Abstract: Heterocyte-forming cyanobacteria form symbiotic relationships with several lineages of plants. Here, twenty (20) strains of endosymbiotic cyanobacteria (cyanobionts) with Nostoc-like morphologies were isolated from the highly specialized coralloid roots of five host species in Cycadales—*Cycas debaoensis*, *C. fairyliuiae*, *C. elongata*, *Ceratozamia robusta*, and *Macrozamia moorei*. Molecular phylogeny based on the 16S rRNA gene placed these strains into seven different taxa within the Nostocaceae, specifically under the genera *Desmonostoc* and *Dendronalium*. The percent dissimilarity and unique patterns in the secondary structures of the D1-D1′, Box-B, V2, and V3 helices, which were based on the 16S–23S rRNA internal transcribed spacer (ITS) regions, supported three distinct species in *Desmonostoc*. These three morphologically distinct novel species are described in this report: *Desmonostoc debaoense* sp. nov., *Desmonostoc meilinense* sp. nov., and *Desmonostoc xianhuense* sp. nov. Other investigated strains were phylogenetically identified as members of the recently discovered genus *Dendronalium* and represent the first report of association of that genus with cycads. Our findings suggest that the order Cycadales hosts diverse species of cyanobionts in their coralloid roots and that many potential unreported or novel taxa are present in cycads occurring in their natural habitat and await discovery.

Keywords: coralloid roots; cyanobiont; *Desmonostoc*; *Dendronalium*; polyphasic approach

1. Introduction

The beneficial cyanobacterial-plant symbioses have long been studied, particularly the associations with nitrogen-fixing cyanobacteria. They are found inhabiting the internal parts of several plant groups, including bryophytes (where they are localized in the thallus) [1], the free-floating aquatic fern *Azolla* (in cavities in the dorsal lobe of the leaf) [2], and angiosperms *Gunnera* (stem glands) [3].

Among the gymnosperms, the order Cycadales, also known as Cycads, are the only group to have developed a mutualistic partnership with endosymbiotic cyanobacteria (cyanobionts) in specialized organs known as coralloid roots [4,5]. These cyanobionts share several characteristics with other cyanobionts from different plant lineages, such as the ability to form (i) short motile filaments known as hormogonia, (ii) differentiated heterocytes and sometimes akinetes, and (iii) filaments embedded in mucilage [6,7]. Different but closely related cyanobiont strains were found in different species of cycads across several habitats. For instance, *Desmonostoc muscorum* strain De was recovered in the coralloid root
of Dioon edule from a botanical garden in Rome, Italy [8], while other unnamed strains (Cc2, Cr3, Ds, PCC9230, and PCC7422), which phylogenetically clustered in the same genus, were recovered from other host species, that is, Cycas cirrata, Cycas revoluta, and Dioon spinulosum, from botanical gardens in Italy and Sweden [8,9]. In our previous paper [10], two new species of cyanobacteria were isolated and described from the green zone of the coralloid root of Cycas fairylakea collected from a botanical garden. These studies suggested the following pattern: different and potentially undescribed species of symbiotic cyanobacteria could live in different cycads, even those grown in botanical gardens.

The taxonomy of cyanobacteria presents a significant challenge to scientists and researchers. Many species belonging to a single taxonomic group display considerable morphological plasticity, whereas in Nostocaceae, there is a little morphological distinction between Nostoc and related non-Nostoc taxa [9], even though these taxa can be shown to be genetically separate lineages based on their 16S rRNA gene sequences [9–12]. Thus, investigators have moved away from the use of traditional classification methods that were based solely on morphological criteria (see papers cited herein) to the application of a multidisciplinary polyphasic approach that integrates life cycle, morphology and ultrastructure, genetic, ecological, and biochemical data, leading to the establishment of a robust taxonomy for cyanobacteria [9,13–18]. Furthermore, the metabolic and physiological characteristics have been shown to provide valuable evidence for understanding the evolutionary perspective and taxonomy of various cyanobacteria [18–20].

The polyphasic approach as a modern taxonomic tool has been consistently adopted in several studies that resulted in the discovery of different monophyletic Nostocacean genera, including Halotia [11], Mojavia [12], Alinostoc [21], Komarekiella [22], Desikacharya [23], Compactonostoc [24], Violetonostoc [25], Parakomarekiella [26], Amazonocrinis, and Atlanticothrix [27], thereby redefining Nostoc as a less polyphyletic genus. Along with these studies, two distinct genera phylogenetically related to Nostoc, Desmonostoc [9], and Dendronalium [27] were also erected.

The genus Desmonostoc is a coherent group representing a diverse group of filamentous cyanobacteria, with members primarily living in freshwater habitats [9,18,19,28]. Some members of the genus were found in terrestrial environments, particularly those species colonizing soil surfaces [9,29,30], rocks, and caves [31,32], which contribute to biological crusts and biofilms in these habitats. Members of Desmonostoc were also recovered from extreme habitats [20] or as associates/symbionts of plants [8–10]. To date, 12 species have been validly described under the genus Desmonostoc, with many unnamed strains still assigned to it. In contrast, Dendronalium, as a newly erected genus, has only one described species, Dendronalium phyllosphericum. This genus was first described in Brazil with reference strains isolated from leaf samples [27].

In the majority of cases, several cyanobionts in association with cycads were identified as Nostoc based on traditional morphological characterization [6,8,33,34] but are now known to belong to Desmonostoc upon comprehensive molecular analyses [9,10]. Therefore, apart from investigating the genetic diversity of cyanobionts associated with the coralloid roots of cycads, there is also a pressing need to characterize them using a polyphasic approach. This study is an extension of our previous work, in which we reported two novel species of Desmonostoc from Cycas fairylakea from a botanical garden [10]. We report an additional 12 strains of cyanobionts isolated from the coralloid roots of the same host species, Cycas fairylakea, collected from the same botanical garden and from a natural habitat in Meilin Nature Reserve. Other cyanobionts were isolated from other cycad species, including Cycas dehaanii, Cycas elongata, Ceratozamia robusta, and Macrozamia moorei, bringing the number of cyanobionts investigated herein to 20. The cyanobionts were extensively studied following a polyphasic approach of morphology-based characterization and analyses of the 16S rRNA gene and the ITS region between the 16S and 23S rRNA. Most of our cycadean cyanobionts are in Desmonostoc, the primary subject of this paper, but we also give the first report of cycad-associated cyanobacteria in Dendronalium. The scientific findings presented
in this paper contribute to the taxonomy of cyanobacteria and hold a broader appeal to individuals interested in plant–bacterial interactions.

2. Materials and Methods

2.1. Sampling and Isolation Protocol

Coralloid root tissues were obtained from different individuals of cycads collected from different sites: a botanical garden, a natural habitat, and a reintroduction site used in ex-situ conservation (refer to [35] for detailed descriptions of these habitats). Coralloid root samples were collected between September 2019 and October 2020. The collection of samples and isolation of cyanobionts follow the methods of Pecundo et al. [10]. Briefly, disease-free coralloid tissues with a healthy, fresh green zone (Figure 1) were collected, placed in an ice chest, and brought to the laboratory for processing. All coralloid root tissues were surface sterilized with sodium hypochlorite (c. 5% active chlorine) and 80% ethyl alcohol prior to the isolation of cyanobionts. Cross- and longitudinal sections of the coralloid roots were observed under a stereomicroscope (Stereozoom S9i, Leica Microsystems, Ltd., Heerbrugg, Switzerland) to get a better view of the green zone within the cortex of the roots (Figure 1). Cyanobacteria were isolated on a nitrogen-reduced BG-11 medium supplemented with cycloheximide to minimize fungal contamination. Isolation plates were maintained inside a growth chamber with conditions set to a temperature of 26 °C ± 2 °C, a photoperiod of 12 h of light and 12 h of dark cycle with white fluorescent lamps, and a photon flux density of 40 μmol m−2 s−1. The living cultures of cyanobacterial strains investigated in this study are maintained in the Cycad Symbiotic Microorganism Laboratory (CSML) at Fairy Lake Botanical Garden, Shenzhen, China. The codes of all the strains under investigation are provided in a supplementary file (Table S1).

Figure 1. The specialized coralloid root of cycads. (a) Coralloid root growing above the surface of the soil. (b) Clusters of coralloid roots excised from Cycas fairylakea; the yellow arrow indicates the green zone visible to the naked eye. (c,d) Stereomicroscopic examination of the green zone within the coralloid roots of Cycas fairylakea (c) and Macrozamia moorei (d). Photos were captured by M.H. Pecundo (Image (a)) and T. Chen (Image (b)).
2.2. Morphological Evaluations

We studied the morphological characteristics and morphometric measurements of the isolated cyanobacteria that were grown on BG-11 medium plates under a Nikon optical microscope (Nikon NI-SS, Tokyo, Japan) at magnifications of 400 to 1000 times. Photomicrographs were captured using a Nikon camera (Nikon Y-TV55, Tokyo, Japan) attached to the microscope. The features of the filaments that were observed include the appearance of the mucilaginous layer, hormogonium, vegetative cells in younger and older colonies, apical cell morphologies, and the presence and appearance of necridia. These were recorded and described for the studied cyanobacteria. Measurements in length (L) and width (W) of vegetative cells were taken from 50 to 100 individual cells. Differentiated cells, such as heterocytes and akinetes, were also observed, measured, and compared to data available for all known species in *Desmonostoc* [10,19,20,28–32] and *Dendronalium* [27].

2.3. Molecular Evaluations

The total genomic DNA was extracted in the same manner as described in Pecundo et al. [10], in which 1 mL of cyanobacterial culture in the exponential phase of growth (ca. 14 days) was harvested by centrifugation for 1 min at 1000 × g. The cells were then rinsed three times with 1 mL of sterile deionized water and centrifuged for 1 min at 1000 × g to expel excess water. Lysing of cells was done by a combined two-fold freeze-thawing method (using liquid nitrogen and a water bath at 60 °C) and shaking on a tissue homogenizer with glass beads (Tissuelyser 24, Jingxin Technology, Shanghai, China) for 60 s. The DNA of the cyanobacteria was extracted using the TIANamp Bacteria DNA Kit (Tiangen Biotech Co. Ltd., Beijing, China) following the manufacturer’s instructions. Amplification of the 16S rRNA gene (~1400 nucleotides) and the ITS region between 16S and 23S rRNA was carried out as previously described using the primer sets 27F/1492R (5′-AGAGTTTGATCCTGGCTCAG-3′/5′-TACGGYTACCTTGTTAYGACTT-3′) and 322F/340R (5′-TGTACACACGCCCAGTC-3′/5′-CTCTGTGTGCCTAGGTATCC-3′) [10,36], respectively. Each reaction contained the following components: 12 µL of 2× Taq PCR Mix (Cat 21401, TOLO Biotech Co., Ltd., Shanghai, China), 1 µL of each primer (10 pmol), 1 µL template DNA, and 10 µL sterile water to bring the final volume to 25 µL. PCR amplification was performed using a BIO-RAD T100 Thermal Cycler with a thermocycling regime of initial denaturation for 5 min at 94 °C, followed by 35 cycles (30 s at 94 °C, 30 s at 52 °C, and 30 s at 72 °C), and a final extension of 10 min at 72 °C. Amplification of the ITS region was carried out following the protocol of Iteman et al. [36]. The quality of the PCR products was checked on a 0.8% agarose gel in 1× TAE buffer. The PCR products obtained for ITS regions were purified using the Tsingke Trelief® DNA Gel Extraction Kit (TSP602-200, Tsingke Biotech Co., Ltd., Beijing, China) following the manufacturer’s instructions. The purified PCR products were then cloned to pClone007 Versatile Simple Vector Mix (Beijing Tsingke Biotech Co., Ltd.) and transformed using the Tsingke Trelief® highly competent cells (Beijing Tsingke Biotech Co., Ltd.). Colonies were grown in LB medium and screened using the blue-white scheme. Subsequently, the DNA from the clones was purified and processed using the Tsingke T5 super PCR Mix (Colony). All samples were sent to Tsingke Biotechnology Co., Ltd. (Guangdong and Wuhan, China) for sequencing on a 3730XL DNA Analyzer (Applied Biosystems, MA, USA). The quality, including the presence of any chimeras in the obtained sequences, was checked using the software toolset DECIPHER available online: http://www2.decipher.codes (accessed on 15 October 2022). The sequences were deposited in the NCBI GenBank database. The accession numbers are provided in the supplemental files (Table S1 for the 16S rRNA gene; Tables S6 and S7 for ITS regions).

2.4. 16S rRNA Gene Analysis

A total of 25 partial 16S rRNA gene sequences from the 20 strains reported herein were generated and deposited in the NCBI GenBank database, with their accession numbers provided in Table S1. One hundred ninety-nine (199) 16S rRNA gene sequences from
cyanobacteria related to the strains analyzed in this study were retrieved from the public database for pairwise alignment. This was performed on Geneious Prime 2020.2 [37] with the default parameters of MAFFT v.450 [38]. The phylogenetic tree was constructed by maximum likelihood (ML), Bayesian inference (BI), and neighbor-joining (NJ) methods. The phylogenetic tree based on ML, BI, and NJ was constructed with 197 nucleotide sequences, including the 25 nearly complete 16S rRNA gene sequences from this study. After pairwise alignment and deletion of gaps, there were 1370 base pairs. The most appropriate fitting model for the aligned sequences was determined under the Bayesian Information Criterion (BIC) using IQ-TREE v6.10 [39] and jModelTest 2.1.1 [40] on XSEDE using the CIPRES Science Gateway [41]. The GTR + I + G evolutionary model was chosen according to BIC and used to infer ML in IQ-TREE, v6.10, using 100 standard and 1000 ultrafast bootstrap analyses to show the robustness of the ML trees and relative support of the branches [39]. Phylogenetic trees analyzed by the BI and NJ methods were generated in Mr. Bayes v3.2.6 [42], applying the model GTR + I + G, and in MEGA version X with the Kimura-2 parameter model [43]. The BI execution involved two runs of four Markov chain Monte Carlo (MCMC) running for 50 million generations using default parameters, sampling after every 1000 generations, and using 25% burn-in. The final average standard deviation of split frequencies between runs was <0.01. All phylogenetic tree consensus files were viewed using FigTree v1.3.1 [44] and rooted with the outgroup Gloeobacter violaceus VP3-01 (NCBI GenBank Accession Number FR798924). The 16S rRNA gene alignments were also used to estimate the degree of similarity between the gene sequences of different cyanobacteria. Using MEGA software v7.0.26 [43], the alignment file was uploaded, and the pairwise distances were estimated. The sequence similarity was then calculated using the formula \((1 - \frac{p}{d}) \times 100\).

2.5. 16S-23S Internal Transcribed Spacer (ITS) Region Analyses

The conserved 16S–23S ITS regions of all strains under investigation were analyzed manually, according to Iteman et al. [36]. The tRNA genes for each ITS operon were also identified manually after alignment on Geneious Prime 2020.2. The 16S–23S rRNA ITS regions were used to predict the secondary structures of different helices, such as the D1-D1’, Box-B, V2, and V3, using the Mfold applications with default settings and untangled loop fix on the online UNAFold Web Server: https://unafold.org (accessed on 5 July 2022) [45]. In addition, lengthwise comparative evaluations of the leader, D1-D1’, Spacer + D2, Spacer + D3 + Spacer, tRNA^{Ile}, Spacer + V2 + Spacer, tRNA^{Ala}, Spacer, Box-B, Spacer + Box-A, D4 + Spacer, V3, and D5 region (end of ITS) were performed for all studied strains and provided in the supplemental files (Tables S6 and S7). Alignment of the entire 16S–23S ITS regions for the studied strains and their close relatives (which were identified according to 16S rRNA gene phylogeny) was conducted separately for those operons containing both tRNA^{Ile} and tRNA^{Ala} genes and those with no tRNA genes. Percent dissimilarity was then calculated using the formula \(100 \times \frac{p}{d}\). Comparison of the 16S–23S ITS percent dissimilarity of our Desmonostoc strains was not performed against species of D. muscorum, D. magnisporum, D. persicum, and D. punense, as the ITS sequences from these strains were either unavailable, incomplete, or incorrectly given.

3. Results

3.1. Host, Locality, and Taxonomic Assessment of the Cyanobionts

Three novel cyanobacteria and 20 additional cyanobacterial strains were recovered from the coralloid roots of various species of cycads that were collected from different habitats (Table S1). The detailed morphological characteristics of the investigated cyanobacteria, except for strain CSM 013-B, were analyzed and compared against their closely related taxa. Descriptions of one or two representative strains from each identified morphospecies are provided. Presented below are the detailed descriptions of the three novel cyanobionts.
3.1.1. Desmonostoc debaense M. Pecundo, N. Li et T. Chen sp. nov. (Figure 2)

Description: Colony on a solid medium grew radially from the middle, tightly clumped. Colonies consisted of small clusters of coccolid cells and filaments. Filaments uniseriate, densely or loosely agglomerated, intertwined, and entangled, forming compact microcolonies up to 200 µm long. Individual filaments sometimes diffuse or strongly constricted, flexuous, or densely coiled and enclosed in a common diffusent, thick, mucilaginous, colorless sheath. Vegetative cells mostly spherical or subspherical to barrel-shaped and compressed globose, bright to dark bluish green, with a prominent cellular granule and distinct chromatoplasm, with dimensions of 2.4–7.8 µm long and 2.3–6.5 µm wide. The apical cells can be spherical, subspherical to oblong. Large spherical cells appearing as akinete-like cells (L: 7.41–10.34 µm; W: 7.59–9.83 µm) were observed within the flexuous trichome during the cell division of vegetative cells. Terminal heterocytes single-pored, conical-rounded or oblong; intercalary heterocytes double-pored, common, spherical or compressed subspherical, colorless or yellow, 2.9–7.6 µm long and 3.5–8.6 µm wide. Akinetes smooth or granulated, occasionally solitary and intercalary, usually in long series, apoheterocytic, spherical, subspherical to oblong, 3.1–9.5 µm long and 4.0–8.1 µm wide, developing from vegetative cells in trichomes. Hormogonia straight with cells longer than wide. Necridia disc-like or irregular in shape.

Holotype here designated: Actively growing cultured material was freeze-dried (metabolically inactive) and was deposited in the herbarium of Shenzhen Fairy Lake Botanical Garden (SZG). It is available under accession number: FLBG-CSM 005.

Isotype here designated: Actively growing cultured material was preserved in 4% formaldehyde and was deposited in the herbarium of Shenzhen Fairy Lake Botanical Garden (SZG), under the same accession number, FLBG-CSM 005.

Habitat/Source: Isolated from the green zone of the coralloid roots of Cycas debaensis collected from a natural karst habitat in Debao County, Baise City, China, 23°29′29.2″ N 106°12′50.8″ E, elevation at 885 m above sea level (masl).

Etymology: The species epithet ‘debaense’ refers to the source locality of Cycas debaensis in Debao County, Guangxi, China.

Reference strain: CSM 005-N. The living culture is maintained at the Cycad Symbiotic Microbiology Laboratory, Fairy Lake Botanical Garden, Shenzhen, China.

Materials analyzed: Strain CSM 005-N.

Nucleotide sequence accession numbers: OM746697 and OM746698 for the 16S rRNA gene; OM746734, OM746735, OR723793, and OR723794 for ITS.

3.1.2. Desmonostoc meilinense M. Pecundo, N. Li et T. Chen sp. nov. (Figure 3)

Description: Colony on a solid medium bright green to dark green, growing radially through hormogonia from the center after inoculation. Filaments dense, occasionally loosely intertwined, sometimes circinate, enclosed in a common diffusent, mucilaginous, colorless sheath. Cells in hormogonia quadratic, mostly longer than wide. Vegetative cells mostly barrel-shaped, spherical to compressed subspherical, bright bluish green, and bright green to olive green in older colonies; chromatoplasm sometimes visible, 1.9–7.1 µm long and 2.4–5.9 µm wide. Apical cells varying in shapes and sizes, from narrowly oblong to spherical and subspherical. Terminal heterocytes single-pored, at one end, spherical to conical-rounded; double-pored intercalary heterocytes common, spherical to subspherical, yellow or colorless, 3.8–6.3 µm long and 3.5–5.6 µm wide. Akinetes common in old cultures, granulated, in long chains, apoheterocytic, cylindrical, subspherical to oblong, 3.7–8.8 µm long and 4.4–7.6 µm wide, developing from vegetative cells in trichomes. Numerous intercalary, colorless heterocytes were observed, solitary or in multiple within the trichomes in old cultures. Necridia between vegetative cells common.

Holotype here designated: Actively growing cultured material was freeze-dried (metabolically inactive) and deposited in the herbarium of Shenzhen Fairy Lake Botanical Garden (SZG). It is available under accession number, FLBG-CSM 017.
Isotype here designated: Actively growing cultured material was preserved in 4% formaldehyde and was deposited in the herbarium of Shenzhen Fairy Lake Botanical Garden (SZG), under the same accession number: FLBG-CSM 017.

Habitat/Source: Isolated from the green zone of the coralloid roots of *Cycas fairylakea* in a natural habitat at Meilin Nature Reserve, Shenzhen, China, 22°34'38.5'' N 114°00'34.2'' E, elevation at 114 masl.

Reference strain: CSM 017-N. The living culture is maintained at the Cycad Symbiotic Microbiology Laboratory, Fairy Lake Botanical Garden, Shenzhen, China.

Materials analyzed: Strains CSM 017-N and CSM 018-N.

Etymology: The species epithet ‘meilinense’ refers to the source locality of *Cycas fairylakea* in Meilin Reservoir Nature Reserve, Shenzhen, China.

Nucleotide sequence accession numbers: OM746704, OM746702, and OM746703 for the 16S rRNA gene; OM746739, OM746738, OR723795, and OR723796 for ITS.

**Figure 2.** Macroscopic and microscopic characteristics of *Desmonostoc debaoense* CSM 005-N. (a) A dark green, clump colony on the culture plate. (b) Agglomerated filaments. (c–e) Filaments with varying shapes of vegetative cells and the presence of necridia, surrounded by a hyaline sheath. (f,g) Formation of large spherical cells (red arrows) in flexuous filaments. (h) Filaments composed of subspherical cells; presence of necridia. (i) Filaments with prominent cellular granules. (j) Akinete formation. (k) Filaments with akinetes, heterocytes, and necridia. Abbreviations: hormogonia (Ho); intercalary (IH) and terminal (TH) heterocytes; sheath (S); necridia (N); akinete (A). Scale bar, 10 μm.
Figure 3. Macroscopic and microscopic characteristics of *Desmonostoc meilinense* CSM 017-N. (a) A dark green colony on the culture plate. (b) Filament showing vegetative cells from isodiametric to wider than long. (c) Filaments in parallel. (d–f) Filaments with spherical to barrel-shaped cells with visible cellular granules. (g) Filament with a double-pored intercalary heterocyte. (h–m) Formation of akinetes. (n) Bright olive-green cells in an older colony. (o) Akinetes in chain; numerous compressed colorless heterocytes and new cells developing from akinetes (arrow). Abbreviations: intercalary (IH) and terminal (TH) heterocytes; sheath (S); apical cell (AC); necridia (N); akinete (A). Scale bar, 10 μm.

3.1.3. *Desmonostoc xianhuense* M. Pecundo, N. Li et T. Chen sp. nov. (Figure 4)

Description: The colony on a solid medium formed a gelatinous mat. After inoculation, they grew radially through the center and were enclosed by a thick mucilage, blue-green at the early stage and olive-green to yellow-brown in aged colonies. The production of spherical thalli on the culture plate was also observed. Trichomes densely entangled, sometimes parallel or circular in younger colonies. Filaments straight to bent and twisted. Vegetative cells isodiametric, barrel-shaped, or compressed subspherical to cylindrical at later stages, 2.9–9.9 μm long, 3.5–6.0 μm wide, and blue-green. Heterocytes solitary when terminal, in multiples of two or more when intercalary, variable in size and shape, spherical
to conical–rounded in terminal or basal position, oblong to spherical and irregularly compressed in flexuous filament or old trichome in intercalary position, yellow to bright green at the young stage, and colorless at the old stage, 4.5–10.9 \( \mu \text{m} \) long and 4.5–9.5 \( \mu \text{m} \) wide. Intercalary heterocytes smooth and granulated. Akinetes occurring in long series, apoheterocytic, barrel-shaped or spherical to oblong, enclosed individually by a very thick sheath, 4.5–12.9 \( \mu \text{m} \) long, 4.8–9.2 \( \mu \text{m} \) wide, developed from vegetative cells in trichomes.

Figure 4. Macroscopic and microscopic characteristics of *Desmonostoc xianhuense* CSM 010-B. (a) A green gelatinous mat on the culture plate. (b) Filaments in parallel and circular arrangements. (c–f) Filaments with heterocytes. (g) Flexuous filaments with barrel-shaped to subspherical cells; presence of 2 or more intercalary heterocytes in a single filament. (h) Akinetes formation. (i) Varying shapes of akinetes; occurring in chains. (j) Reproduction from akinetes (arrows). Abbreviations: intercalary (IH) and terminal (TH) heterocytes; necridia (N); akinete (A). Scale bar, 10 \( \mu \text{m} \).

Holotype here designated: Actively growing cultured material was freeze-dried (metabolically inactive) and deposited in the herbarium of Shenzhen Fairy Lake Botanical Garden (SZG). It is available under accession number: FLBG-CSM 010.

Isotype here designated: Actively growing cultured material was preserved in 4% formaldehyde and was deposited in the herbarium of Shenzhen Fairy Lake Botanical Garden (SZG), under the same accession number, FLBG-CSM 010.

Habitat/Source: Isolated from the green zone of the coralloid roots of *Ceratozamia robusta* and *Cycas fairylakea* in a botanic garden habitat at Fairy Lake Botanical Garden, Shenzhen, China, 22°34’38.5” N 114°00’34.2” E, elevation at 114 masl.
Reference strain: CSM 010-B. The living culture is maintained at the Cycad Symbiotic Microbiology Laboratory, Fairy Lake Botanical Garden, Shenzhen.

Materials analyzed: Strains CSM 009-B and CSM 010-B.

Etymology: The species epithet ‘xianhuense’ refers to the source locality of its hosts, Ceratozamia robusta and Cycas fairylykea, in Fairy Lake Botanical Garden, also known locally as Xianhu Botanical Garden in Shenzhen, Guangdong, China.

Nucleotide sequence accession numbers: OM746715, OM746714, OM746712, and OM746713 for the 16S rRNA gene; OM746749, OR723797, OR723798, OM746747, and OM746748 for ITS.

3.1.4. Distinction of the Novel Cyanobacterial Strains from Known Species

All Desmonostoc strains investigated in this study displayed long (>200 µm) and entangled trichomes enclosed by a thick or thin transparent mucilage layer, heterocytes in basal or intercalary positions, and akinetes solitary or in chains. They were also observed with dead cells in between the vegetative cells of the filaments. In culture and under light microscopy, the features of the novel species Desmonostoc debaoense differed from the descriptions of other Desmonostoc species, specifically from its closely related taxon, D. aggregatum. D. debaoense was blue-green in younger colonies, becoming bright green in later stages, with entangled and densely coiled trichomes, mostly spherical cells, and does not exhibit aggregate cells in older colonies (Figure 2, Table 1). This species showed an even more compact colony in culture than D. aggregatum. The akinetes of D. debaoense were also longer (up to 9.5 µm), while the closest relative, D. aggregatum, was reported to have a complete absence of akinetes throughout its lifecycle. Despite the high gene sequence similarity values in 16S rRNA against several strains in Desmonostoc, significant differences in the dissimilarity values and secondary structures of the D1-D1′, Box-B, V2, and V3 helices drawn from the 16S–23S ITS region were observed for D. debaoense. The same could be said for the morphological characters of D. meilinense, which showed similarities to the descriptions of D. danxiaense in filaments (e.g., densely entangled) and cellular shapes except that D. meilinense produces longer (up to 7.1 µm long) and wider (up to 5.9 µm wide) vegetative cells (Figure 3 and Table 1). The heterocytes of D. meilinense ranged from spherical to compressed subspherical and slightly larger (up to 6.3 µm long and 5.6 µm wide) as opposed to D. danxiaense, with oval-shaped and small heterocytes (3.97 µm long and 3.54 µm wide). D. meilinense also showed slightly longer akinetes (8.8 µm) compared to D. danxiaense (7.8 µm), but they were slightly smaller in comparison to the akinetes of the close relative D. debaoense. Furthermore, this novel species was delimited by strong molecular and phylogenetic analyses based on the data from the 16S rRNA gene sequence. The 16S rRNA sequence similarity of D. meilinense and D. danxiaense was 98%, and they were placed in separate, distinct nodes in the 16S rRNA phylogeny. The secondary structures of the D1-D1′, Box-B, and V2 helices of this strain were also distinct from those of other Desmonostoc species, including that of D. danxiaense, and the three taxa were in the same clade. The morphology of the filaments, vegetative cells, heterocytes, and akinetes in D. xianhuense closely resembles that of D. muscorum, both in appearance and shape. Only the size range of the cells exhibited variations between the two species, with D. xianhuense having slightly larger cells compared to D. muscorum (Table 1). Meanwhile, based on the pattern in the secondary structures of the ITS region, D. xianhuense and D. geniculatum shared identical structures in the D1-D1′ helix but differed in Box-B, V2, and V3 helices. The combined evidence from the morphology and genetic data supports the separation of D. debaoense, D. meilinense, and D. xianhuense as new species within the genus Desmonostoc.
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Table 1. Morphological characteristics of the strains under investigation and the known species in *Desmonostoc*. The source, locality, and habitat condition (if available) for each strain are provided, as well as their placement in the 16S rRNA gene phylogeny.

<table>
<thead>
<tr>
<th>Species</th>
<th>Vegetative Cells Shape and Size (µm)</th>
<th>Heterocytes Shape and Size (µm)</th>
<th>Akinetes Shape and Size (µm)</th>
<th>Source—Locality (Habitat Condition)</th>
<th>Phylogenetic Position/Reference</th>
</tr>
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<tbody>
<tr>
<td><em>D. dehaenei</em> CSM 005-N</td>
<td>Spherical to subspherical W: 2.3–6.5; L: 2.4–7.8</td>
<td>Spherical to subspherical W: 3.5–8.6; L: 2.9–7.6</td>
<td>Spherical, subspherical to oval, W: 4–8.1; L: 3.1–9.5</td>
<td>Coralloid root of <em>C. robusta</em> from a natural karst habitat, Debao County</td>
<td>Cluster E (D1); this study</td>
</tr>
<tr>
<td><em>D. meilandense</em> CSM 017-N</td>
<td>Barreled-shaped to subspherical W: 2.4–5.9; L: 1.9–7.1</td>
<td>Spherical to subspherical W: 3.5–5.6; L: 3.8–6.3</td>
<td>Spherical to subspherical to oval, W: 4–4.7; L: 3.7–8.8</td>
<td>Coralloid root of <em>C. robusta</em> from natural moist forest, Meilin Nature Reserve</td>
<td>Cluster A (D1); this study</td>
</tr>
<tr>
<td><em>D. aggregatum</em> CSM 008-B</td>
<td>Barreled-shaped to spherical to subspherical W: 3.5–6.6; L: 2.9–9.9</td>
<td>Spherical to subspherical W: 4.5–9.5; L: 4.5–10.9</td>
<td>Spherical to subspherical to oval, W: 4.8–9.2; L: 4.5–12.4</td>
<td>Coralloid root of <em>C. fairylakea</em> from an ex-situ conservation site in Xianhu/Fairy Lake botanical garden</td>
<td>Cluster F (D2); this study</td>
</tr>
<tr>
<td><em>D. meilandense</em> CSM 010-B</td>
<td>Barreled-shaped to spherical to subspherical W: 3.5–5.9; L: 2.9–9.8</td>
<td>Spherical to subspherical W: 5–9; L: 4.5–10.7</td>
<td>Spherical to subspherical to oval, W: 5–9; L: 4.8–12.9</td>
<td>Coralloid root of <em>C. fairylakea</em> from Xianhu/Fairy Lake botanical garden</td>
<td>Xianhu/Fairy Lake botanical garden</td>
</tr>
<tr>
<td><em>D. aggregatum</em> CSM 022-B</td>
<td>Spherical to subspherical to barreled-shaped W: 2–5.5; L: 1.8–4.2</td>
<td>Conical to spherical to subspherical W: 4–5.5; L: 3.7–6.5</td>
<td>Not observed</td>
<td>Coralloid root of <em>C. fairylakea</em> from Xianhu/Fairy Lake botanical garden</td>
<td>Cluster C (D1); this study</td>
</tr>
<tr>
<td>CF06</td>
<td>Barreled-shaped to subspherical W: 2.3–5.6; L: 2.3–4.7</td>
<td>Conical to spherical W: 4.1–5.6; L: 3.8–6.7</td>
<td>Subspherical to oval, W: 3.9–6.0; L: 4.8–6.9</td>
<td>Coralloid root of <em>C. fairylakea</em> from Xianhu/Fairy Lake botanical garden</td>
<td>Cluster C (D1); [10]</td>
</tr>
<tr>
<td><em>D. lechangense</em> CSM 014-B</td>
<td>Barreled-shaped to spherical to oval W: 2.5–4.9; L: 2.5–6.1</td>
<td>Conical to spherical W: 3.8–5.3; L: 4.1–5.9</td>
<td>Spherical to oval/subspherical W: 4.1–5.6; L: 3.8–6.7</td>
<td>Coralloid root of <em>C. elongata</em> from Xianhu/Fairy Lake botanical garden</td>
<td>Cluster B (D1); this study</td>
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<tr>
<td>CF01</td>
<td>Barreled-shaped to subspherical W: 2.5–4.9; L: 2.5–6.1</td>
<td>Conical to spherical W: 4–4.7; L: 3.7–10.2</td>
<td>Subspherical to oval, W: 3.9–6.0; L: 4.8–6.9</td>
<td>Coralloid root of <em>C. fairylakea</em>, initially grown from a natural habitat in northern Guangdong and later introduced to Xianhu Botanical Garden</td>
<td>Cluster B (D1); [10]</td>
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<td><em>D. duxiaeense</em> CHARS686</td>
<td>Barreled-shaped to oval W: 2.65–3.9; L: 2.48–4.4</td>
<td>Conical to spherical W: 3.54; L: 3.97</td>
<td>Spherical to oval W: 5.59; L: 7.94</td>
<td>Rocky wall in Danxia Mountain, Guangdong</td>
<td>D1 subcluster; [32]</td>
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<tr>
<td>NIVA-CYA818 (TYPE SPECIES)</td>
<td>Barreled-shaped to cylindrical W: 4–5.9; L: 2.8–6.8</td>
<td>Barreled-shaped to subspherical W: 4.4–7.2; L: 5.3–10.2</td>
<td>Spherical or hemispherical with homogeneous content W: 3.4–6.3; L: 4.5–9.4</td>
<td>Soil, arable field in Douhua, Czech Republic</td>
<td>D2 subcluster; [9]</td>
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<td><em>D. genticulatum</em> HAA3440-L1</td>
<td>Barreled-shaped to oval W: 3.7–4.6; L: 2.8–6.3</td>
<td>Barreled-shaped to subspherical W: 3.5–8.3; L: 8.3–10</td>
<td>Subspherical to oval W: 5.3–7; L: 3.7–7.5</td>
<td>Dry wall of Maniniholo Cave in Kilaeua, Hawaii</td>
<td>D2 subcluster; [31]</td>
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<td><em>D. salinus</em> CCM-UFOV59</td>
<td>Barreled-shaped to subspherical to oval W: 3.3–4.5; L: 3.7–5.4</td>
<td>Subspherical to subspherical W: 3.7–4.6; L: 5.8–8.3</td>
<td>Spherical or subspherical W: 5.8–3; L: 8.3–10</td>
<td>Periphytic microbial mats in a saline-alkaline lake (H2O temperature 6°C, pH9.5), Laguna Amarga, Torres del Paine National Park, Chile</td>
<td>D1 subcluster; [20]</td>
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<td><em>D. magnispisseri</em> AR6-P5</td>
<td>Barreled-shaped, rarely isodiametric W: 4.1–4.7; L: 4–4.5</td>
<td>Barreled-shaped to oval W: 4.8–5.6; L: 5.3–6</td>
<td>Spherical to oval W: 5.7–57; L: 11.8–13.4</td>
<td>Freshwater habitat (temperature 9.8°C, pH 7.3, clay-lean) Amol, Mazandaran, Iran</td>
<td>D1 subcluster; [28]</td>
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<td><em>D. persicum</em> SA14</td>
<td>Barreled-shaped to subspherical W: 2.2–5.4; L: 1.9–5.5</td>
<td>Barreled-shaped to oval W: 4.5–7.1; L: 4.6–7.1</td>
<td>Oblong to oval W: 3.9–5.2; L: 5.5–8.8</td>
<td>Freshwater habitat in Pune, India</td>
<td>D1 subcluster; [29]</td>
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<td><em>D. puentei MCC 2741</em></td>
<td>Barreled-shaped W: 3.1–4.9; L: 3.5–4.5</td>
<td>Barreled-shaped to oval W: 3.7–5.1; L: 4.7–5.5</td>
<td>Oblong to oval W: 3.8–4.5; L: 6.5–8</td>
<td>Meadow subalpine soil in the Greater Caucasus (an altitude of 2100 masl), Russia</td>
<td>D1 subcluster; [30]</td>
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<td><em>D. caucasicum</em> MZ-C154</td>
<td>Barreled-shaped to subspherical W: 3.1–4.7; L: 2.9–4.5</td>
<td>Barreled-shaped to oval W: 3.1–4.4; L: 3.4–6</td>
<td>Not observed</td>
<td>Freshwater from the qanat water supply system (pH 7.1, nitrate 0.09 mg/l, phosphate 0.1 mg/l), Iran</td>
<td>D1 subcluster; [19]</td>
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<td><em>D. alborizicum</em> 1387</td>
<td>Barreled-shaped to subspherical W: 0.7–4.2; L: 2.5–8.5</td>
<td>Barreled-shaped to subspherical W: 2–2.5; L: 1.2–6</td>
<td>Oblong to elliptoidal W: 5.6–7; L: 7.5–9</td>
<td>With mixed algae on the right-hand wall of Waikapala’s Cave in Hawaii</td>
<td>See Figure 5, [31]</td>
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<td><em>D. ziminense</em> HA7617-LM4</td>
<td>Compressed, globose to longer than broad W: 0.5–1.6; L: 2–4</td>
<td>Spherical to compressed W: 2.5–3; L: 2.5–3.5</td>
<td>Granular W: 3–7; L: 3–6.7</td>
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3.2. 16S rRNA Gene Sequence Similarity and Phylogenetic Analyses

The 16S rRNA similarity matrix of cyanobacterial sequences was generated after pairwise alignment with the deletion of gaps. Analysis of p-distance based on the 16S rRNA gene showed that the cyanobacterial strains reported herein were closest to the
Nostocacean genera Desmonostoc and Dendronalium. Fifteen of the 20 strains shared a greater sequence similarity to members of Desmonostoc (95.8%–99.7%, Table S2) with the exception of D. vinosum (with similarity values of 94.4%–96.3%, Table S2) than to members of other Nostocacean genera, that is, the type genus Nostoc (93.8%–95.4%), Dendronalium (95.6%–96.7%), Mojavia (94.4%–94.9%), and other Nostoc-like genera (Aliinostoc, Desikacharya, Komarekiella, Violetonostoc, and Amazonocrinis; 92.5%–95.7%) (Table S3). Interestingly, D. debaoense and D. meilinense showed maximum 16S rRNA gene sequence similarities to Desmonostoc sp. 111_CR4_BG11B at 98.8% and 98.3%, respectively (Table S2). D. xianhuense showed high 16S rRNA gene sequence similarities to D. muscorum, D. geniculatum, and strains 7N/81-NMI_ANAB at 99.6%, 99%, and 99.6%, respectively (Table S2). However, other strains belonging to different species in this genus showed the same range of similarity or were like each other. For example, D. muscorum strain Lukesova 1/87 and D. geniculatum HA4340-LM1 showed 16S rRNA gene sequence similarity at 99.2%, and D. magnisporum AR6-PS showed 99.1% similarity to D. punense MCC 2741 (Table S2).

The BI and ML phylogenetic analyses based on 16S rRNA gene sequence shared the same topology (Figure 5), but the topology for the NJ analysis differed (Figure S3). Fifteen strains clustered with Desmonostoc sensu stricto, and five clustered with Dendronalium (Figure 5). Three (CSM 016-B, 014-B, 015-B) and four (CSM 019-B, 021-B, 020-B, 022-B) cyanobacterial strains grouped in particular with the recently described species D. lechangense (Figure 5, cluster B) and D. aggregatum (Figure 5, cluster C), respectively, with high bootstrap support values (ML = 100, BPP = 1.0 for D. lechangense, ML = 93, BPP = 0.97 for D. aggregatum). D. meilinense (cluster A; ML = 100, BPP = 1.0) formed a basal node close to the cluster containing a mixture of free-living and symbiotic cyanobacterial strains. The unnamed strains CSM 013-B (cluster D) and D. debaoense (cluster E; ML = 99, BPP = 1.0) formed unique clades in sister position to D. aggregatum, all in the previously reported D1 subcluster in this lineage [9]. D. xianhuense (Figure 5, cluster F) was in the D2 internal subcluster together with D. geniculatum and a tight group of D. muscorum strains (Figure 5), some of which were the basis for the original description of Desmonostoc [9].

Five strains from this study clustered within the Dendronalium clade (Figure 5, cluster G). The Dendronalium clade can be further subdivided into four distinct subclades (G-I to G-IV). The first subclade G-I holds the type species D. phyllosphericum CENA369 and strain CENA358, the second subclade G-II contains two strains CENA389 and CENA388, and the third subclade G-III is formed by a single strain D. phyllosphericum CENA73. Our strains formed the fourth subclade (G-IV) in a sister position to the subclade containing the type D. phyllosphericum CENA69 (G-I) for this genus. Moreover, our strains shared 16S rRNA sequence similarity of 98.7%–99.9% (Table S4) and showed higher similarity to Dendronalium (98%–99.7%) than to other genera in the Nostocaceae: Desmonostoc (96.9%–97.7%), Nostoc (94.9%–95.7%), Amazonocrinis (96.6%–97.7%), Mojavia (96.2%–96.8%), Desikacharya, Aliinostoc, Komarekiella, and Violetonostoc (93.6%–96%) (Table S4). The 16S rRNA gene sequences of Dendronalium strains investigated in this study shared 98.7%–99.6% with the reference strain for D. phyllosphericum, CENA369, and 98%–99.7% with other members of the Dendronalium clade, including CENA358, CENA73, and CENA389 (Table S4). The strains of Dendronalium investigated herein also showed 16S rRNA gene sequence similarities of 97.4%–98.3% with strain NIES-26, which has the uncorrected name Nostoc minutum (Table S4).
Figure 5. Maximum likelihood (ML) tree based on 16S rRNA gene sequences showing the phylogenetic positions of cyanobacterial strains under investigation and other closely related taxa (224 16S rRNA gene sequences). Numbers on nodes indicate bootstrap values and posterior probability (>50%/>0.50) obtained from ML and Bayesian analyses, respectively. OTU indicates operational taxonomic unit. Black bars denote bootstrap values of 100% for ML and posterior probabilities of 1.0 for Bayesian analysis on the same node. The investigated strains (positioned in subclades A–G) are in bold font and highlighted in gray. The novel species are in clades A and E in the internal D1 subcluster and clade F in the D2 subcluster derived from [9]. An asterisk marked the reference strain. Mislabeled strains of *Desmonostoc muscorum* are marked with three asterisks (***). The scale corresponds to substitutions/site.
3.3. 16S–23S rRNA ITS Region Analyses

Two distinct kinds of ITS operon, one containing both tRNA\textsuperscript{Ile} and tRNA\textsuperscript{Ala} and one with no tRNA genes, were found in one species assigned to Desmonostoc, D. geniculatum (Table S6). Most of the complete ITS sequence data of Desmonostoc deposited in the public database (e.g., NCBI) have operons either with both tRNA genes or with no tRNA genes (Table S6). The investigated strains that clustered with Desmonostoc according to 16S rRNA phylogeny were obtained with ITS sequence data having both operons. Therefore, apart from D. geniculatum, the Desmonostoc strains reported in this paper have both ITS operons, indicating the presence of quite different ITS transcripts in the Desmonostoc lineage. The length of the ITS region of the investigated strains ranged from 508 to 609 nt for operons with both tRNA genes and from 254 to 335 nt for operons with no tRNA genes (Table S6). The percent dissimilarity based on the ITS regions for the strains under investigation was calculated and compared with their close relatives. The several OTUs that clustered with D. lechangense and D. aggregatum in the 16S rRNA phylogeny showed ITS dissimilarity values of less than 3% against reference strains for these species, CF01 and CF06, respectively (Tables 2 and 3). The two novel species, D. debaoense and D. meilinense, and other Desmonostoc strains were dissimilar at 10–28.8% in operons with both tRNA genes (Table 2), while dissimilar at 6.1–14.0% in operons with no tRNA genes (Table 3). The dissimilarity between D. xianhuense and the closest relative, D. geniculatum, was 9.4% in operons with tRNA (Table 2) and 2.9% in operons with no tRNA (Table 3). The ITS dissimilarity values of >7% (in operons with both tRNA genes) are considered good evidence of lineage separation between the three novel species and the closely related taxa, therefore worthy of taxonomic recognition. The dissimilarity within-species for the different strains of the three novel species of Desmonostoc was between 0.0 and 2.1% for both operons (Tables 2 and 3).

### Table 2. Percent dissimilarity for the 16S-23S ITS region for Desmonostoc strains for which the ITS sequence contains both tRNA genes. The level of sequence divergence for intraspecies is shaded gray.

<table>
<thead>
<tr>
<th>Strains</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
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<th>10</th>
<th>11</th>
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</thead>
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### Table 3. Percent dissimilarity for the 16S-23S ITS region for Desmonostoc strains for which the ITS sequence contains no tRNA genes. The level of sequence divergence for intraspecies is shaded gray.

<table>
<thead>
<tr>
<th>Strains</th>
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<tr>
<td>7 D. salinum CCM-UFV059</td>
<td>12.9</td>
<td>11.1</td>
<td>15.5</td>
<td>8.8</td>
<td>13.3</td>
<td>11.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8 D. geniculatum HA4340-LM1 clone 37A/37C</td>
<td>14.0</td>
<td>13.4</td>
<td>2.9</td>
<td>11.5</td>
<td>13.8</td>
<td>14.4</td>
<td>7.0</td>
<td>0.5</td>
</tr>
<tr>
<td>9 Desmonostoc sp. 111_CR4_BG11B</td>
<td>13.1</td>
<td>11.3</td>
<td>15.5</td>
<td>8.8</td>
<td>13.8</td>
<td>13.1</td>
<td>0.4</td>
<td>6.9</td>
</tr>
</tbody>
</table>

The secondary structures of the conserved domains (e.g., D1-D1', Box-B, V2, and V3) of 16S–23S ITS regions were drawn and compared against the structures of known strains of Desmonostoc and Dendronalium. For D. lechangense and D. aggregatum, almost identical secondary ITS helices (with substitution of only 1–2 bases in either D1-D1' or V3 helices
Diversity geniculatum was closely similar to which the operon contained both tRNA genes, differed markedly in length (54 nt) but were characterized by eight-base symmetrical internal loops, a 3 bp stem region, and D. debaoense were different between the two operon types (Figure 7). For with the cultural and morphological features (Figure S1) observed with the strains from this study when compared with the previously reported strains. For instance, the strains of D. lechangense from this study were all characterized by less entangled filaments in younger cultures, subspherical to barrel-shaped vegetative cells, and chains (short and long) of less granulated cells similar to D. lechangense CF01. The heterocytes were also observed to be rare throughout the life cycle of the present strains. Interestingly, D. lechangense CSM 014-B was observed to have the unique feature of having filaments with narrow, elongated apical cells (Figure S1b–e), which were not reported in the reference strain CF01. This adds to the features reported for D. lechangense, thereby expanding our understanding of the morphological variation within this species. Other strains (e.g., CSM 019-B, 021-B, 020-B, and 022-B), which were strongly supported by genetic data to be the same species as D. aggregatum, interestingly showed the same cultural and morphologic appearance (Figure S1g–j) as D. aggregatum CF06 except for the strains reported herein, which appeared to have quite smaller and more spherical vegetative cells (Figure S1h–j and Table 1). However, as in strain CF06, our new strains still failed to produce dormant akinetes even after prolonged incubation and observation.

The secondary structures of the conserved domains of the ITS regions differed largely among novel strains of Desmonostoc and other known species and/or strains of Desmonostoc. D. lechangense and the unnamed strain CSM 013-B showed different D1-D1′ helices between the two operon types (Figure 6). The D1-D1′ helix of all strains of Desmonostoc were very similar in length (65–69 nt) with consistent basal regions of six base pairs (5′-GACCUA-UAGGUC-3′), except for D. vinosum with only a 5 bp helix in the basal region. The D1-D1′ region of D. debaoense and D. meilinense was structurally closest to D. aggregatum, D. danxiaense, and D. lechangense, being 65 nt long and differing in sequence by 1–5 nucleotides (Figure 6). The D1-D1′ helices of D. xianhuense and its close relatives, D. geniculatum and Desmonostoc strains 7N and 81-NMI_ANAB, were identical in length and structure (Figure 6). Other ITS regions also differed in lengths but were quite similar between species (Table S6). For example, the D2 region with a spacer at the beginning ranged from 31 to 36 nt for the two kinds of operons, while the D3 region with a spacer at the beginning and end ranged from 14 to 16 nt for operons with both tRNA genes (Table S6).

The basal structure of all Box-B helices shown consists of 5 bp and is identical in sequence for this region among all species. The Box-B helices of the three novel species were different between the two operon types (Figure 7). For D. debaoense, the Box-B helices were characterized by eight-base symmetrical internal loops, a 3 bp stem region, and five-residue terminal loops (AUUUU in the operon with both tRNA and UUUUA in the operon with no tRNA) (Figure 7). The Box-B helix of D. meilinense contained a 4 nt insert in the operon with no tRNA genes (Figure 7). The Box-B helices of D. xianhuense, of which the operon contained both tRNA genes, differed markedly in length (54 nt) but was closely similar to D. geniculatum clone37D and strains 7N and 81-NMI_ANAB (50 nt long) (with both tRNA genes) (Table S6 and Figure S5). D. xianhuense was characterized by an identical 5 bp basal region, a six-base symmetrical internal loop, followed by a six-bp stem region, a five-base asymmetrical internal loop, an eight-long bp stem, and a five-residue terminal loop in comparison to D. geniculatum, which had a seven-bp stem region below the three-residue terminal loop. Both species have a shorter Box-B region, 28 nt long (Figures 7 and S5), in their operons with no tRNA genes. D. vinosum and the unnamed Desmonostoc strain from this study (CSM 013-B) showed an additional 3–11 bases (32–40 nt) in their Box-B regions in comparison to most Desmonostoc strains that were only 29 nt long (Figure 7). The V2 helices were only drawn for strains for which ITS operons contain both the tRNA genes (Figure 8), and thus the V2 helices for D. salinum and strain CR4_BG11B were missing. As seen in Figure 8, the V2 helices were highly variable in length and structure between taxa; even the sequences in the basal region were different. The length of V2 helices for strains in Desmonostoc was common, between 70 and 81 nt long, except for D. caucasicum, which was only 24 nt long.
Figure 6. Predicted secondary structures of conserved D1-D1′ helices of the 16S–23S ITS region among Desmonostoc strains investigated in this study (in bold font) were compared to 15 strains in Desmonostoc (Figure S4). The novel species are marked with an asterisk beside the name.
On the contrary, the V3 helices showed some consistency in the sequence of the basal region between taxa but markedly varied in their overall structures (Figure 9). The V3 helix of *D. debaoense*, which is 31 nt long (identical in both operons), is also quite unique from those *Desmonostoc* strains compared here. *D. meillinense* shared the same V3 helix with *D. danxiaense* and *D. lechangense* in the operon with tRNA and no tRNA genes, respectively (Figure 9). *D. xianhuense*, along with *D. geniculatum*, *D. caucasicum*, strains 7N and 81-NMI_ANAB, markedly differed in length (76–91 nt) in comparison to other *Desmonostoc* strains with a shorter V3 region of about 31–42 nt long (Figures 9 and S6). The terminal loop in the V3 helix, in operons with tRNA genes, of *D. xianhuense* had an 8-base residue (AUUAAAAC; Figure 9), in contrast to the 4-base residue (UA(G/A)A) in *D. geniculatum* and strains 7N and 8-NMI_ANAB (Figure S6). These differences can therefore serve as additional distinguishing features between *D. xianhuense* and its closest relatives in *Desmonostoc*. *D. alborizicum* and the strain CCIBT3489, so far, have the shortest V3 region in this lineage, which is only 18 nt long (Figure S6). Only the D1-D1’ helix was drawn for *D. magnisporum* and the undescribed strain SK7A_PS (Figures 6 and S4), while other ITS domains such as Box-B, V2, and V3 helices were missing due to an incomplete ITS sequence available for these taxa.
For strains that were phylogenetically assigned to Dendronalium, the ITS operon contained both tRNA genes and a length that ranged from 534 to 540 nt (Table S7). The ITS sequence of the only known species, Dendronalium phyllosphericum strain CENA369, also contained both tRNA genes. The percent dissimilarity of the five strains in this study is either 4.3% or 10.6% in comparison to the ITS sequence of the reference strain CENA369 (Table S5). The lengths and structures of the ITS-conserved regions were also predicted among these strains (Table S7). The D1-D1′ helix of the five strains we investigated is composed of 64 nt in comparison to CENA369, which was presented with a D1-D1′ helix of 65 nt and a 6 bp helix basal region (5′-GACCUA-UAGGUC-3′) (Figure 10). The D1-D1′ helix of our strains is unique in having a 5 bp basal region and a longer terminal loop consisting of 17-base residue (Figure 10). Furthermore, the Box-B helix of our strains, when compared again to the reference strain CENA369, was 11 nt longer (Figure 10). One (CSM 004-B) of the five studied strains shared an identical V2 helix to CENA369, while all strains herein and CENA369 displayed the same V3 helix. Finally, our strains showed greater morphological resemblance to the genus Dendronalium than to other related Nostocacean genera (Table 4 and Figure S1k–o).

**Figure 9.** Predicted secondary structures of conserved V3 helices of the 16S–23S ITS region among Desmonostoc investigated in this study (in bold font) were compared to 15 strains in Desmonostoc (Figure S6). The novel species are marked with an asterisk beside the name. Arrowhead marks the place of variable base, while an arrow marks the location of insertion of base in homologous operons of D. xianhuense.

**Figure 10.** Predicted secondary structures of conserved D1-D1′, Box-B, V2 and V3 helices of 16S–23S ITS region containing both tRNA genes in five Dendronalium strains investigated in this study and the closest relative Dendronalium phyllosphericum CENA369.
**4. Discussion**

Genetic diversity of cyanobacteria in association with cycads has been conducted mainly in the genera *Encephalartos* [34], *Macrozamia* [46,47], *Ceratozamia*, *Cycas*, and *Zamia* [48–50]. In this study, we isolated 20 cyanobionts from the green zone of the coralloid roots of *Cycas fairylakea*, *C. debaoensis*, *C. elongata*, *Ceratozamia robusta*, and *Macrozamia moorei*. They were morphologically characterized, and their phylogenetic relationships were inferred. Seven strains were distinct from existing strains of Nostocaceae and were proposed as three different novel species following extensive polyphasic analyses.

The taxonomic advancements in the polyphasic approach and technological advancements in molecular biology have enabled researchers to identify and characterize new species of cyanobacteria. This has also paved the way to narrowly refine cyanobacterial genera with polyphyletic origins, including several members of the Nostocaceae family [9,12]. Komárek [14,16] has recommended that to define a monophyletic genus, strains with similar morphology but in a phylogenetically distinct clade should be described and classified into different genera. This has resulted in a great improvement in the status of *Nostoc* in the last decade following the discovery of multiple monophyletic genera [9,11,12,27,51,52]. A monophyletic branch and a cut-off value of 95% in 16S rRNA gene sequence similarity were set as the criteria for species to be treated within the same genus [15]. Initially, a threshold value of <98.8% sequence similarity in the 16S rRNA gene was established to separate cyanobacterial taxa at the species level [53], but a more stringent threshold of <99% for strains in Nostocales was later suggested [54]. Moreover, genetic distance based on the 16S–23S ITS region and the folding of the secondary structures of the conserved domains of the ITS region have provided evidence of species separation in many members of Nostocales [27,28,55–57] and have further contributed to the overall resolution of cyanobacterial taxonomy. All the strains examined in our study had 16S rRNA sequence similarities of 96–99% and monophyletic branches in *Desmonostoc* and *Dendronalium*, thereby confirming their inclusion into these groups. Indeed, *Desmonostoc* has emerged as a diverse genus within the family Nostocaceae [18]. Over the past decade, there has been significant progress in describing new species and assigning numerous strains to *Desmonostoc*, with currently 12 species validly described and more than 50 unnamed strains (see Figure S2 for the uncollapsed version of the phylogenetic tree and records in the GenBank database). This highlights the growing recognition and understanding of the genetic diversity within this genus. Among these, two species were known as symbionts, namely, *D. lechangense* and *D. aggregatum* [10], and were isolated from the coralloid roots of *Cycas fairylakea*. This paper reports 15 strains with *Nostoc*-like morphologies that were organized into six distinctive clusters within *Desmonostoc sensu stricto*. Five of those clades were in the D1 subcluster and one in the D2 subcluster, as previously described by Hrouzek et al. [9]. There was also a slightly visible genetic separation between the symbiotic and free-living strains, as exemplified in our study, showing that most of our strains

### Table 4. Morphological characteristics of *Dendronalium* strains under investigation and the only known species, *Dendronalium phyllosphericum* CENA369.

<table>
<thead>
<tr>
<th>Species</th>
<th>Vegetative Cells Shape and Size (µm)</th>
<th>Heterocytes Shape and Size (µm)</th>
<th>Akinetes Shape and Size (µm)</th>
<th>Source—Locality</th>
<th>Phylogenetic Position/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Dendronalium</em> sp. CSM 003-B</td>
<td>Barrel-shaped to spherical to compressed subepithelial, or quadratic W: 2.7–5.6; L: 2.3–5.2</td>
<td>Spherical to subepithelial W: 4.1–5.6; L: 3.8–6.7</td>
<td>Spherical to subepithelial to oval W: 3.5–7.3; L: 5.2–9</td>
<td>Coraloid root of <em>C. fairylakea</em> from Xianhu/Fairy Lake botanical garden</td>
<td>Cluster G (G-IV); this study</td>
</tr>
<tr>
<td><em>Dendronalium</em> sp. CSM 024-R</td>
<td>Spherical to subepithelial to cylindrical or oval W: 2.1–4.8; L: 2.9–6.7</td>
<td>Cylindrical, rounded to subspherical W: 4.1–5.6; L: 3.8–6.7</td>
<td>Spherical to subepithelial to oval W: 3.5–7.3; L: 5.2–9</td>
<td>Coraloid root of <em>C. debaoensis</em> from the reintroduction site in Debao County</td>
<td>Cluster G (G-IV); this study</td>
</tr>
<tr>
<td><em>Dendronalium phyllosphericum</em> CENA369</td>
<td>Conical-rounded to subspherical W: 1.6–3.1; L: 1.2–5</td>
<td>Cylindrical, rounded to elliptical W: 2.9–3.9; L: 3.5–5</td>
<td></td>
<td>Leaves of <em>Euterpe edulis</em> from Atlantic Forest in Brazil</td>
<td>Cluster G (G-4); [27]</td>
</tr>
</tbody>
</table>
were phylogenetically organized in clades distinct from previously reported free-living strains (Figure 5).

The cultured materials of Desmonostoc species were also diverse in appearance and texture, ranging from amorphous and soft-gelatinous mats to densely clumped colonies [10,19], as were also observed from different Desmonostoc strains reported in this study. This variability suggests that Desmonostoc species produce different growth patterns, which can be attributed to various factors, including conditions of the environment, available nutrients, and variations in genes among different strains or species. The proposal of the three novel species was strongly supported by the morphological examination and molecular analyses of the 16S rRNA gene phylogeny, percent dissimilarity values, as well as secondary structures of the 16S–23S ITS regions. Taxonomic studies by 16S rRNA phylogeny and molecular distance in the 16S–23S ITS regions have resolved several cryptic species in Nostoc and other phylogenetically related taxa [13,56–60]. These previous studies also reported that differences of more than 7% on the 16S-23S rRNA ITS sequence can be used to delineate species of cyanobacteria, while differences between strains of the same species should not be more than 3%. Similar taxonomic principles were used in the analyses of all strains investigated in this work. Our novel species Desmonostoc debaoense, D. meilinense, and the unnamed strain CSM 013-B formed distinct clades sister to D. aggregatum and close to a branch containing a mixture of free-living (recovered from a pond, soil, or lake) and symbiotic strains (i.e., of the hosts Cycas and Gunnera) [8,9,20,31,61].

Based on the molecular analysis, we conclude that the unnamed strain CSM 013-B is different from D. aggregatum and D. debaoense, but the absence of a morphologic analysis of CSM 013-B has prohibited a separate taxonomic status for it. Interestingly, D. debaoense is different from D. aggregatum in that it has 97.9% 16S rRNA gene sequence similarity, ITS dissimilarity values of >8% (in both operons), and different ITS secondary structures. Morphologically, D. debaoense in culture formed a more tightly clumped colony and was microscopically observed with diffuse trichomes, spherical cells, compressed subspherical heterocytes, and the presence of akinetes that differ significantly from the features of D. aggregatum, which is characterized by strongly compartmentalized cells in older colonies, aggregated cells, dividing in two planes, small heterocytes (average of 5 μm long, 4.75 μm wide), and the absence of akinetes [10]. The morphology of D. meilinense, on the other hand, resembled that of D. lechangense and D. daxiaense in filaments, except that D. meilinense showed bright blue-green vegetative cells, lighter than the two mentioned species. In addition, D. meilinense showed subspherical and slightly larger heterocytes (Table 1) than D. lechangense and D. daxiaense, which exhibited slightly smaller (Table 1) and oval heterocytes, respectively. The apical cells of D. meilinense were subspherical to conical-round in shape, contrary to the diverse and distinct morphology of the apical cells of D. lechangense (Figure S1), which ranged from subspherical to narrowly elongated.

Moreover, additional novel strains (CSM 008-B, 009-B, 010-B, and 011-B) named Desmonotoc xianhuense from this work clustered in a clade containing different species, namely, D. muscorum, D. geniculatum, and the strain 81 NML_ANAB/7N clone NC3C, and showed a high 16S rRNA sequence similarity with these strains. Their placement in the 16S rRNA phylogeny with high support values may suggest close genetic relatedness. However, it is noteworthy that the slight variations in morphology and additional information from other genetic markers, that is, ITS regions, have provided strong evidence to support their distinctness as separate species. In addition, the lack of the 16S–23S ITS sequence for validly described D. muscorum strains II/NIVACYA817/818 prevents a comprehensive comparison with our novel strains. Therefore, the ITS secondary structure of our strains was closely similar to that of D. geniculatum and strains 7N/81-NML_ANAB, but the dissimilarity value of 9.4% in the ITS sequence data, which contained both tRNA and the different morphology of D. geniculatum (e.g., coccoid clusters; Figs. 48–52 in [31]), clearly indicated that the symbiotic D. xianhuense was distinct from the free-living D. geniculatum isolated from caves in Kauai [31]. Based on morphological examinations, D. xianhuense showed mostly barrel-shaped to subspherical cells to cylindrical cells (at the old stage) occasionally arranged in parallel or densely entangled trichomes (Figure 4), a clearly recognizable long chain (typically with more than 10 cells), and strongly granulated akinetes (Figure 4), closely similar to those observed in D. muscorum II/NIVACYA817/818, isolated
from soil [9]. Interestingly, D. xianhuense showed frequent occurrence and diverse appearances of heterocytes and larger vegetative and differentiated cells than strains D. muscorum II/NIVACYA817/818 (Table 1). It is apparent, however, that comparison of the ITS sequence with strains of D. muscorum has been problematic, as many sequences in the public database (e.g., NCBI) were under incorrect names. For example, several strains (ACSS1/ACSSL149, TAUMAC, DRSCY01, and Ind33) under the name D. muscorum with available 16S rRNA sequences were not in fact within the tight group clade of D. muscorum (Figure 5) or even in the Desmonostoc lineage, for example, D. muscorum CENA18 (GenBank Accession number: AY218827; [62]), SN455 (KR709143, unpublished), CENA61 (AY218828; [62]), and SAG (KM019938, unpublished, NCBI), to cite a few. We believe that the correct sequence of the 16S–23S ITS region from a correctly identified D. muscorum is lacking since the ITS sequences available in the database were not from strains of D. muscorum sensu stricto. In addition, when Hrouzek et al. [9] described the genus, they did not provide ITS sequence data for the type species of D. muscorum, and this species is polyphyletic as currently understood. For example, the strain CENA18, isolated from the Amazon floodplain [62] with the 16S–23S ITS sequence originally designated as D. muscorum and used as a comparative strain in several studies reporting new species (e.g., in Compactonostoc [24]; Desmonostoc [10]), was found in a cluster of the recently described novel species, Amazonocrinis nigriterrae, based on 16S rRNA phylogeny [27]. The same can be said for D. muscorum CENA61 (AY218828) [62], which joined the monophyletic Pseudohalinostoc cluster in the 16S rRNA phylogeny (Figure S2). The 16S rRNA gene from the whole genome sequence of the strain LEGE 12446, under the name Desmonostec muscorum (isolated from Cycas revoluta from a botanical garden in Portugal), was extracted, and a distinct node in the D2 subclade of Desmonostoc was formed, which was slightly distant from the cluster of Desmonostoc muscorum sensu stricto. This highlights the fact that despite revisions, there are still many sequences in GenBank with incorrect names, and therefore, they must be carefully evaluated prior to use. Taxonomic revisions are still very much needed. Our study further reinforces the need for reexamination within Desmonostoc, particularly of the several strains labeled as D. muscorum, as several mislabeled strains of Desmonostoc are hereby noted. In summary, robust criteria from polyphasic analyses have resulted in the description of these three new species in Desmonostoc.

Similar to what has been observed in the Nostoc-like genus Amazonocrinis [63], our study also revealed genetic diversity within the genus Dendronalium, highlighting the potential genetic divergence and subgroups within this genus. Accordingly, the phylogenetic analyses based on the 16S rRNA gene sequences formed four distinct subclades (G-I to G-IV) within the genus Dendronalium. The strains we studied clustered with the type species D. phyllosphericum CENA369. In this clade, the strain CENA73 (MW327027) collected in 2004 from anthropogenic dark earth (anthropogenic soil) exhibits an early divergence compared to the closest relatives. Despite the striking morphological resemblance of our investigated strains to D. phyllosphericum and the high sequence similarity observed in their 16S rRNA gene sequences, it is important to highlight the dissimilarity values obtained from the comparisons of their complete ITS sequences. The dissimilarity values of our five strains are between 4.3% and 10.6% when compared to the reference strain CENA369, overlapping with the non-definitive threshold of 7% typically used to reconsider species classification [51,59,60]. Additionally, among the ITS secondary structures, only the V3 helix of the CENA369 [27] matches with our strains, further suggesting potential differences at the species level. However, we are unable to confidently propose them as new species within the genus Dendronalium at this time. Further investigations, that is, the availability of whole genome sequences from the representatives of the investigated strains and the comparison to CENA369, would greatly aid in the thorough assessment of their taxonomic status and determination if they represent new species within the genus Dendronalium. However, we highlight that the current strains assigned to D. phyllosphericum may require subdivision or re-evaluation as new research and taxonomic revisions unfold.

Interestingly, most of the characterized strains in the genus Dendronalium have been isolated from the phyllosphere [27], except for CENA73. For instance, the reference strain CENA369 was recovered from Euterpe edulis, while other strains, namely, CENA358
and CENA388/389, were recovered from *Garcinia macrophylla* and *Guapira opposita*, respectively [27]. The consistent observation of these strains in close proximity to plants, along with the five strains investigated in this study and isolated from the coralloid roots of two *Cycas* species (*C. debaoensis* and *C. fairylakea*), strongly suggests a clear association between *Dendronalium* and host plants. This further suggests the potential symbiotic nature of this cyanobacterial genus and offers opportunities for the study of plant–cyanobacterial interaction.

As the most primitive living seed plant group and a living fossil, cycads exhibited and maintained specialized relationships with microbes, especially with the rich cyanobacterial community that now exists in the endosphere of the coralloid roots, to withstand poor-nutrient soils [4,5]. The recovery of different cyanobacterial strains isolated from cycads collected from various habitats, as supported by evidence from old studies [8,9,49] and recent studies with several described species new to science [10], is a noteworthy discovery, suggesting that a diverse community of cyanobacteria may colonize the internal layers of the roots. This may also provide insights to better understand the context of coevolution between cycads and cyanobacteria that were engaged in symbiosis for millions of years. This study highlighted the importance of the isolation of cyanobacteria outside of their natural hosts, which can provide specimens that are useful for taxonomic and genomic studies. This study also presented the discovery of different strains from unexplored habitat sites, with the description of new taxa, for example, *D. meilinense* from *C. fairylakea*, which was collected from its natural habitat under a canopy forest, and *D. debaoense* from *C. debaoensis* living in a karst limestone forest. Furthermore, several members of the new genus *Dendronalium*, which were mostly isolated from natural habitat, lead to interesting patterns of many potentially novel or unreported strains of cyanobionts that await discovery. Therefore, the isolation and description of new strains based on morphology and genetic data must be regularly conducted. Additional research on heterocyte-forming symbiotic cyanobacteria associated with cycadean plants, specifically from unexplored habitats, is still needed.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d15111132/s1, Figure S1: Macroscopic and microscopic characteristics of other strains of cyanobacteria investigated in this study; Figure S2: Uncollapsed maximum likelihood (ML) tree based on 16S rRNA gene sequences showing the phylogenetic positions of cyanobacterial strains under investigation and closely related taxa; Figure S3: Neighbor-joining (NJ) tree based on 16S rRNA gene sequences showing the phylogenetic positions of the twenty cyanobacterial strains recovered from this study and other cyanobacterial taxa; Figure S4: Predicted secondary structures of conserved D1-D1′ helices of the 16S–23S ITS region among *Desmonostoc* strains; Figure S5: Predicted secondary structures of conserved Box-B helices of the 16S–23S ITS region among *Desmonostoc* strains; and Figure S6: Predicted secondary structures of conserved V3 helices of the 16S–23S ITS region among *Desmonostoc* strains. Table S1: List of the 20 cyanobacterial strains examined in this study; Table S2: Comparison of the 16S rRNA gene sequence similarity between *Desmonostoc* strains under investigation and closely related taxa; Table S3: Comparison of the 16S rRNA gene sequence similarity between *Desmonostoc* strains under investigation and species of other genera in Nostocaceae; Table S4: Comparison of the 16S rRNA gene sequence similarity between *Dendronalium* strains under investigation and closely related taxa; Table S5: Percent dissimilarity for the 16S–23S ITS region for *Dendronalium* strains; Table S6: Analyses of the 16S–23S ITS region among *Desmonostoc* strains for which ITS sequences are complete; and Table S7: Analyses of the 16S–23S ITS region among *Dendronalium* strains.

**Author Contributions:** Conceptualization, M.H.P., T.C., H.C. and N.L.; methodology (sample collections), M.H.P., N.L. and T.C., (isolation of cyanobacteria and morphological and molecular evaluations), software, formal analysis, investigation, data curation, M.H.P.; validation, T.E.E.d.C. and M.P.G.; writing—original draft preparation, M.H.P.; writing—review and editing, T.E.E.d.C., T.C., M.P.G. and M.H.P.; project administration, M.H.P. and T.C.; supervision, Z.H., H.C. and N.L.; funding acquisition, N.L., H.C. and Z.H. All authors have read and agreed to the published version of the manuscript.
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**Data Availability Statement:** All nucleotide sequences used in this study are available from NCBI, with their accession numbers provided in supplementary files.

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