harbor novel cyanobacterial strains with unique and unexplored physiological traits. To this end, a filamentous heterocytous cyanobacterial strain isolated from a saline–alkaline lake in Patagonia was characterized applying a polyphasic approach, by using morphological characters, phylogeny of 16S rRNA gene, 16S–23S internal transcribed spacer (ITS) secondary structure, coupled with ecological and physiologic data (growth response to saline conditions and its fatty acid profile determination). Our results allowed the description of the first *Desmonostoc* strain isolated from a saline–alkaline lake, to our knowledge, culminating in the proposal of the novel species, *Desmonostoc salinum* sp. nov. Furthermore, it was demonstrated that this strain is able to thrive at salt concentrations higher than 0.25 M of NaCl.

The cyanobacterial strain analyzed in this study was isolated from periphytic microbial mats collected at Laguna Amarga, Torres del Paine National Park (50° 29′ 00″ S and 72° 45′ 00″ W), Chile, during February 2011 (Fig. 1a, b). Water pH and temperature were measured at the sampling site using a portable Digimed DM-2 meter (Digimed Analítica, Digicrom Analítica) calibrated with buffer solutions of pH 4.0 and 7.0; the temperature was compensated using automatic temperature compensation. Water and periphytic samples were stored in polyethylene bottles and kept on ice for further physical–chemical analysis and isolation procedures. Alkalinity was measured by titrating an aliquot of the solution to pH 8.3 to determine phenolphthalein alkalinity and to a pH near 4.5. Alkalinity is reported as mg $CaCO_3$ l^{-1} equivalent, which can be interpreted as the equivalent amount of calcite needed to consume the amount of acid titrated. A subset of elements was also quantified and are presented in Table S1 (available in the online version of this article). Following these analyses, Laguna Amarga presents high amounts of ions in the water, showing deposits of white sediments in the shoreline (Fig. 1). At sampling, the water temperature was around 6 °C, and the pH was 9.5, presenting a sodium concentration of 28.77 g l^{-1} (equivalent to 1.25 M). The ions SO_4^{2-}, K^+, S^{2-} and Mg^{2+} were also found in high abundance. Carbonate (CO_3^{2-}) and bicarbonate (HCO_3^-) concentrations were 2.67 g l^{-1} (0.044 M) and 0.95 g l^{-1} (0,015 M), respectively (Table S1).

Samples of the periphytic microbial mats (Fig. 1) collected from the shoreline were fragmented and spread on BG-11_0 plates (BG-11 without nitrogen sources) [31]. In the first rounds of the isolation, the plates were maintained at low temperatures, starting at 10±2 °C, which were successively increased, up to 24±2 °C. The plates were maintained under light intensity of 40 µmol m^{-2} s^{-1} and photoperiod of 16/8 h (light/dark). The biomass was constantly analyzed under the microscope and successive streaking was performed until unicyanobacterial colonies were obtained. The strain CCM-UFV059 was the only filamentous heterocyte-forming cyanobacterium isolated from the collected mat. After isolation, the non-axenic unicyanobacterial culture was

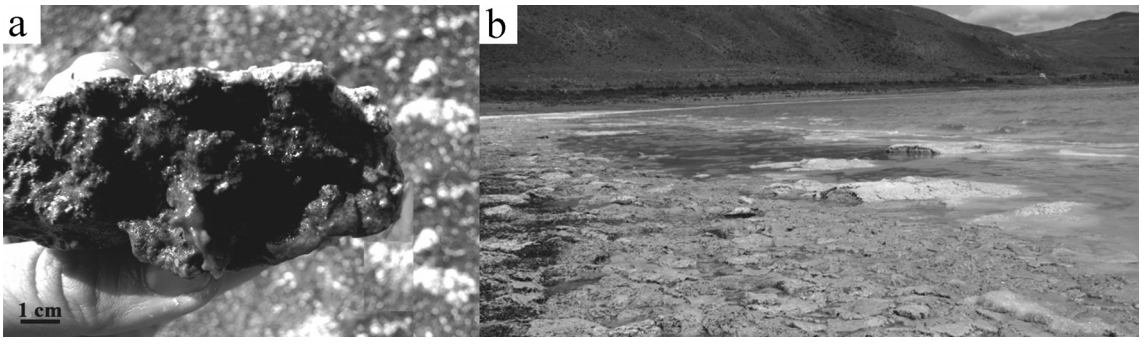

Fig. 1. Samples of periphytic microbial mat adhered to clayey material (a) collected from Laguna Amarga shoreline in January of 2011(b).

maintained in liquid ($BG\text{-}11_0$) medium under the same conditions described above, at the Collection of Cyanobacteria and Microalgae (CCM–UFV), in the Laboratory of Phycology and Molecular Biology, Plant Biology Department at the Universidade Federal de Viçosa.

Morphological characterization was performed as previously described [32, 33], using a Zeiss Axioskop 40 optical light microscope equipped with an AxioVision LE 4.6 digital imaging system (Carl Zeiss) [34]. The morphology of colonies, vegatative filaments and hormogonia were observed; the presence or absence of sheaths was recorded and the size of vegetative cells, heterocytes and akinetes was measured.

For molecular characterization, total genomic DNA was extracted from a 14-day-old culture using the UltraClean Microbial DNA Isolation Kit (MoBio). The 16S and the 16S–23S ITS gene region were PCR-amplified using the primer set 27F1 [35] and 23S30R [36], following steps described previously [12]. The amplicons were, then, cloned into a pGEM-T Easy Vector System (Promega, Madison) according to the manufacturer's manual. Competent *Escherichia coli* DH5α cells were transformed and recombinant plasmids were purified from white colonies using the UltraClean Standard Mini Plasmid Prep Kit (MoBio). Plasmids containing the fragments of interest were sequenced using the M13F/R primer set and the 16S rRNA internal primer sets 341–357F, 357–341R, 685–704F, 704–685R, 1099–1114F, and 1114–1099R [37], exactly as described by Genuário *et al.* [12]. The sequenced fragments were assembled into one contig using the software Phred/Phrap/Consed (Philip Green, University of Washington, Seattle, USA) and only bases with >20 quality were considered.

The 16S rRNA gene sequence obtained in this study and related ones retrieved from GenBank were aligned using CLUSTAL W from MEGA version 5 [38] and trimmed (16S rRNA gene matrix with a 1446 bp length). A total of 108 sequences were considered and used to infer the phylogeny based on the maximum likelihood (ML) method. The general time reversible evolutionary model of substitution with gamma distribution and with an estimate of proportion of invariable sites (GTR+G+I) was selected as the best fitting model, applying the model-testing function in MEGA version 5 [38] and jModelTest 2.1.1 program [39]. The robustness of the phylogenetic trees was estimated by bootstrap analysis using 1000 replications. Bayesian inference was also conducted using MrBayes 3.2 [40], applying two separate runs with four chains each and 50 000 000 Markov chain Monte Carlo generations. Posterior probabilities (PP) were calculated at the conclusion of the Markov Chain Monte Carlo analysis and a traditional burn-in on the first 25 % of the trees was performed. The tree was viewed in FigTree 1.3.1 (http://tree.bio.ed.ac.uk/software/figtree). Given that ML and Bayesian methods resulted in nearly identical topologies, only the ML tree is presented, with indications of ML bootstrap values and Bayesian posterior probabilities.

The 16S–23S ITS gene region obtained was further used for analysis based on secondary structure folding. The selected 16S–23S ITS sequences were aligned with CLUSTAL W from the Mega package [38] (Fig. S1). Their ITS regions (D1–D1′, D2, V2, Box-B, Box-A, D4, V3 and D5) and tRNA genes were found using LocARNA-Alignment and Folding [41, 42] and tRNAscan-SE Search Server (1.21) [43], respectively. The D1–D1′ and V2 secondary structures were folded using the Mfold WebServer with the default conditions except for applying untangle with loop fix as structure draw mode [44]. The sequence of 16S and the 16S–23S ITS gene region obtained from the isolated strain was deposited in the NCBI GenBank database under the accession number KX787933.

To screen microcystin and saxitoxin synthetase genes, three molecular markers were selected from each gene cluster, respectively: *mcyD*, *mcyE* and *mcyG* as well *sxtA*, *sxtB* and *sxtI*. The PCR amplifications of *mcyD* (818 bp) and *mcyE* (809 bp) were conducted using the specific primers designed previously [45] whereas *mcyG* (534 bp) was amplified using the primer set described by Fewer *et al.* [46]. The PCR amplifications were performed as described by Genuário *et al.* [12]. For saxitoxin synthetase genes, the PCR amplification was conducted using the specific primers (*sxtA* – 200 bp; *sxtB* – 400 bp; and *sxtI* – primer set OCT-F/R, 900 bp) and the conditions designed by Hoff-Risseti *et al.* [47].

Given that Laguna Amarga is characterized by a high salt concentration (Table S1), the lethal dose concentration (LD_{50}) of NaCl for CCM-UFV059 was determined. The LD_{50} is the minimum concentration of a specific agent capable of killing 50 % of cells after 72 h of exposure, according to Organization for Economic Co-operation and Development (OECD) Guidelines [48]. Briefly, independent growth curves were performed, in triplicate, using different NaCl concentrations (0, 0.05, 0.075, 0.1, 0.25, 0.5, 0.75, 1, 1.75 M), with CCM-UFV059 being maintained under a photoperiod of 16/8 h (light/dark), 24±2 °C, light intensity of 70 µmol m^2 s^{-1} and constant shaking (30 *g*), for 10 days. Growth evaluation was performed by measuring the number of cells in a Neubauer chamber (Optik Labor), ashes-free dry mass and OD_{750}. Likewise, growth curves using the same salt concentrations were conducted for *Nostoc* sp. PCC7120, which was selected based on its non-NaCl-tolerance (negative control) [34].

The fatty acid profile of this strain was analyzed after cultivation in conditions without salt. Briefly, 10 mg lyophilized cells were independently harvested at four sampling points along the exponential phase of a growth curve (0, start point of exponential phase; 2, 8 and 24 h). The derivatization was conducted according to the Sherlock Microbial Identification MIDI System, using HCl–methanol 6 % (v/v) and hexane (http://www.midi-inc.com/ Technical note #101). A total of 0.05 ml of the non-polar fraction was taken for analysis on model 7890 gas chromatograph (Agilent) equipped with an HP-ultra 2 column (25 m, 0.20 mm ID, 00 : 33 µM film thickness). The MIDI Sherlock version 6.2 (MIDI) software was

used to adjust the operational parameters and for recognition, quantification and comparison with the reference libraries. The results are expressed as percentages in relation to the total response obtained in the chromatogram.

The non-axenic filamentous heterocytous strain, obtained after the isolation procedures, was named as Nostocaceae CCM-UFV059, once its main morphological traits had been matched with those found in members of this family. Under standard conditions (BG-11_0 medium, without NaCl), microscopic inspection revealed that CCM-UFV059 had straight trichomes at the beginning of development, as hormogonia or young trichome, and later was organized in a set of parallel trichomes, enclosed by a diffluent mucilage (Fig. 2a, c). This parallel organization of trichomes, observed in early and middle phases of culture, could be lost later, as a consequence of akinete differentiation. Both intercalary and terminal heterocytes were always present in vegetative trichomes, with the terminal ones being differentiated during the early stages of trichome development. Whilst in the presence of NaCl akinetes were easily found, under standard conditions they were not often observed. Initially, the akinetes were organized in chains, which could be broken, releasing isolated mature akinetes (Fig. 2b, d). Macroscopically, the strain grew as a free-floating gelatinous biomass, which occasionally formed aggregated colonies. Motile hormogonia were frequent and abundant in earlier growth phases, usually containing more than 20 cells, without heterocytes. Vegetative cells presented a dark-green colour, were barrel-shaped, sub-spherical or longer than they were wide (3.7–5.4 µm long and 3.3–4.5 µm wide). Heterocytes were sub-spherical, longer than they were wide (5.8–8.3 µm long and 3.7–4.6 µm wide) with a slight-green or yellowish colour. The akinetes were spherical or sub-spherical (8.3–10.0 µm long and 5.0–8.3 µm wide), with green to yellowish colour and conspicuous granulation (Fig. 2 and Table 1).

The nearly complete 16S rRNA gene (1413 bp) and the 16S–23S ITS gene region (327 bp) were sequenced from the CCM-UFV059 strain. The 16S rRNA gene sequence obtained showed identity higher than 98.5 % to related sequences available in Genbank (Table 2). It is important to mention that among the related sequences, seven were from strains assigned to the genus *Desmonostoc* or belonging to its corresponding phylogenetic clade [30], and seven sequences were related to uncultured bacterium clones harvested from the gut microbial communities of zebrafishes

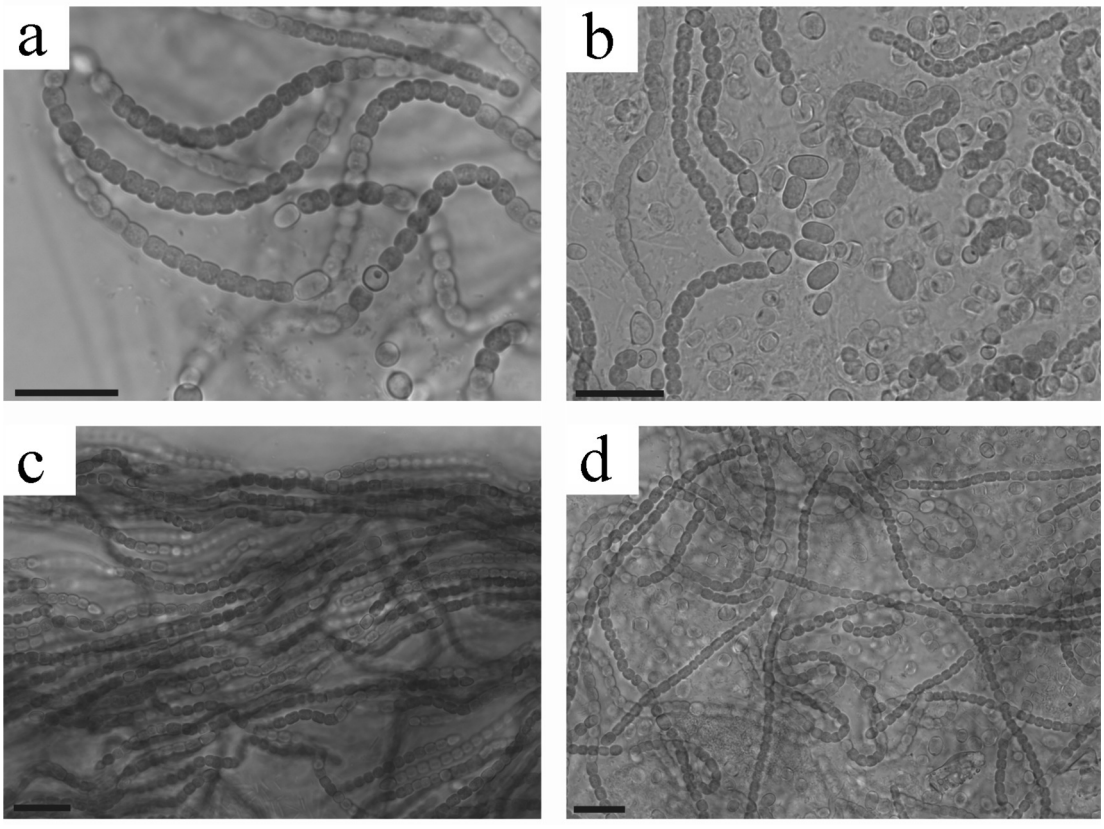

Fig. 2. Photomicrographs of *Desmonostoc salinum* CCM-UFV059. a and c: Vegetative trichomes grown in BG-11_0 without NaCl. b and d: Trichomes grown in BG-11_0 supplemented with 890 mM NaCl. b Presence of akinetes after 96 h of cultivation in BG-11_0 with 890 mM NaCl. Bars, 20 µm.

Table 1. Morphometric characteristics of *Desmonostoc salinum* CCM-UFV059

All measurements [mean, (range)] are given in µm.

Cyanobacterial strain	Vegetative cells		Heterocytes		Akinetes		
	Length	Width	Length	Width	Length	Width	Shape
Desmonostoc salinum CCM-UFV059	4.5 (3.7–5.4)	4.2 (3.3–4.5)	7.0 (5.8–8.3)	4.2 (3.7–4.6)	9.0 (8.3–10.0)	7.0 (5.0–8.3)	Oval

[49] (Table 2). In the phylogenetic reconstruction based on 16S rRNA gene sequences, the sequence of CCM-UFV059, grouped in a robust clade (94 % bootstrap value, ML; and posterior probability of 1, BI) with other sequences of *Desmonostoc* (Cluster *Desmonostoc sensu stricto* – *Desmonostoc* spp., Figs 3 and S4). The sequences assigned to the genus *Desmonostoc* characterized by Hrouzek *et al.* [30] and the recently described *D. geniculatum* [50] were all included in this cluster (Figs 3 and S4). Additionally, the comparison of the 16S rRNA gene sequence of CCM-UFV059 with a subset of selected sequences used for phylogenetic reconstruction, including those from members of the genera *Nostoc sensu stricto*, *Mojavia* and *Halotia* (Table S2) showed identities ranging from 97.5 to 99.8 % against sequences of members of the genus *Desmonostoc* and from 94.6 to 96.6 % against those of members of the other selected genera (Table S2). Furthermore, the 16S rRNA gene sequence of CCM-UFV059 has an identity of 97.9 % against the sequence of *Desmonostoc muscorum* Lukesova 1/87 (AM711523), the type-species of the genus *Desmonostoc* [30], and 97.5 % against the sequence from *Desmonostoc geniculatum* HA4340-LM1 (KU161660), which have been previously described [50] (Table S2).

The novel 16S–23S ITS spanned the D1–D1′, D2, V2 and D5 regions and the secondary structures of D1–D1′ and V2 regions were folded. The length and secondary structures of the novel 16S–23S were compared against sequences from those strains of the genus *Desmonostoc* available and the type strains of the type species of the genera *Nostoc*, *Mojavia* and *Halotia* (Table S3; Figs 4, 5, S2 and S3). Within *Desmonostoc* 16S–23S ITS sequences, the length of D1–D1′, V2 and D5 regions were 65–69, 29–62 and 16–21 bp, respectively (Table S3). For the remaining analysed genera, these regions presented a wider variation 60–67, 27–76 and 14–21 bp for D1–D1′, V2 and D5 regions, respectively. Considering the sequence length, all the regions from CCM-UFV059 coincided with those of *Demonostoc* sp. 111_CR4_BG11B and *Desmonostoc* sp. 111_CR4_BG11N, indicating that they might represent the same species (Table S3). Similarly, their D1–D1′ and V2 secondary structures precisely matched (Figs 4, 5). Eight D1–D1′ helical patterns were recognized among the 16S–

Table 2. Sequence identity (%) of 16S rRNA gene fragments among *Desmonostoc salinum* CCM-UFV059 and other cyanobacterial strains available in GenBank

Strain	Length (bp)	Closest match (Accession number)	C* (%)	I† (%)
Desmonostoc salinum CCM-UFV059	1413	*Desmonostoc* sp. 8964:3 (AM711541)‡	100	99.8
		Desmonostoc entophytum IAM M-267 (AB093490)‡	100	99.7
		Uncultured bacterium JFR0702_jaa51e01 (HM780037)‡	100	99.7
		Desmonostoc sp. PCC9231 (AY742452)‡	99	99.7
		Desmonostoc sp. PCC8306 (HG004584)‡	100	99.6
		Uncultured bacterium JFR0702_jaa37g03 (HM780016)‡	100	99.4
		Uncultured bacterium JFR0702_jaa50a03 (HM780005)‡	100	99.3
		Uncultured bacterium JFR0702_jaa38d08 (HM780003)‡	100	99.2
		Uncultured bacterium JFR0702_jaa37c06 (HM780249)‡	100	99.1
		Uncultured bacterium JFR0702_jaa37g03 (HM780016)‡	100	99.4
		Uncultured bacterium JFR0702_jaa50a03 (HM780005)‡	100	99.3
		Uncultured bacterium JFR0702_jaa37d05 (HM780163)‡	100	99.0
		Uncultured bacterium JFR0702_jaa40g08 (HM780211)‡	99	99.0
		Desmonostoc sp. PCC8107 (HG004583)‡	100	98.5
		Nostoc linckia var. *arvense* IAM M-30 (AB325907)‡	100	98.5
		Desmonostoc sp. Cr4 (AM711533)‡	100	98.5

*Coverage.

†Identity.

‡Published sequence.

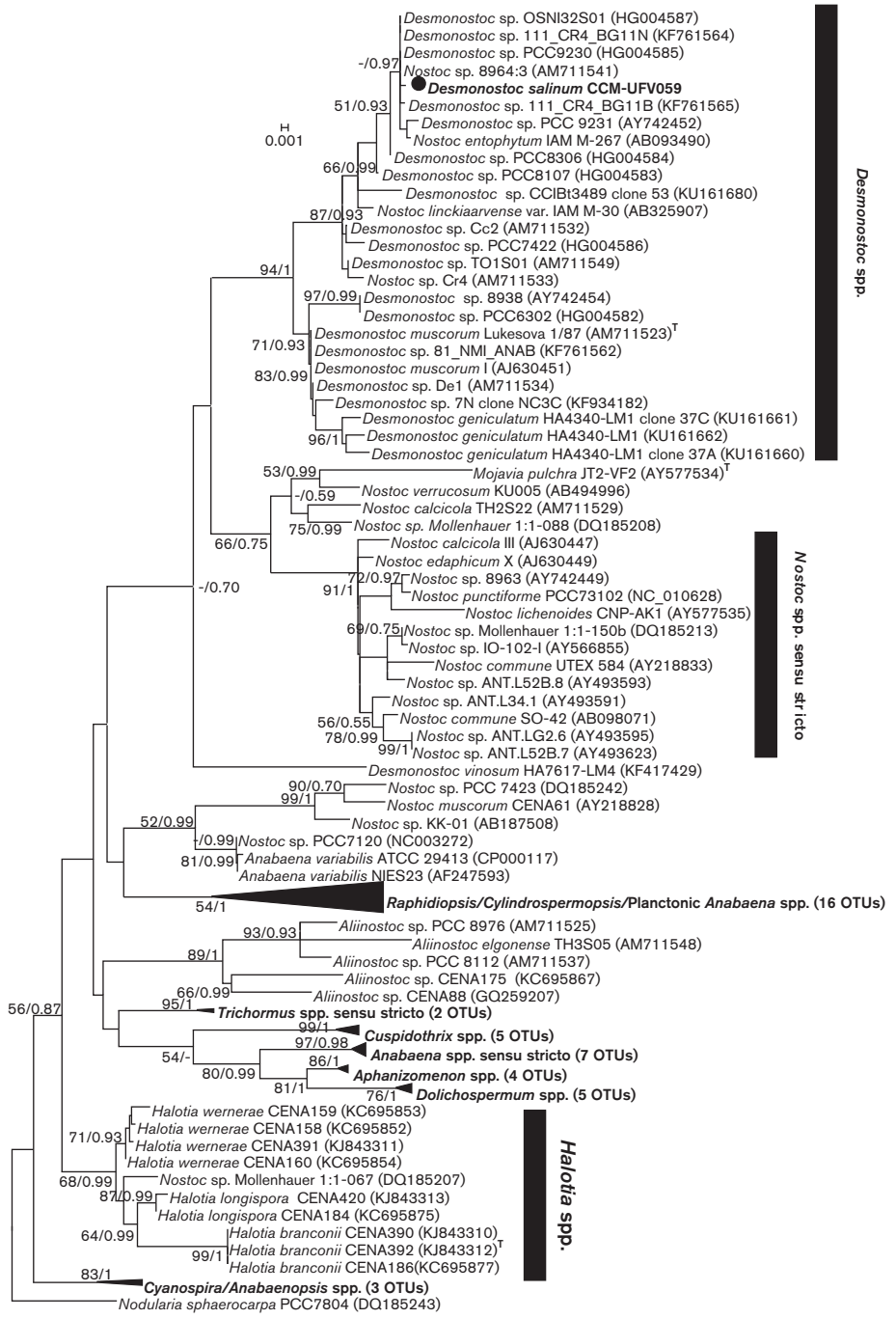

Fig. 3. Maximum likelihood phylogenetic tree based on the 16S rRNA gene sequences of nostocacean cyanobacteria. The sequence of *Desmonostoc salinum* CCM-UFV059 is shown in bold type with a black circle. Bootstrap (greater than 50 %) and probability values, obtained from ML and Bayesian inference, respectively, are displayed in front of the relevant nodes.

23S sequences analyzed and all of them shared the same basal stem structure (GACCU–AGGUC) (Figs 4 and S2). Interestingly, the secondary structure from CCM-UFV059 coincided only with those from *Desmonostoc* sp. 111_CR4_BG11B and *Desmonostoc* sp. 111_CR4_BG11N helixes (Fig. 4). Altogether, one single unpaired nucleotide located in first internal loop after the basal stem and two unpaired nucleotides in the lateral bulge are the main differences when compared with the remaining helical patterns (Fig. 4). The number of V2 helical patterns was slightly higher among the 16S–23S sequences analyzed and they did not share the same sequence in their basal stems

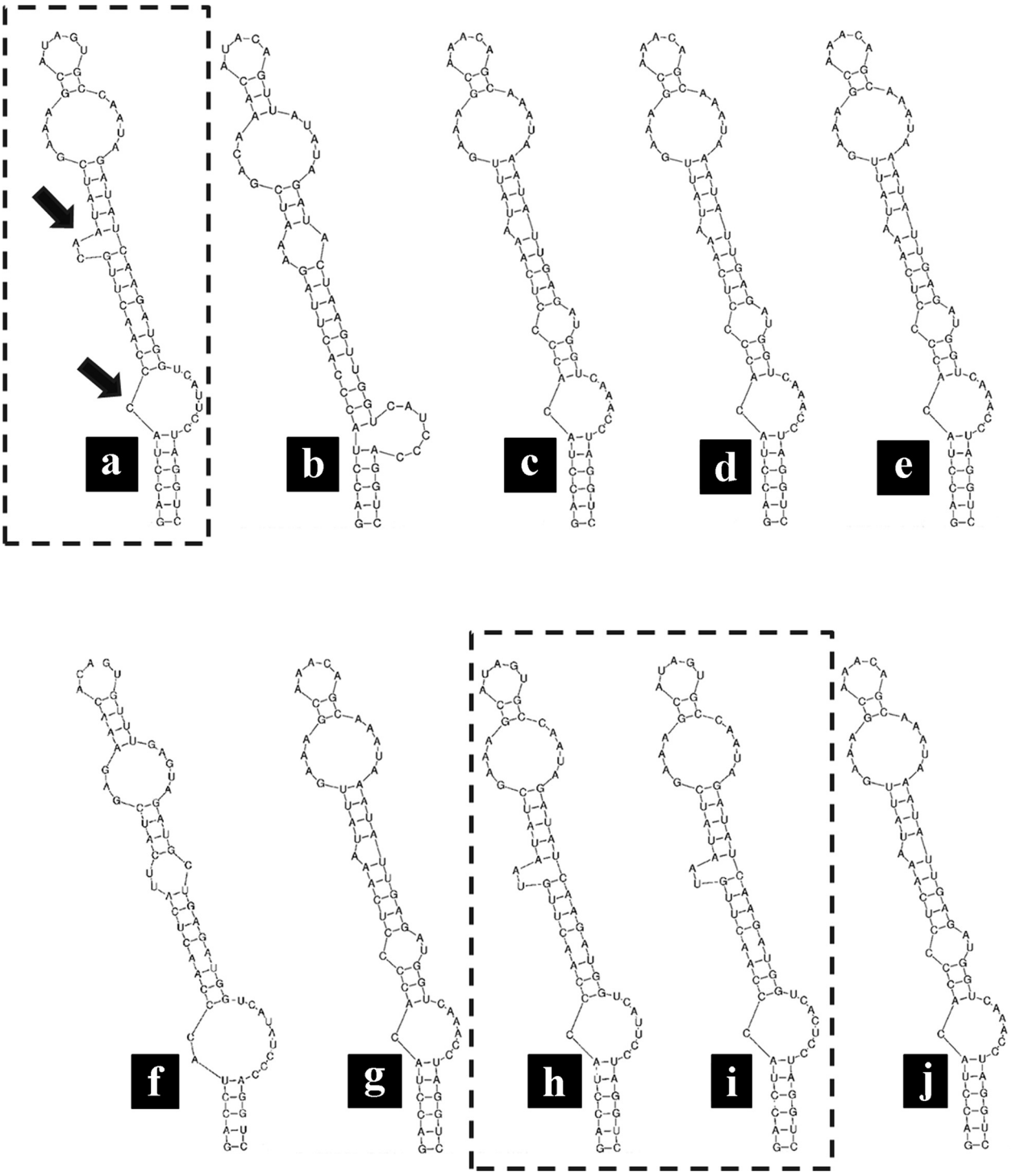

Fig. 4. D1–D1′ secondary structures of the 16S–23S ITS sequences from selected strains of members of the genus *Desmonostoc*. a - *Desmonostoc salinum* CCM-UFV059; b - *Desmonostoc vinosum* HA7617-LM4 (KF417429); c - *Desmonostoc geniculatum* HA4340-LM1 (KU161662); d - *Desmonostoc geniculatum* HA4340-LM1 (KU161661); e - *Desmonostoc geniculatum* HA4340-LM1 (KU161660); f - *Desmonostoc* sp. CCIBt3489 (KU161680); g - *Desmonostoc* sp. 7N (KF934182); h - *Desmonostoc* sp. 111_CR4_BG11B (KF761565); i - *Desmonostoc* sp. 111_CR4_BG11N (KF761564) and j - *Desmonostoc* sp. 81_NMI_ANAB (KF761562). The structures presented in a, h and i are the most similar. The arrows indicate common specific traits in these secondary structures.

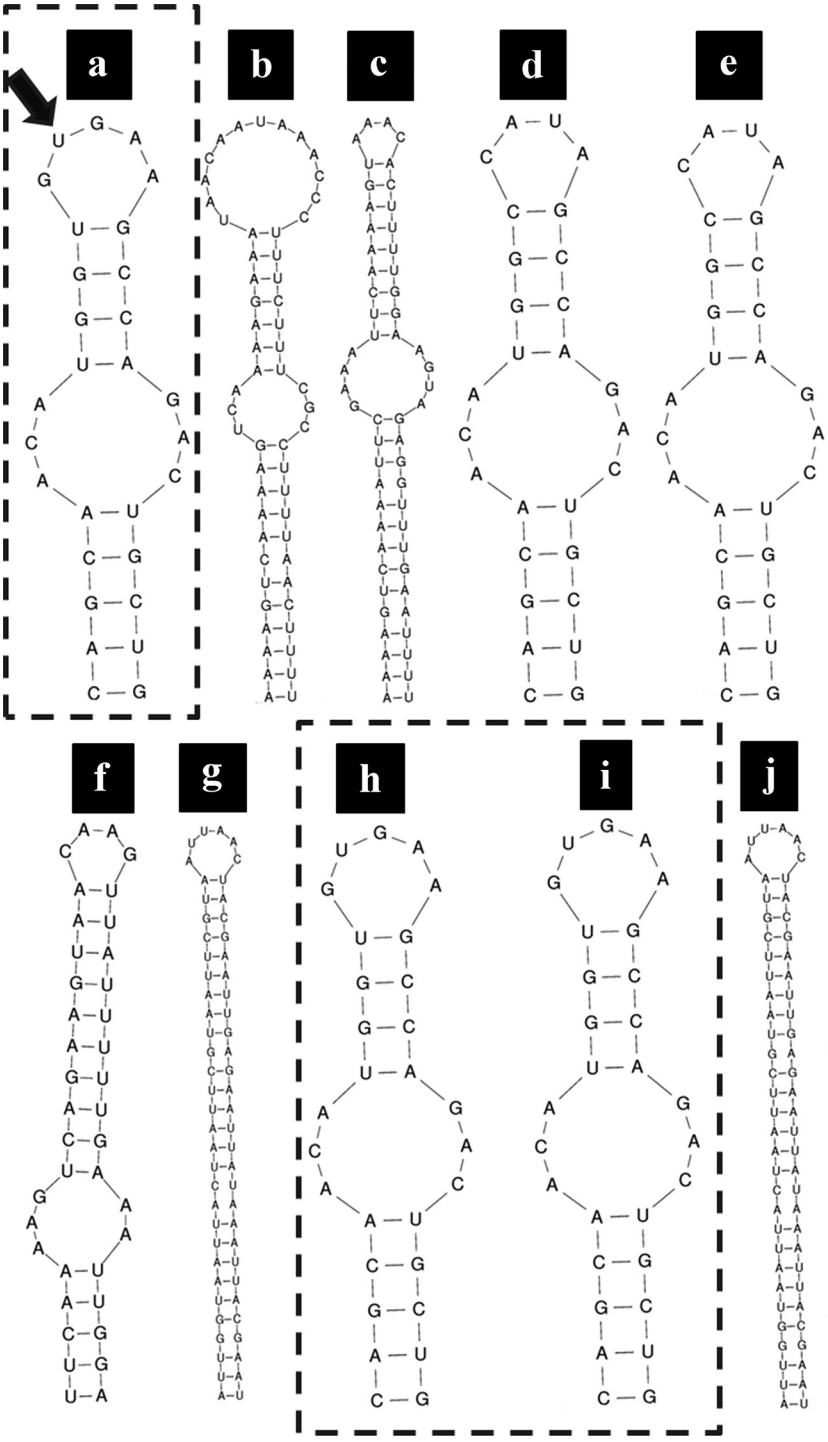

Fig. 5. V2 secondary structures of the 16S–23S ITS sequences from selected strains of members of the genus *Desmonostoc*. a - *Desmonostoc salinum* CCM-UFV059; b - *Desmonostoc vinosum* HA7617-LM4 (KF417429); c - *Desmonostoc geniculatum* HA4340-LM1 (KU161662); d - *Desmonostoc geniculatum* HA4340-LM1 (KU161661); e - *Desmonostoc geniculatum* HA4340-LM1 (KU161660); f - *Desmonostoc* sp. CCIBt3489 (KU161680); g - *Desmonostoc* sp. 7N (KF934182); h - *Desmonostoc* sp. 111_CR4_BG11B (KF761565); i *Desmonostoc* sp. 111_CR4_BG11N (KF761564) and j - *Desmonostoc* sp. 81_NMI_ANAB (KF761562). The structures presented in a, h and i are the most similar. The arrow indicates common specific trait in these secondary structures.

even when compared only among the sequences from members of the genus *Desmonostoc* (Figs 5 and S3). Again, the V2 secondary structure from CCM-UFV059 agreed only with those from *Desmonostoc* sp. 111_CR4_BG11B and *Desmonostoc* sp. 111_CR4_BG11N helixes mainly by their six unpaired nucleotide located at the terminal loop (Fig. 5). All this information interpreted altogether indicates that the novel CCM-UFV059, *Desmonostoc* sp. 111_CR4_BG11B and *Desmonostoc* sp. 111_CR4_BG11N represent a single species.

The molecular screening for genes involved in the biosynthesis of microcystin and saxitoxin did not produce any positive reaction, indicating that CCM-UFV059 does not have the genetic potential to produce these toxins. Moreover, the chemical analysis conducted using its biomass aiming to access these toxins did not detect their presence (data not shown). It should be mentioned that, to date, there is no register of strains of members of the genus *Desmonostoc* capable of producing such cyanotoxins.

The LD_{50} observed for CCM-UFV059 was 0.89 M whereas it was only 0.1 M for PCC7120 (Fig. S5), indicating that this novel strain can tolerate high NaCl concentrations. Next, the growth of CCM-UFV059 was evaluated in culture media applying NaCl concentrations below the LD_{50} dose. By increasing the salt concentration, clear reductions in the growth of CCM-UFV059 were observed, measured both by OD_{750} and ashes-free dry mass (Fig. S6). In the absence of NaCl (standard conditions), CCM-UFV059 showed a six-day exponential phase (from the second to the eighth days) (Fig. S6a) and the maximum dry mass was achieved after 8 days of cultivation (Fig. S6). A longer lag phase and a shorter exponential phase were observed in the presence of 0.25 M NaCl, leading to a reduction of 31 % in biomass production. Although treatments with 0.5 and 0.75 M NaCl did not show any expressive growth (Fig. S6), it was possible to observe, through microscopic analysis, the differentiation of vegetative cells into akinetes. The differentiation started at the second day of exposure to 0.75 M NaCl, similarly to the cultures growing at the LD_{50} (Fig. 2b, d).

The fatty acid methyl esters (FAMEs) profile showed a major abundance of palmitic acid (16 : 0) (28 %), palmitoleic acid (16 : 1ω7*c*) (18 %) and oleic acid (18 : 1ω7*c*) (33 %). Myristic acid (14 : 0 anteiso) (3 %), linoleic acid (18 : 2ω6,9*c*) (7 %) and other FAMEs were also found in minor amounts. The FAMEs profile did not present significant variation among the four sampling points, which encompassed a complete day into the exponential phase, indicating that the FAMEs profile must be seen as an important taxonomic marker (Fig. 6) [51]. The FAMEs profile of CCM-UFV059 presented clear differences from those described by Temina *et al.* [52] in a study analyzing a subset of *Nostoc*-like strains. However, a deeper comparison was hampered since no other strain belonging to a member of the genus *Desmonostoc* has been analyzed to date by means of fatty acid profile. Nevertheless, this result serves as a foundation for comparison in further studies aiming to characterize the intra- and inter-generic diversity of strains belonging to *Nostoc sensu lato*.

Laguna Amarga, from which the novel species *Desmonostoc salinum* CCM-UFV059 was isolated, showed high pH (>9.0), high dissolved content of Na^+, K^+, Mg^+ and SO_4^{2-} and high content of carbonates, as sodium carbonate/bicarbonate precipitates (Table S1). Such harsh environmental conditions can be a limiting factor for many organisms, including cyanobacteria. In fact, only a few organisms can grow in saline–alkaline lakes, like Laguna Amarga [4, 53]. Taking into account morphological results, CCM-UFV059 resembles the morphotypes of the related genera, *Nostoc sensu stricto*, *Desmonostoc* and *Halotia*. That being said, the identification of diacritical morphological features among these genera and species is apparently highly complicated [23]. However, it is important to mention that akinete differentiation observed under both standard culture conditions and high salt concentrations, the dark green of vegetative cells, the production of large amounts of exopolysaccharides during the whole life cycle and the parallel organization of trichomes can be assumed to be important morphological traits distiguishing members of the genus *Desmonostoc*.

The phylogenetic analyses presented here (Tables 2 and S2; Figs 3 and S4) coupled with the cut off values for genus and species delimitation (95 and 97.5 %, respectively) [54, 55], clearly indicates that CCM-UFV059 represents a member of the genus *Desmonostoc* (identity $\geq$95 %). More importantly, our results are suggestive of a novel specific entity. Although these last identity values are slightly higher than the ones proposed for species delimitation, this numeric percentage value cannot be used as a robust diacritical trait for species separation, especially for nostocacean taxa, for which broader limits are accepted [23, 56, 57]. In addition, on the basis of phylogenetic reconstruction, the *Desmonostoc* cluster presented two major subclusters in which the first subcluster harbored the 16S rRNA gene sequence retrieved from the type species (*Desmonostoc muscorum* Lukesova 1/87) and other sequences from other members of the genus (*D. muscorum* I, *Desmonostoc* sp. PCC6302 and *Desmonostoc* sp. 8938). Notably, this cluster corresponds to the *Desmonostoc* cluster D2 presented by Hrouzek *et al.* [30], and represents the 'type' cluster of *D. muscorum*. In addition, from this subcluster emerged a subdivision containing three sequences of *Desmonostoc geniculatum* HA4340-LM1, a recently described species from Hawaiian cave walls [50]. The second subcluster accommodated the novel 16S rRNA gene sequence retrieved from the CCM-UFV059 and other sequences assigned to the *Desmonostoc* and also corresponded to the cluster D1 revealed by Hrouzek *et al.* [30]. Internally, the 16S rRNA gene sequence from CCM-UFV059 clustered together with eight other sequences and these sequences shared more than 99.5 % identity among

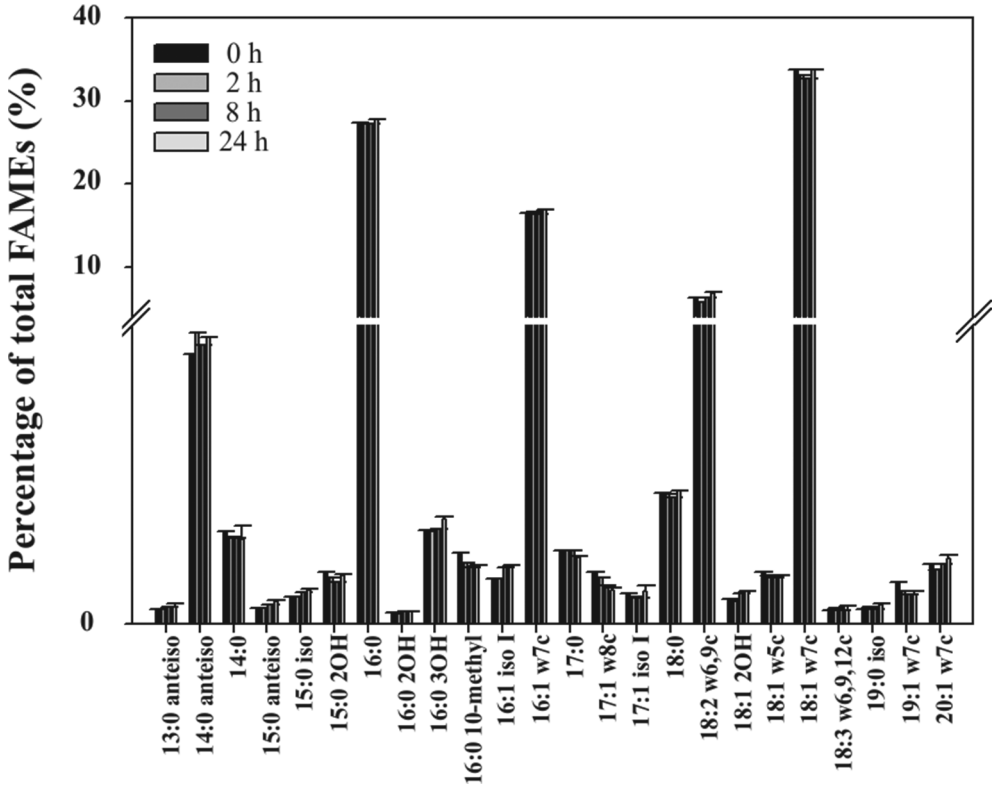

Fig. 6. Fatty acid profile, as percentage of each fatty acid with respect to total fatty acid fraction, for *Desmonostoc salinum* CCM-UFV059. Values are expressed as means of three replicates.

themselves, indicating that they must represent the same species.

Analysis of 16S–23S ITS secondary structures has been considered to be a powerful tool for delimitation of cyanobacterial species [58], among which V2 helix is the most variable and D1–D1′ more important for separation of strains in different species [23, 27]. Length comparison among the regions within the 16S–23S sequences showed that the D1–D1′, V2 and D5 regions from CCM-UFV059 and those of *Desmonostoc* sp. 111_CR4_BG11B and *Desmonostoc* sp. 111_Cr4_BG_11N coincided and that their D1–D1′ and V2 secondary structures matched precisely (Figs 4, 5). These similarities indicate that CCM-UFV059 and two strains (*Desmonostoc* sp. 111_CR4_BG11B and *Desmonostoc* sp. 111_CR4 _BG11N), which had been isolated from the walls of caves on Kauai, Hawaii, for which detailed information about their sampling sites are lacking [50], represent a single species. Likewise, ecological information from the remaining strains in the *D. salinum* sub cluster (Fig. 3) is not precise. More importantly, these sequences do not have their 16S–23 ITS available, impairing a better comparison about their relatedness at species level.

Strains assigned to the genus *Desmonostoc* are normally found in non-extreme environments, characterized by moist or wet meadow, field and forest soils [30] and more recently, strains of species of the genus *Desmonostoc* have also been recovered from caves on Kauai, Hawaii [50]. It is worthy of mention that these strains are rarely found in periphyton, biofilms as well as in deserts; however, some strains are symbiotic to *Cycas* and *Gunnera* [30, 59]. To our knowledge, CCM-UFV059 is the first strain of a member of the genus *Desmonostoc* isolated from a saline–alkaline lake, an extreme environment considering both the high pH and salinity of the sampled site. When considered together, the ecological features of this novel strain, which is highly distinct from previously described species, coupled with molecular and phylogenetic data obtained here, make it seem reasonable to propose the description of *Desmonostoc salinum* sp. nov.

The strain *Desmonostoc salinum* CCM-UFV059 was able to grow in presence of different NaCl concentrations, showing a LD_{50} of 0.89 M, a value eight times higher than found in others filamentous heterocytous species [34]. Given that Na^+ is the most abundant ion in Laguna Amarga (Table S1), it was used for simulating the natural conditions faced by this strain. It is well known that cyanobacterial strains can grow in marine waters (salinity approximately 3.5 %) and other saline environments such as saline–alkaline lakes, soda lakes or saltworks [4, 6, 53, 60]. This fact apart, the

classification of these cyanobacteria as halotolerant and halophilic has been widely discussed [61–64] and despite being able to grow in presence of high salt concentrations (up to 0.25M NaCl), *Desmonostoc salinum* CCM-UFV059 may be classified as halotolerant, as it does not require specific salts or higher salt concentrations to survive. Accordingly, at concentrations exceeding 0.25 M NaCl CCM-UFV059 decreased or even ceased its growth by differentiating akinetes, which are again viable when inoculated in fresh medium. Recently, a novel nostocacean genus, *Halotia*, has been described based not only on the ecological origin of the isolated strains (intertidal zones, mangroves and seashores from Brazilian mangroves and Antarctica), but also on their physiological responses of its members to saline conditions and its phylogenetic placement apart from the *Nostoc* cluster *sensu stricto*, as well as the novel 16S–23S ITS sequences and secondary structures of its members [23]. Given the combination of ecological (sampling sites) and physiological information (capability of growth in saline culture media) for cyanobacterial taxonomy, the genus *Halotia* was therefore described as halotolerant. In contrast, *Oxynema thaianum* [60] and *Acaryochloris marina* [65] were described as halophilic, requiring NaCl concentrations of up to 15 % (2.58 M) and from 1–5 % (0.17–0.85 M), respectively, for their growth. In general, physiological investigations have been left out of taxonomic studies conducted with cyanobacterial strains; however, ecological data seems to be of pivotal importance as a feature to be considered, providing a wider resolution of cyanobacteria's diversity.

By applying a polyphasic approach, based on morphological, molecular, ecological, and physiological data, we present here compelling evidence for the description of *Desmonostoc salinum* sp. nov. In summary, the work presented here is, to our knowledge, the first description of a halotolerant species belonging to the genus *Desmonostoc*. Remarkably, our molecular results (16S rRNA gene phylogeny and 16S–23S ITS secondary structure) coupled with ecological data demonstrated the potential of a polyphasic approach as a tool for the precise recognition of the novel specific entity. Although complete genome sequencing was not performed for *Desmonostoc salinum* CCM-UFV059, it seems clear that further investigation in order to obtain a better understanding about the molecular mechanisms behind its halotolerance is required. It is also worth pointing out that more genomic data will certainly improve the public genome database for taxonomic and physiological purposes. Accordingly, a deeper investigation of the intriguing aspects associated with the ecophysiology of this novel strain should be targeted for future research aiming to enhance our understanding of salt tolerance in cyanobacteria.

Taken together, the results presented in this study allowed the proposal of the novel species *Desmonostoc salinum* sp. nov., with *Desmonostoc salinum* CCM-UFV059 as reference strain. This proposal is given under the provisions of the International Code of Nomenclature for algae, fungi and plants.

DIAGNOSIS FOR *DESMONOSTOC SALINUM* SP. NOV.

Desmonostoc salinum sp. nov. is described under the provisions of the International Code of Nomenclature for algae, fungi and plants [66].

DESMONOSTOC SALINUM (ALVARENGA *ET AL.*) SP. NOV.

Phylum: Cyanobacteria

Order: Nostocales

Family: Nostocaceae according to Hoffmann *et al.* [18]

Description: The sampled periphytic microbial mat used for cyanobacterial isolation presented a thickness of 4.0 cm, without laminated structure, with a gray layer on the surface and greenish inside, occurring at the edge of Laguna Amarga. The trichomes are straight at the beginning of development, as hormogonia or young trichomes, and later organized in parallel, enclosed by a diffluent mucilage. This parallel organization of trichomes observed in later phases of cultures can be lost as a consequence of akinete differentiation. Both intercalary and terminal heterocytes were always present in vegetative trichomes, with the terminal ones having been differentiated in the early stages of trichome development. Initially, the akinetes were organized in chains, which can be broken, releasing isolated mature akinetes. Macroscopically, the strain grows as a gelatinous free-floating biomass, which occasionally forms aggregated colonies. Motile hormogonia are frequent and abundant in earlier growth phases, usually containing more than 20 cells. Vegetative cells present a dark-green colour and are barrel shaped, sub-spherical, longer than they are wide (3.7–5.4 µm long and 3.3–4.5 µm wide). Heterocytes are subspherical, longer than they are wide (5.8–8.3 µm long and 3.7–4.6 µm wide) with a light green or yellowish colour. The akinetes are spherical or subspherical (8.3–10.0 µm long and 5.0–8.3 µm wide), with green to yellowish colouration and conspicuous granulation. Reproduction occurs via hormogonia differentiation. This species is typical from saline–alkaline environments.

Diagnosis: This novel species was defined according to molecular (16S rRNA gene identity, 16S–23S ITS secondary structure folding), phylogenetic position (based on 16S rRNA gene) and physiological/ecological traits.

Etymology: (sa.li′num. N.L. neut. adj. *sal*, *salinum*, salty, salted, saline) The specific epithet '*salinum*' (N. L. neut. adj.) refers to the salinity of the sampling site, Laguna Amarga.

Type locality: Periphytic mats in Laguna Amarga, Torres del Paine National Park, Chile (50° 29′ 00″ S and 72° 45′ 00″ W).

Holotype: Freeze-dried sample of strain CCM-UFV059 deposited at Herbarium VIC ('Herbário VIC': Universidade Federal de Viçosa, Viçosa, Minas Gerais State, Brazil) – voucher 47.756.

Reference strain: *Desmonostoc salinum* CCM-UFV059.

Living culture: Living cultures of the type strain (*Desmonostoc salinum* CCM-UFV059), from which the holotype was derived, are available at Collection of Cyanobacteria and Microalgae at Universidade Federal de Viçosa (CCM-UFV), under the internal code CCM-UFV059.

DNA sequence available: 16S rRNA gene and 16–23S ITS region: NCBI accession number KX787933.

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

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