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Genomic Insights into the Taxonomy and Metabolism of the Cyanobacterium *Pannus brasiliensis* CCIBt3594

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Abstract: The freshwater *Pannus* genus comprises cyanobacterial unicellular species with a particular morphology, forming free-floating rounded colonies with thin, homogenous, and colorless colonial mucilage. There is little literature on the taxonomy of the *Pannus* and none on its metabolism. This study presents the first genomic characterization of a *Pannus* strain isolated from Pantanal Biome, Brazil. The genome was assembled into 117 contigs with a total size of 5.1 Mb and 99.12% completeness. It contained 4988 protein-encoding genes, including some involved in secondary metabolite biosynthesis, such as cyanobactin and terpenes. Interestingly, *P. brasiliensis* CCIBt3594 has a complete set of nitrogen fixation genes and is a non-heterocytou unicellular cyanobacterium. Finally, the phylogenomic analyses revealed the lack of information on closely related strains and anchored the genus *Pannus* within the order Chroococcales, Microcystaceae family, closest to *Microcystis* spp. representatives. This work presents novel evidence concerning a sparsely characterized genus of the Cyanobacteria phylum and contributes to elucidating taxonomic and systematic issues within the group of unicellular cyanobacteria.

Keywords: diversity; genome assembly; genome mining; phylogenomics; taxonomy

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1. Introduction

Cyanobacteria is a group of ancient oxygenic photoautotrophic bacteria, considered the predecessors of plant chloroplasts and responsible for the shift to an oxygen-rich atmosphere about 2 billion years ago [1,2]. These organisms' distribution encompasses aquatic and terrestrial ecosystems [3–5] due to their diverse morphological and metabolic diversity, including extreme habitats like cold polar regions [6] and hot thermal springs [7]. Cyanobacteria can also establish symbiotic relationships with plants [8–10], diatoms [11], fungi [12], and marine animals [13,14]. They influence life on earth as important primary producers, capable of fixing atmospheric carbon and, sometimes, nitrogen [15,16]. Known for producing toxins in freshwater, cyanobacteria constitute a fascinating group for bioprospection as producers of various secondary metabolites with commercial applications [17–19].

Historically, cyanobacterial systematics and classification relied on morphological characteristics. The genus *Pannus* was first described using morphological features by Hickel [20] in natural material collected from plankton in a brackish cove in the Baltic Sea, in Northern Germany. However, recognizing the constraints of morphological classification, a polyphasic approach now integrates ecological, molecular, and ultrastructural characteristics to enhance the comprehension of their phylogenetic relationships [21]. Twenty-one years after the description of the *Pannus* genus, a new species, *Pannus brasiliensis* CCIBt3594,

was isolated and characterized from a macroscopic greenish biofilm collected in a puddle in Mato Grosso do Sul State, Brazil [22]. Currently, the genus *Pannus* adheres to the original concept and includes only three species: *P. spumosus* (the type species), *P. punctiferus*, and the newly discovered *P. brasiliensis*. A phylogenetic analysis placed the *Pannus* spp. in a clade closely related to *Microcystis* spp. representatives. As far as we know, the CCIBt3594 strain is the only *Pannus* representative isolated and maintained in culture collections nowadays, and a recent floristic survey has found representatives of the genus in Southern Brazil [23].

The *Pannus brasiliensis* CCCIBt3594 strain discovered thriving within a biofilm composed of Oscillatoriacean filaments and green algae in a water pool in the Brazilian Pantanal distinguishes itself from the type species (*P. spumosus*). Notable differences include a larger cell size (up to three times), the absence of individual mucilage, and a distinct habitat. Malone et al. [22] conducted a detailed characterization of the morphology of the type strain of *P. brasiliensis* CCIBT3594 from the molecular and ultrastructural point of view. The cellular arrangement of strain CCIBt3594 is a single-layered structure at the mucilage surface. The developmental progression of the species commences with rounded clathrate colonies characterized by densely arranged cells. Then, colony expansion begins through a protrusion containing multiple cells, in line with the observations made by Hickel [20]. Mature colonies attain a considerable size, display an irregular morphology, and frequently adopt open and undulating configurations. In aging colonies, cells assume a loosely organized arrangement, forming lines or ribbons. Remarkably, gas vesicles have never been observed in the cells of the strain CCIBt3594. Cell division consistently occurs in two planes. An ultrastructural analysis of *P. brasiliensis* CCIBt3594 revealed the presence of fasciculated thylakoids initiating along the cell wall and extending towards the center of the protoplast, occasionally forming a loop.

Intra-specific variations are observed in cyanobacteria due to their morphological and genomic plasticity, contributing to their adaptability. This inherent flexibility, however, poses a challenge in the taxonomic identification of the clade [24]. Nevertheless, studies involving genomic sequences have assisted researchers in this process [25–27]. There are only 43 cyanobacterial genomes, out of more than 120 species and 3000 strains belonging to the order Chroococcales, available in the National Center for Biotechnology Information (NCBI) databases. Despite observing a disparity between the described species and the quantity of genomes, whole-genome sequencing and annotation can significantly enhance species identification. This approach elucidates the functional profiles of taxonomic groups, offering a better resolution to distinguish between closely related species when compared to single-gene approaches. Additionally, it facilitates the discovery of unique and novel features among different groups of cyanobacteria [25,28,29].

Given the significant impact of Cyanobacteria on ecosystems and the taxonomic complexities within this phylum, this study aimed to provide genomic insights on the *P. brasiliensis* CCIBt3594 in order to enhance our comprehension of cyanobacterial diversity and their ecological importance.

2. Materials and Methods

2.1. Culture and DNA Extraction

The freshwater unicellular cyanobacterium *P. brasiliensis* CCIBt3594 was isolated and described by Malone et al. [22]. The strain is maintained in the Molecular and Cellular Biology Laboratory collection (CENA/USP), Piracicaba, São Paulo State, Brazil. The strain was grown into 125 mL Erlenmeyer flasks containing 50 mL of BG-11 and kept under a 14:10 h light:dark (L:D) light cycle and under constant light (3–15 $\mu mol \cdot m^{-2} \cdot s^{-1}$) at 20 to 25 °C for 30 days. The culture was concentrated by centrifugation at 5000× *g* for 10 min. The concentrated biomass was used for total DNA extraction using the AxyPrep Bacterial Genomic DNA Miniprep Kit (Axygen Biosciences, Union City, CA, USA) according to the manufacturer's requirements. The quality of the DNA was confirmed using 1% (m/w)

of agarose gel. DNA quantification was performed with a Qubit 2.0 Fluorometer, using a Qubit dsDNA BR Assay Kit (Life Technologies, Carlsbad, CA, USA).

2.2. Genome Sequencing and Assembly

An amount of 1 µg of DNA was used to prepare paired-ends libraries with the Nextera XT Sample Prep Kit (Illumina, San Diego, CA, USA), which was sequenced in a platform MiSeq (Illumina) using the MiSeq Reagent Kit v3 600 cycle (Illumina) following the manufacturer's instructions.

The quality of the reads was verified with FastQC 0.11.8 (www.bioinformatics.babraham.ac.uk/projects/fastqc/). Sequences with qualities lower than Phred 20, lengths shorter than 50 bp, and adapters were removed with Trimmomatic v.0.38 [30]. De novo genome assembly was performed with SPAdes v.3.15.1 [31] with meta parameters according to Alvarenga et al. [32]. Sequences presenting similarity to cyanobacterial references were identified among the assemblies with Kraken2 v.2.1.2 [33], and contamination sequences were removed with KrakenTools v.1.2 [34]. Assembly statistics were obtained with QUAST v.5.0.2 [35] and CheckM v1.0.18 [36]. The assembled sequence obtained in the study was deposited in NCBI under the following BioProject ID: PRJNA1068625.

2.3. Functional Annotation

Prokka 1.13 [37] and the NCBI Prokaryotic Genome Annotation Pipeline [38] were used for automatic annotation. Functional analyses and metabolic pathways were identified with the BlastKOALA [39] web interface querying against the genus of prokaryotes database. The genome was analyzed with PHASTER to identify the presence of prophages regions [40]. Also, the antiSMASH 7.0.1 [41] software was used for predicting biosynthetic gene clusters (BGCs) of secondary metabolism. Geneious Prime 2022.2.2 (https://www.geneious.com) was used for manual curation of the putative genes.

2.4. Potential for Nitrogen Fixation

A comparative analysis was made upon the identification of genes associated with biological nitrogen fixation through automatic annotation. Primarily, nucleotide sequences of the *nif*H gene were curated against in the database, and the sequences exhibiting robust identification were used for constructing the phylogenetic tree. The sequences were aligned with MUSCLE v.5 [42] in Geneious Prime 2022.2.2 (https://www.geneious.com). Additionally, the operon of *nif* sequences from genomes more closely related was compared with deposited sequences through the Clinker v.1 software using the default settings [43].

2.5. 16S rRNA Gene Phylogeny

The 16S rDNA gene extracted from the *P. brasiliensis* CCIBt3594 genome was compared with 16S rDNA gene sequences deposited in the databases to update the phylogenetic tree initially presented by Malone et al. [22]. A maximum-likelihood tree was constructed using 74 nucleotide sequences (>1100 bp) obtained from cyanobacterial strains of the orders Chroococcales and Synechococcales retrieved from NCBI (https://www.ncbi.nlm.nih.gov/genome/; accessed on 20 September 2023), which were aligned with MUSCLE v.5 [42] in Geneious Prime 2022.2.2 (https://www.geneious.com). Bayesian inferences were performed with MrBayes 3.2 [44] using two separate runs with four chains each and 5,000,000 Markhov Chain Monte Carlo generations. The tree was visualized with iTOL v.6.8.1 (http://itol.embl.de/; accessed on 22 September 2023) and edited with Inkscape v.1.3 (https://inkscape.org/).

2.6. Phylogenomic Analysis

A data set covering Chroococcales and Synechococcales reference genomes was created using publicly available genomes from NCBI (https://www.ncbi.nlm.nih.gov/genome/; accessed on 2 October 2023). A set of 45 genomes, supplemented with an outgroup cyanobacterial genome, was used for the phylogenomic analysis based on the alignment of

120 bacterial single-copy conserved marker proteins with GTDB-tk v.0.32 [45]. A maximum-likelihood tree was generated using IQ-TREE v.2 [46], with the LG + F + R5 model selected by ModelFinder v.1 [47] and 1000 bootstraps. The tree was visualized with iTOL v.6.8.1 (http://itol.embl.de/; accessed on 3 October 2023) [48] and edited with Inkscape v.1.3 (https://inkscape.org/).

Average nucleotide identity (ANI) by orthology was calculated between the genome of *Pannus brasiliensis* and the reference genomes of the *Microcystis* spp. using OAT v.1 software [49,50], and the average amino acids identity (AAI) was estimated by an AAI calculator (http://enve-omics.ce.gatech.edu/aai/; accessed on 23 January 2024). Digital DNA–DNA hybridization (DDH) values were calculated using the Genome-To-Genome Distance Calculator (GGDC 3.0) server [51,52]. Identification and visualization of orthologous clusters of the genomes were carried out using OrthoVenn3 v.3 [53].

3. Results and Discussion

3.1. Genome Assembly and Annotation

Genome sequencing confers numerous advantages to the taxonomic identification of species, particularly microorganisms that are difficult to describe by morphological traits or that exhibit polyphyletic clades. Using complete genomes creates opportunities for the development of innovative tools in phylogenetic reconstruction and evolutionary analyses [54]. The *P. brasiliensis* genome was sequenced for the first time, providing a glimpse of its complexity and allowing for its comparison with the closest-related species. The draft genome assembled of *P. brasiliensis* CCIBt3594 had a 5.1 Mb total size and 117 contigs, a completeness of 99.12% and, in the quality control, presented a low contamination (0.29%) and a genomic GC content of 52.04% (Table 1). Fragmented assemblies represent a large part of the currently deposited cyanobacterial genomes [55]. The G + C content of *Pannus brasiliensis* was higher than that observed for the most phylogenetically related genus, *Microcystis* (approximately 42%) [56–58]. The automatic annotation of genes using Prokka (1.13) predicted 4833 protein-encoding genes. Conversely, the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) presented a broad annotation, identifying 5040 total genes, including 4988 protein-encoding genes, 38 pseudogenes, and 51 RNA sequences comprising three rRNA, 44 tRNA, and four ncRNA.

Table 1. Features of *Pannus brasiliensis* CCIBt3594 genome assembly.

Attributes	Value
Genome size (bp)	5,152,646
G + C content (%)	52.04
Completeness (%)	99.12
Contamination (%)	0.29
Contigs	117
Largest contig (bp)	248,692
N50 length (bp)	83,624
Protein encoding gene	4988
Total tRNAs	44
16S rRNA copies	2

Functional annotation by the BlastKOALA tool on the KEGG Orthology (KO) database revealed energy metabolism (10.2%), genetic information processing (19.6%), carbohydrate metabolism (9.2%), signaling and cellular processes (8.7%), metabolism of cofactors and vitamins (7.7%), and environmental processing (7.6%) as the most represented categories (Figure S1). The existence of unclassified categories (14.4%) may indicate an uncharted and untapped potential within the metabolism and genetic processing of *P. brasiliensis*. Remarkably, the PHASTER web server identified a prophage region in the contig 17, with 25.1 Kb and G + C content of 51.83%; genes of Phage-like protein, integrase, and a tail shaft were annotated (Figure S2). Bacteriophages play a crucial role in the evolution and ecology

of the Cyanobacteria group, serving not only as a means of population control but also facilitating the transfer of genes between species [59]. The integrase gene is responsible for phage integration and excision of the host's chromosome [60,61]. The presence of this gene may suggest the potential ability of the prophage region identified in the genome to establish lysogenic relationships with *P. brasiliensis* CCIBt3594, with further analyses required to elucidate this relationship.

3.2. Identification of Genes Involved in Secondary Metabolites Products

AntiSMASH (7.0.1) predicted five secondary metabolite gene clusters, two clusters for terpene compounds corresponding to the genes of phytoene synthase (*PSY*) and terpene cyclase (*TPS*), in addition to BGCs encoding for T1PKS: Type 1 polyketide synthase, NRPS, RiPPs, and ribosomal cyclic peptides produced by cyanobacteria (cyanobactins) (Table 2).

Table 2. Identification of secondary metabolite biosynthesis gene clusters in *P. brasiliensis* CCIBT3594 genome sequence.

Contig	Location (nt)	Product Type	Most Similar Known Cluster	Similarity
8	80,421–126,963	T1PKS; NRPS	1-heptadecene	100%
22	5103–76,035	Terpene	Phytoene synthase	31%
51	1–19,543	Terpene	Terpene cyclase	11%
57	12,658–28,163	RRE-Containing	RiPP	12%
76	1–13,978	Cyanobactin	RiPP	13%

Cyanobacteria synthesize a diverse array of natural products, which include photoprotective mycosporine-like amino acids, complex cyanotoxins, alkaloids, polyketides, flavonoids, peptides, and more [62], exhibiting neurotoxic, antibacterial, anticancer, and immunosuppressive properties [63,64]. This metabolic versatility stems from remarkable morphological, biochemical, and physiological diversity. They are often produced in response to environmental stressors, providing cyanobacteria the means to thrive in diverse and extreme habitats [65]. Among the natural products synthesized by Cyanobacteria, peptides are the most representative class. These molecules are biosynthesized through the activities of nonribosomal peptide synthetases (NRPSs) and ribosomally synthesized and post-translationally modified peptides (RiPPs) [17,66].

The polyketide 1-Heptadecene is a prevalent alkane produced by cyanobacteria and is regarded as crucial for biofuel production [67–69]. Heptadecene is commonly studied for alkane production through intermediate pathway genes' overexpression in cyanobacteria, such as *Synechocystis* sp. PCC6803 [70]. Moreover, cyanobactins, prevalent peptides within this taxon, exhibit diverse natural functions, ranging from chelating metals to demonstrating cytotoxic activity [71].

For the pathway of terpene compounds, the gene squalene-hopene cyclase (*shc*) responsible for the biosynthesis of hopanoids was predicted. These compounds are associated with the previously predicted terpene cyclase BCG. Squalene synthesis by cyanobacteria has previously been reported in the *Thermosynechococcus* sp. in marine environments [72,73], in the *Synechocystis* sp. PCC6803 in freshwater [74], and in *Phormidium autumnale* in industrial wastewater [75]. The latter two have been studied to provide a new source for squalene production [76,77], as, currently, the primary source for the high commercial demand of this product is shark liver, endangering marine ecosystems [78]. These genes may be particularly important for *P. brasiliensis* because hopanoids also play a crucial role in bacterial stress resistance, enabling bacteria to adapt to adverse environmental conditions, including high temperatures, pH levels, and salinity gradients through the modulation of the cell membrane [79,80].

Phytoene synthase-related genes, *cruA*, *crtB*, *crtH*, *crtO*, *crtP*, *crtQ*, *crtR*, and *crtW* for the synthesis of pigments were predicted through the reconstruction of metabolic pathways using the BlastKoala tool, with the potential of producing beta-carotene, beta-cryptoxanthin,

echinenone, canthaxanthin, antheraxanthin, and zeaxanthin, as well as compounds from the myxol group. This variety of accessory pigments extends the range of absorbed wavelengths, allows for their survival under variable light environments [81], and contributes to their ecological success [82]. Additionally, as active oxy-photosynthetic organisms, cyanobacteria generate reactive oxygen species (ROS), damaging cellular components like DNA, proteins, and lipids [83]. Pigments act as antioxidants to mitigate oxidative stress's harmful effects by neutralizing ROS [84]. Thus, the broad pigment repertoire predicted in the *P. brasiliensis* CCIBt3594 genome not only helps to prevent the detrimental repercussions of ROS but also enhances light absorption efficiency. It may also reduce competition for light within the aquatic environment, given their absence of gas vesicles, benthic growth habits, and fluctuations in the water levels, resulting in varying wavelengths capable of penetrating water in the Pantanal region [22,85].

3.3. Genes Involved in Nitrogen Fixation

After the automatic prediction of some genes related to nitrogen fixation in the previous analyses, a genomic screening of the nitrogen fixation operon (*nif*) was performed on *P. brasiliensis* CCIBt3594. We report here, for the first time, this genus as a potential diazotrophic unicellular cyanobacterium. Some groups of cyanobacteria carry out nitrogen fixation. The best-known is the Nostocales order, which has specialized cells called heterocytes to isolate the N_2 fixation process from possible damage caused by the oxygen released during the photosynthesis process in vegetative cells [86,87]. Non-heterocytous cyanobacteria can perform atmospheric nitrogen fixation through spatial (microzones with low levels of oxygen) and temporal (in the night) mechanisms [87–90]. This mechanism is particularly significant for oceanic cyanobacteria, allowing them to conduct nitrogen fixation simultaneously with photosynthesis [91,92]. The first species of unicellular non-heterocytous cyanobacteria described as nitrogen-fixing was the *Gloeothece* sp. [93], followed by the *Synechococcus* sp. [94].

The 16 nif genes in cyanobacteria can be identified as forming distinct operons (*nif*BSU, *nif*ENXW, *nif*HDK, and *nif*VZT). These operons exhibit a high degree of conservation, with minimal translocations and insertions [95,96]. Furthermore, it is usual to find more than one operon in some strains [97]. In the *P. brasiliensis* CCIBt3594 genome, the genes *nifK*, *nifH*, *nifD*, *nifB*, *nifS*, and *nifJ* were identified, as well as *isc*U and *fdx* within the *nif* gene cluster, in the same order previously reported for *Anabaena variabilis* ATCC29413 [98] and other strains (Figure 1). The gene *fdx* plays a crucial role in heterocytous-forming cyanobacteria. It encodes a distinct [2Fe-2S]-type ferredoxin that is particularly adapted for electron donation to nitrogenase [99]. Remarkably, *isc*U translation showed high identity scores with the *nif*U protein from *Rippkaea orientalis* PCC8801 (77.7%) and the *Crocosphaera* sp. (78.4%). The other genes in the cluster showed a high similarity to sequences deposited in the database (Table S1). In addition, nitrogen-fixing related genes *isc*S, *hes*A, *hes*B, and *nif*W [100] and genes involved in environmental nitrogen absorption and metabolism were also identified (Table S1). The central enzyme in nitrogen fixation is nitrogenase, which has two parts: dinitrogenase, a FeMo protein encoded by the genes *nif*D (α-subunit) and *nif*K (β-subunit), which is organized in a α2β2 tetramer associated with two FeMo cofactors (FeMo-co) and two P-clusters; and dinitrogenase reductase, a homodimer with one [4Fe-4S] cluster (Fe Protein) encoded by *nif*H [95,101,102].

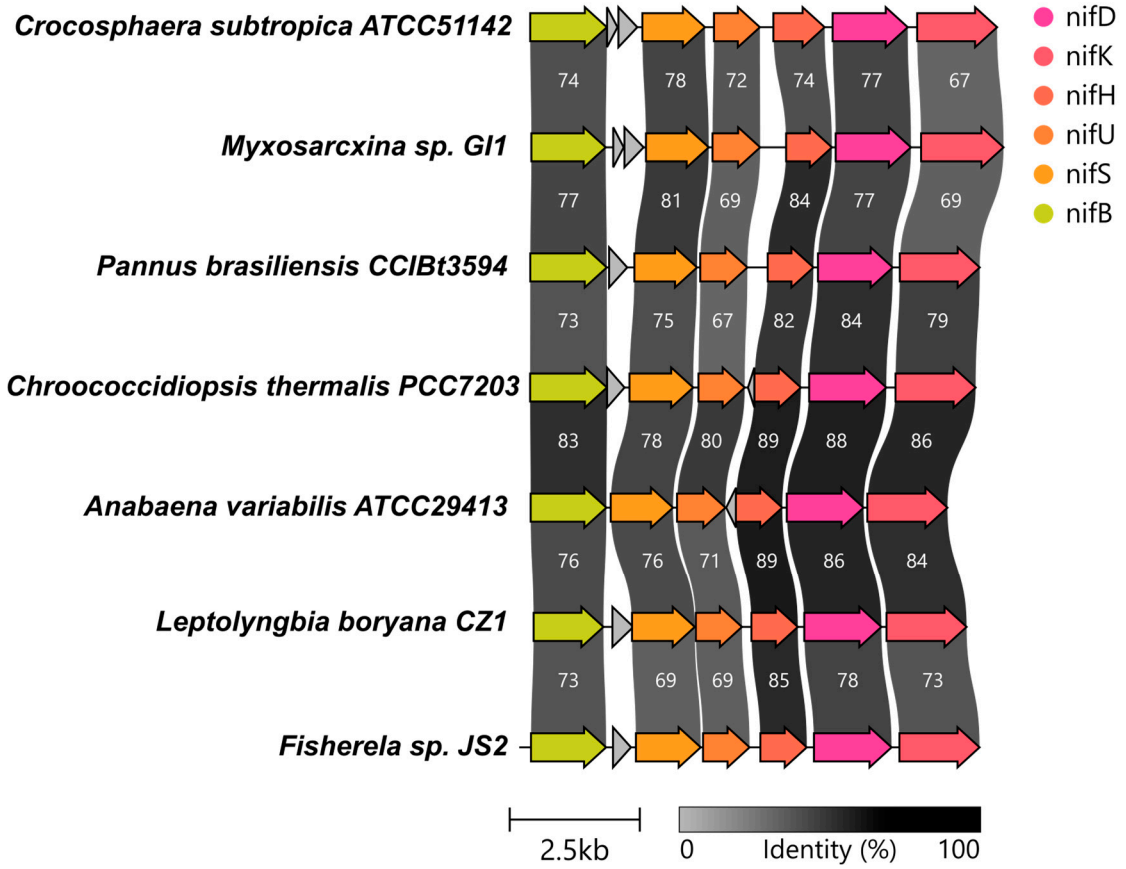

Figure 1. Synteny analysis showing the *nif*HDK and *nif*BSU operon in *Pannus brasiliensis* CCIBt3594 genome and the most related sequences in the database.

Considering the conservation pattern of *nif* genes, a phylogenetic tree was constructed based on *nif*H (Figure S3), regarded as one of the most conserved genes and a structural protein [88]. Through a phylogenetic analysis, it was observed that, despite *Pannus* forming an exclusive clade, the closest genus to *P. brasiliensis* was the *Myxosacina* sp. and *Chroococcidiopsis thermalis*, as seen in Figure 1 for the operon. However, this gene did not provide conclusive evolutionary inferences, suggesting the possibility of the absence of a monophyletic clade for this gene among unicellular lineages, in contrast to the established pattern for heterocytic Cyanobacteria [103].

3.4. Phylogenetic Analyses

The evaluation of 16S rDNA phylogeny was performed to update the previous one observed by Malone et al. [22] in the description of this species in 2014 (Figures S4 and S5). In both trees, *P. brasiliensis* CCIBt3594 anchored isolated, with the nearest genus being *Microcystis*, sharing a maximum 16S sequence identity of 94.53% with the cyanobacterium *Microcystis ichthyoblabe* VN213. Morphologically, *Microcystis* and *Pannus* are unicellular, colony-forming, mucilage-producing freshwater genera. However, different from *Pannus*, *Microcystis* is known as cosmopolitan and bloom-forming and, mainly, for its toxigenic potential, for producing toxins like microcystin [104]. The genomic comparisons between *Pannus* and *Microcystis* revealed a 73.49% similarity to the cyanobacterium *Microcystis aeruginosa* NIES-88, determined through the average nucleotide identity (ANI). Additionally, the average nucleotide identity (AAI) analysis indicated a 72% similarity across all genomes within the *Microcystis* genera (Figure S6). In silico, the DDH values with reference genomes from the *Microcystis* genera ranged from 20.8 to 21.2%, and the G + C content ranged from 9.31 to 9.69%, indicating that the genomes do not belong to the same species (Table S2). The analyses revealed that the strain closest to *P. brasiliensis* CCIBt3594 is *M. aeruginosa*

NIES-88, showing a similarity of less than 75%. *M. aeruginosa* NIES-88 was isolated from a freshwater lake in Japan and assembled into 262 contigs with remarkable completeness (99.47%) and minimal contamination (0.35%). These characteristics closely resemble the genomic attributes of *P. brasiliensis* CCIBT3594.

The phylogenomic assessment applied to validate phylogenetic positions was based on the alignment of 120 conserved bacterial single-copy marker proteins (Figure 2). A similar result to the 16S rDNA trees was obtained, with *Microcystis* being the genus most closely related to the *Pannus* sp., grouping in a monophyletic clade *Microcystis*, *Pannus*, *Aphanothece,* and *Gloeothece*, all members of the Microcystaceae family [105] with 99% bootstrap. The *Pannus* genus was originally described as a member of the family Microcystaceae, of the Chroococcales order [20]. Afterwards, Komárek and Anagnostidis [106] transferred this genus to the family Merismopediaceae due to its typical two planes of cell division. In 2005, the proposal for a cyanobacterial classification system by Hoffmann et al. [107] placed the genus *Pannus* in the family Synechococcaceae based on phenotypic features. In 2014, Komárek et al. [21] returned the *Pannus* genus into the Merismopediaceae, a classification which is used nowadays by the NCBI and CyanoDB databases. However, the most recent proposal for a cyanobacterial classification system considers the genera as belonging to the order Chroococcales, of the Microcystaceae family [105]. The phylogenomic evaluation presented corroborates the most recent proposal for *Pannus* placement into cyanobacterial taxonomy.

The primary challenge in analyzing whole genomes is the smaller presence of genome data in public databases. Known cyanobacterial genomes deposited in the databases correspond to cultivated strains belonging to more than 100 genera [108], as opposed to the broader representation found in known 16S rDNA sequences. The most represented genera in the genome database correspond to *Nostoc*, *Leptolyngbya*, *Prochlorococcus*, *Synechococcus*, *Planktothrix*, *Microcystis*, and *Calothrix* [108]. While this is the first genome of a *Pannus* representative, there are around 200 genomes from different *Microcystis* morphospecies released into public databases [109]. The low identities and phylogenetic distribution obtained showed that there are no described lineages closely related to the genus *Pannus* and emphasized that generating new sequences for type species and recently isolated species of Cyanobacteria will significantly advance the field of taxonomy within this clade. Furthermore, it illustrated the need for efforts to isolate and characterize traditionally uncultivated Cyanobacteria strains by sampling underexplored habitats and locations [110–112].

Assessing homological relationships between sequences has considerable meaning in genome annotation and phylogenetic inference. In the orthology analyses, when considering the genomes of five *Microcystis* sp. and the genome of *P. brasiliensis*, 2406 clusters of orthologous genes were identified. This set represents the core genome shared between the species, and the large number of shared genes suggests a common evolutionary history with conserved functions between the genera (Figure 3). These analyses aid in discerning whether a pair of homologous genes are orthologues (speciation) or paralogs (gene duplication) [113]. The stable core is a collection of genes characterized by comparable evolutionary trajectories exhibiting high conservation. This core encompasses genes responsible for main metabolic functions and the ribosomal apparatus, and it is resistant to horizontal gene transfer (HGT) events [24].

The accessory genome of *P. brasiliensis* CCIBt3594 includes 91 singletons, representing genes encoding proteins exclusive to this lineage. Among these, genes associated with biological nitrogen fixation stand out, an attribute absent in the other evaluated genomes. This observation suggests that this characteristic originated from an evolutionary path distinct from the most closely related lineages. Selective pressures predominantly shape the evolution of the accessory genome. Diversity within this genome is maintained through selection, acting on the acquisition of genes via horizontal transfer and the loss of genes possessing metabolic advantages [114,115]. The differences between genomes can be used to distinguish and separate closely related species, thereby identifying phylogenetic patterns [116]. This is especially relevant because there is a tendency for even the same

species to have similar core and accessory genomes, supporting the hypothesis that the evaluated species are phylogenetically distinct.

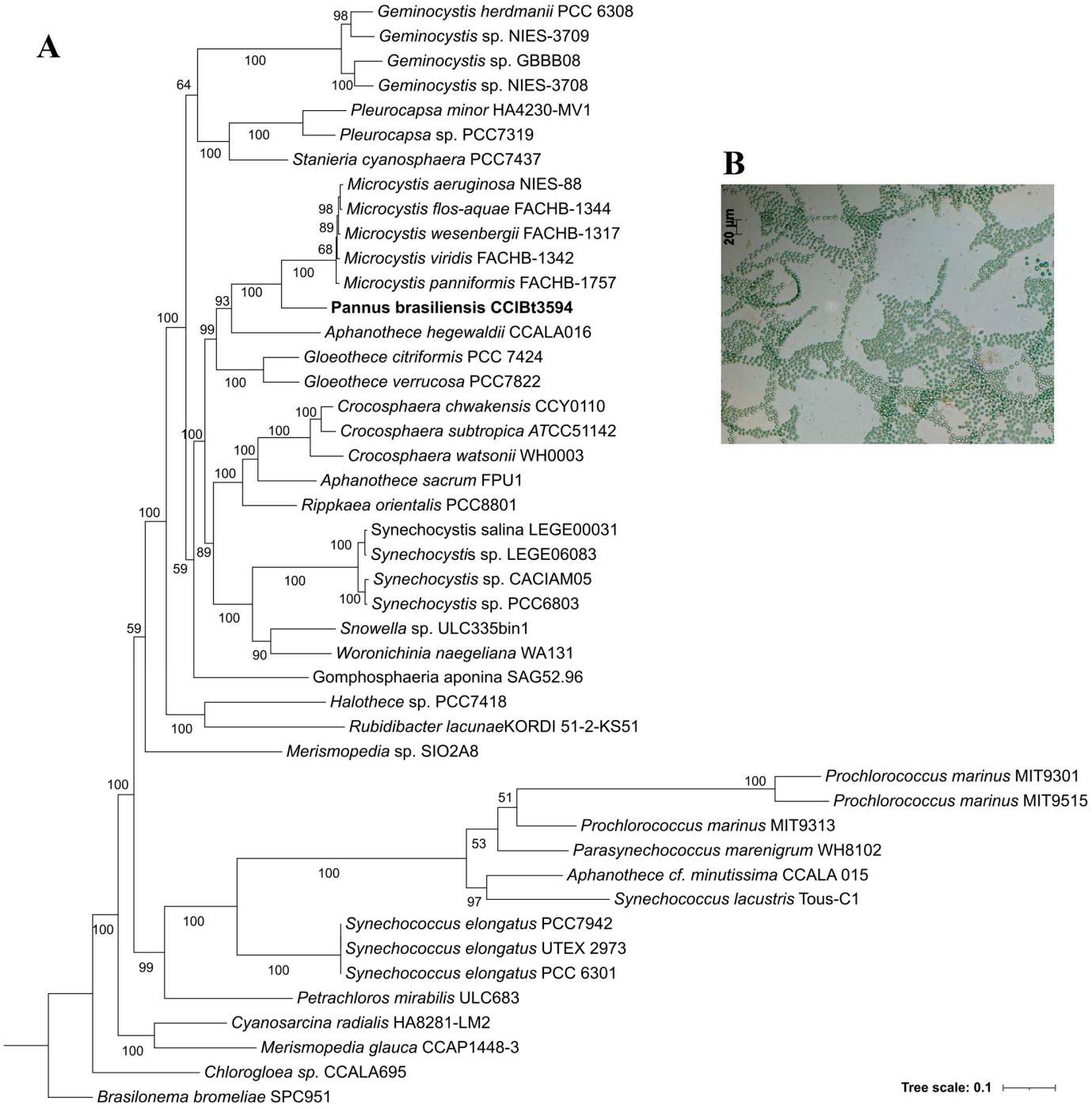

Figure 2. (**A**) Maximum-likelihood phylogenomic tree based on the alignment of 120 bacterial single-copy conserved marker proteins using GTDB-tk. A 1000 resampling bootstrap test was performed. Bootstrap values > 50 are displayed at nodes. (**B**) Photomicrograph of *P. brasiliensis* CCIBt3594 cells grouped by a mucilaginous structure.

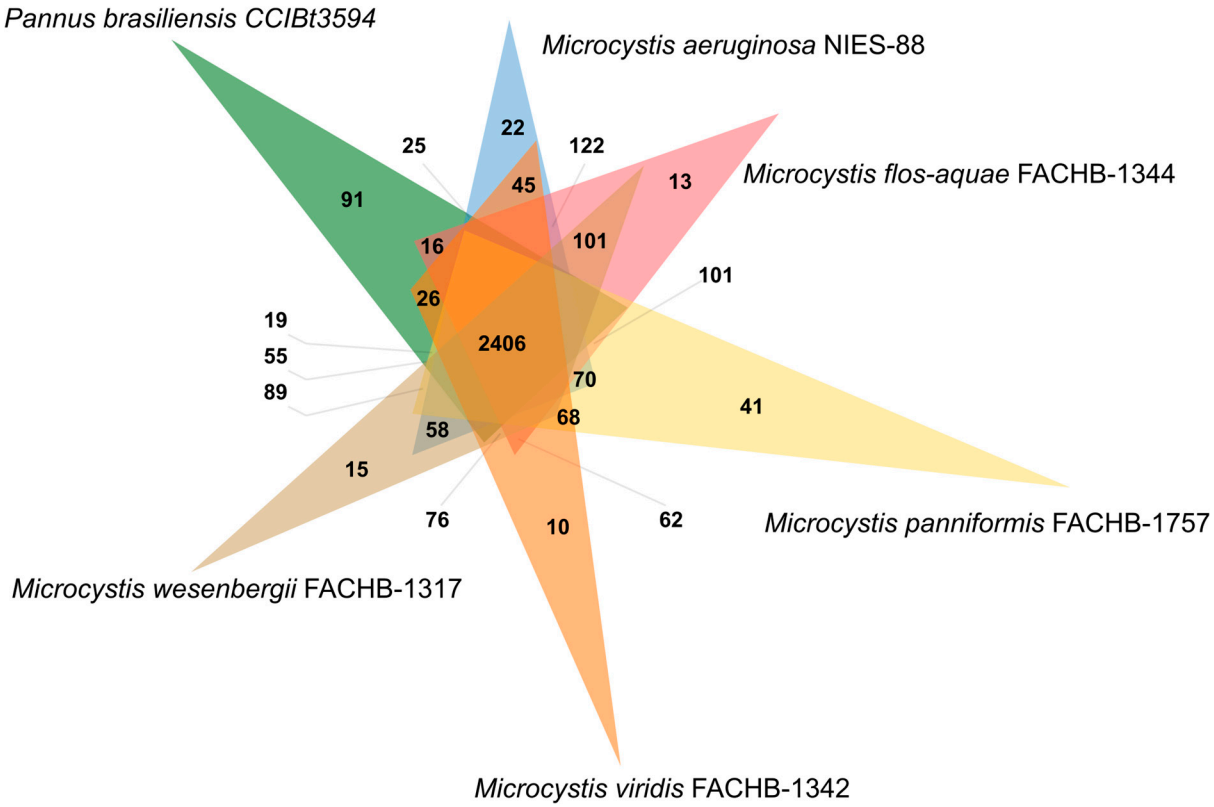

Figure 3. Venn diagrams for gene orthology between *Pannus brasiliensis* CCIBt3594 and *Microcystis* spp., emphasizing the number of genes that structure the core genome (2406) shared among the genera as well as highlighting the exclusive characteristics within the accessory genomes (91) of *P. brasiliensis* CCIBt3594.

4. Conclusions

This study presented the first genome of a *Pannus* genus representative and identified *P. brasiliensis* CCIBT 3594 as a potential diazotrophic cyanobacterium. The phylogenomic analyses anchored *Pannus* to the order Chroococcales, of the family Microcystaceae. These findings contribute to the taxonomy of this poorly reported genus. The presence of multiple secondary metabolite gene clusters in the genome of *Pannus brasiliensis* CCIBT3594 adds to the organism's metabolic versatility and ecological adaptability, including the synthesis of pigments with photoprotective and antioxidant properties, which may have applications in the industry. Further studies are needed to evaluate the nitrogen-fixing mechanisms associated with *P. brasiliensis* CCIBt3594, adding to the understanding of nutrient cycling and primary production in the natural environment.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/taxonomy4010010/s1, Figure S1: Functional classification of the genes annotated in the genome of *Pannus brasiliensis* CCIBt3594; Figure S2: Prophage region identified in the contig number 17 in the genome of *Pannus brasiliensis* CCIBt3594. PLP: Phage-like Protein; *Sha*: Tail Shaft; *Hyp*: Hypothetical Protein; *Int*: Integrase; Figure S3: Maximum-likelihood Phylogenetic tree using *nif*H gene displaying the relationship of *Pannus brasiliensis* CCIBt3594 (in bold). 1000 resampling bootstrap test was performed. Bootstrap values 50% are displayed at nodes; Figure S4: Phylogenetic tree using 16S rDNA gene displaying the relationship of *Pannus brasiliensis* CCIBt3594 (in bold) with other species of order Synechococcales and Chroococcales available in NCBI. Maximum-likelihood and 1000 resampling bootstrap test was performed. Bootstrap values 50% are displayed at nodes; Figure S5: Phylogenetic tree reconstructed by the Bayesian Inference method displaying the relationship of *Pannus brasiliensis* CCIBt3594 (in bold) with other species of order Synechococcales and Chroococcales available in NCBI. Bayesian posterior probabilities are represented on each node;

Figure S6: Heatmap showing the Average Nucleotide Identity (ANI) and the Average Amino acids Identity (AAI) among the genomes of *Pannus brasiliensis* CCIBT3594 and related species of the genus *Microcystis* spp.; Table S1: Identity of the amino acid sequences of the of *nif*, *isc*, *fdx* and *hsc* genes in *Pannus brasiliensis* CCIBt3594 genome; Table S2: The in silico DDH values for *Pannus brasiliensis* CCIBt3594 and selected reference genomes of *Microcystis* genera.

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