Article

Description of *Pseudocalidococcus azoricus* gen. sp. nov. (Thermosynechococcaceae, Cyanobacteria), a Rare but Widely Distributed Coccoid Cyanobacteria

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Abstract: Coccoid cyanobacteria represent an important part of cyanobacterial freshwater diversity, with many studied strains in public databases identified as *Synechococcus*. This is a diverse genus, both morphologically and ecologically, with a global distribution. However, many of the so-called *Synechococcus*-like cyanobacteria strains could represent several independent genera that require further studies. In this work, four strains of a *Synechococcus*-like cyanobacteria isolated from freshwater lakes and terrestrial atmophytic habitats on São Miguel and Flores Islands (Azores archipelago) were studied genetically using the 16S rRNA and 16S–23S rRNA ITS, morphologically with light and transmission electron microscopy, and ecologically. A draft genome was produced from the reference strain by Illumina sequencing, which allowed a more complete phylogenetic study and a deeper taxonomic analysis, revealing a divergent phylogenetic evolution and low ANI and AAI values (69.4% and 66.3%, respectively) to *Thermosynechococcus*, the closest phylogenetic genus. Although morphologically similar to *Synechococcus*, the 16S rRNA and genome phylogenetic analysis placed the studied strains in a clade sister to *Thermosynechococcus*, inside the Thermosynechococcaceae. Thus, *Pseudocalidococcus azoricus* gen. sp. nov. is described as a new coccoid freshwater genus and species from the Azores archipelago. A detailed comparison with similar morphological taxa is provided, supporting the separation of the new genus. The 16S rRNA with a high genetic similarity to other strains from several continents identified as *Synechococcus* sp. suggests that the new genus probably has a worldwide distribution. Future studies should be performed to clarify the taxonomic identity of those strains.

Keywords: AAI; ANI; Azores; coccoid cyanobacteria; DDH; genome; new genus; phylogeny; *Synechococcus*; 16S rRNA

1. Introduction

Cyanobacteria are one of the most ancient organisms [1], which arose around 3500 million years ago [2]. They are present in a wide diversity of habitats, in terrestrial and aquatic ecosystems [3–5], and are common inhabitants of extreme environments [5–7]. Recent studies on cyanobacteria diversity in the Azores Islands, a remote oceanic archipelago with a great variety of suitable habitats [6,8], allowed the description of several new taxa [9,10].

Therefore, many cyanobacteria species may still be unknown, and increased sampling efforts should be taken in these remote areas, especially in less-studied habitats such as terrestrial atmophytic habitats.

The morphology of coccoid cyanobacteria is poorly characterized, compromising their taxonomical classification, which was until recently based mainly on morphological characteristics [11,12]. In recent years, a significant effort to improve the coccoid cyanobacteria taxonomy has been made using a polyphasic approach with molecular and morphological data for the description of new taxa or its reassessment [13,14], and more recently using genomic data [11,15].

Synechococcus Nägeli represents a large role, with a recognized polyphyletic nature from the most commonly studied cyanobacteria [16]. However, many strains lack apomorphic features that could aid in the morphologic identification, which often resulted only in the classification of *Synechococcus*-like cyanobacteria [17]. *Synechococcus* was traditionally classified as a benthic freshwater rod-like cyanobacteria, according to Nägeli (1849) [18], yet with several described species over the years with an increasing ecological range. Recent molecular studies have confirmed the polyphyletic nature of the genus [11,19], such as *Thermosynechococcus* Katoh, Itoh, Shen, and Ikeuchi [11,20], which was initially based on its ecophysiological and biochemical features, since all its strains were thermophilic [20], and was recently validated [11].

An important shift in cyanobacteria taxonomy was observed in the twentieth century, with many authors relying more on genetic data [9,12,21,22]. More recently, the fast growth of cyanobacteria genomic data has allowed for a more robust analysis [11,23–25], not only for taxonomic resolution [26,27] but also for ecological studies [28,29]. The relevance of this new taxonomical tool is well represented in the last cyanobacterial order and family classification update, with its results based on the available genomic data [23].

In this work, we applied a polyphasic approach to study four *Synechococcus*-like cyanobacteria strains from BACA (Bank of Algae and Cyanobacteria of the Azores), assigning *Pseudocalidococcus azoricus* gen. sp. nov. to the Thermosynechococcaceae Komárek, Strunecký, and Johansen, according to its phylogenetic placement. More importantly, *Pseudocalidococcus azoricus* was defined as a new genus through a combination of molecular data (genomic data, 16S rRNA, and 16S–23S rRNA ITS), morphological characters (by light and transmission electron microscopy), and ecological data. The obtained draft genome and the taxonomic analysis allowed for a better knowledge of the *Synechococcus* polyphyletic nature. The description of the new taxa followed the International Code of Nomenclature for algae, fungi, and plants [30].

2. Materials and Methods

2.1. Strains and Morphological Characterization

The studied strains (Table 1) were isolated from lakes and a rock wall in São Miguel Island and Flores Island (Azores archipelago), and maintained in BG11 medium at 19 °C and a 14/10 h photoperiod in the BACA culture collection. For the morphological descriptions, at least 50 cells per strain were examined using a Leica DM4 B microscope equipped with a digital camera, the Leica MC 190 HD (Leica, Wetzlar, Germany). Morphological data from the four strains were combined for taxa description.

The transmission electron microscopy (TEM), biomass was preserved in 2.5% glutaraldehyde and 0.1 M cacodylate buffer, and postfixed with 2% osmium tetroxide; then, it was dehydrated in an acetone series (30%, 50%, 70%, 80%, 90%, 95%, and 100%) and embedded in Spurr's resin [31]. Ultra-thin sections (70 nm) were placed on formvar-coated grids, contrasted by uranyl acetate and lead citrate, and analyzed with a JEOL JEM 1010 microscope.

Table 1. Strain location of the sampling in the Azores archipelago, Portugal, and GenBank accession codes of the *Pseudocalidococcus* strains.

Strain	Taxonomy	Local	Sampling Date	Habitat	Coordinates	GenBank
BACA0433	*P. azoricus*	Furnas, São Miguel Island	1 August 2017	Aquatic	37°46′18.3″ N 25°18′42.7″ W	OM732237
BACA0444	*P. azoricus*	Lagoa Comprida, Flores Island	27 September 2017	Aquatic	39°26′26.1″ N 31°13′19.0″ W	OM732240
BACA0446	*P. azoricus*	Lagoa das Empadadas Norte, São Miguel Island	12 July 2017	Aquatic	37°49′32.5″ N 25°44′54.9″ W	OM732241
BACA0781	*P. azoricus*	Ribeira Grande, São Miguel Island	6 September 2022	Atmophytic	37°47′35.9″ N 25°29′05.7″ W	OR725120

2.2. DNA Extraction, Gene Amplification, and Sequencing

The PureLink® Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA, USA) was used for the DNA extraction following the protocol recommended by the manufacturer for Gram-negative bacteria.

For the 16S rRNA and 16S–23S rRNA ITS region amplification, the primers 27F and 23S30R [32,33] were used in a polymerase chain reaction following the protocol described by Luz et al. (2023) [9]. Thermal cycling reactions were carried out in a ProFlex™ 3 × 32-well PCR System (Thermo Fisher, Waltham, Massachusetts, USA) using the same conditions as Taton et al. (2003) [34]. Visualization and purification of the amplified sequences followed Luz et al. (2023) [9], and amplicon sequencing was conducted as a commercial service at MACROGEN (Madrid, Spain) using the 27F, 781F, 781R, CSIF, and 23S30R primers [32,33,35–37].

2.3. Genome Sequencing and Assembly

The chosen reference strain (BACA0444) was produced in 50 mL cultures in BG11 medium for three weeks, and biomass was recovered through centrifugation at 7000 RCF. The same kit was used to extract DNA as for the 16S rRNA and 16S–23S rRNA ITS amplicons. However, for the elution, DNAse- and RNAse-free water was used. Sequencing was performed on an Illumina platform in NovoGene (Cambridge, UK) using NovaSeq, producing 1 G of data output.

The draft genome was assembled using the GEN-ERA assembly pipeline 2.0 [38] with SPades v3.15.3 [39] and with the metagenomics option selected. Binning was performed by CONCOCT v1.1.0 [40], and the produced binned genome was assessed for quality using CheckM v1.2.2 [41], BUSCO v5.5.0 [42], and CheckM2 v1.0.2 [43].

2.4. 16S rRNA Phylogenetic Analysis

The sequences of the studied strains were aligned with 96 sequences from other cyanobacteria retrieved from GenBank using BLAST or from the literature. Sequence alignment was carried out using MAFFT v7.520 with the G-INS-i method [44]. The final alignment contained 1076 columns. The best-fit nucleotide model was assessed using ModelFinder [45], with the selection of the GTR + G4 + I + F evolution model according to the Bayesian information criterion.

Phylogenetic trees were constructed using Bayesian inference (BI) with MrBayes v3.2.7a [46] on XSEDE through the CIPRES Science Gateway, and maximum likelihood (ML) using the IQ-Tree online version v1.6.12 [47]. *Gloeobacter violaceus* PCC 8105 was used as an outgroup. The following conditions were used in the BI analysis: 2.5×10^6 generations, with two runs of four Markov chains, custom parameters (temp = 0.01), sampling every 1000 generations, and a 0.25 burn-in rate (the final average standard deviation of split frequencies was equal to 0.004500). The ML analysis was carried out using the GTR + G4 + I + F model with 1000 ultrafast bootstrap replicates [48]. Trees were visualized

in FigTree v1.4.4 [49], and the final composite tree from the maximum likelihood with the addition of the posterior probabilities values from the BI was redrawn with Inkscape v1.3.

2.5. Genome Analysis

Genomes were selected following a literature review and their availability in GenBank. For quality control, an analysis using BUSCO v5.5.0 [42] was performed in all retrieved genomes with the cyanobacteria_odb10 dataset lineage selected (date: 23 February 2021; number_of_BUSCOs: 773) and CheckM v1.2.2. The number of identified genes was analyzed, and genomes with low-conserved genes were removed, along with genomes with fragment genes (5 >; BUSCO analysis) and less than 95% completeness (CheckM analysis). A final dataset of 114 genomes was then used for further analysis.

An adapted and custom python pipeline based on Jamie McGowan (https://github.com/jamiemcg/BUSCO_phylogenomics.git, accessed on 31 August 2023) was used for the phylogenomic analyses, modified to better fit a prokaryote analysis and taking into account updated software, here presented as KABOOM (https://github.com/rubenluz/KABOOM.git, accessed on 31 August 2023). This python pipeline, working in a Conda environment, takes assembled genomes as input (.fasta and .fna), performs BUSCO analyses for the identification of conserved BUSCO genes, trims them, concatenates common and single-copy genes, and performs a phylogenetic analysis based on nucleotides or amino acid sequences. Briefly, common genes to all the selected genomes were retrieved and then aligned using MAFFT v7.520 [44], trimmed using trimAl v1.4.1 [50], and concatenated. Phylogenetic analysis was performed on the final concatenated alignment of 217 genes, with 65,463 columns of amino acids, using IQTREE 2.2.3 [51] with the automatic selection of the LG + F + I + R10 best-fit model according to the Bayesian information criterion by ModelFinder [45] and 1000 ultra-fast bootstrap [48]. The final bootstrap correlation coefficient of split occurrence frequencies was 1 after 102 iterations. The same approach was applied using the nucleotide option for the phylogenomic inference, with a final concatenated alignment of 217 genes with 198,274 columns of nucleotides. The model was selected by ModelFinder [45], with the best-fit model SYM + I + R10 chosen according to Bayesian information criterion. The final bootstrap correlation coefficient of split occurrence frequencies was 0.998 after 200 iterations. Trees were visualized in FigTree v1.4.4 [49] and redrawn with Inkscape v1.3.

The average nucleotide identity (ANI) and the average amino acid identity (AAI) to the closest phylogenetic and morphological taxa were calculated using orthoANI [52] and EzAAI [53], respectively. Digital DNA–DNA hybridization (DDH) was calculated using the genome-to-genome distance calculator [54].

2.6. Analyses of the 16S–23S rRNA ITS Region

For the 16S–23S rRNA ITS secondary-structure identification, M-fold was used, applying the default parameter settings [55]. The D1–D1′, Box-B, and V3 helix sequences were identified after the M-fold results and the published literature. Final structures were redrawn with Inkscape v1.3.

3. Results

Pseudocalidococcus azoricus R.F.S. Luz, J. Kaštovský, V. Gonçalves gen. sp. nov. (Figure 1).

Diagnosis: Morphologically similar to *Synechococcus*, but with a distinct phylogenetic placement in the Thermosynechococcaceae by the 16S rRNA phylogeny and phylogenomic analysis. Differs from *Thermosynechococcus* ecologically, as *Pseudocalidococcus* is a freshwater and *Thermosynechococcus* is strictly thermal, and genomically, both in its phylogenetic position and low AAI (66.3%) and ANI (69.4%).

Description: Cells solitary or arranged in small clusters. Without mucilage or any evident envelops. Cells blue-green, cylindrical, rod-shaped (sometimes slightly arcuate) to elongated cylindrical, and occasionally slightly widened at both ends. Cells 1.6–6.5 μm in length (mean = 2.9 μm) and 0.8–2.0 μm wide (mean = 1.4 μm), with a length/width ratio of 1.1–6.3 (mean = 2.2). Observed elongated cells were up to 45 μm in length. Cell division perpendicular to the long axis of the cells. Thylakoids present in a parietal arrangement; up to five.
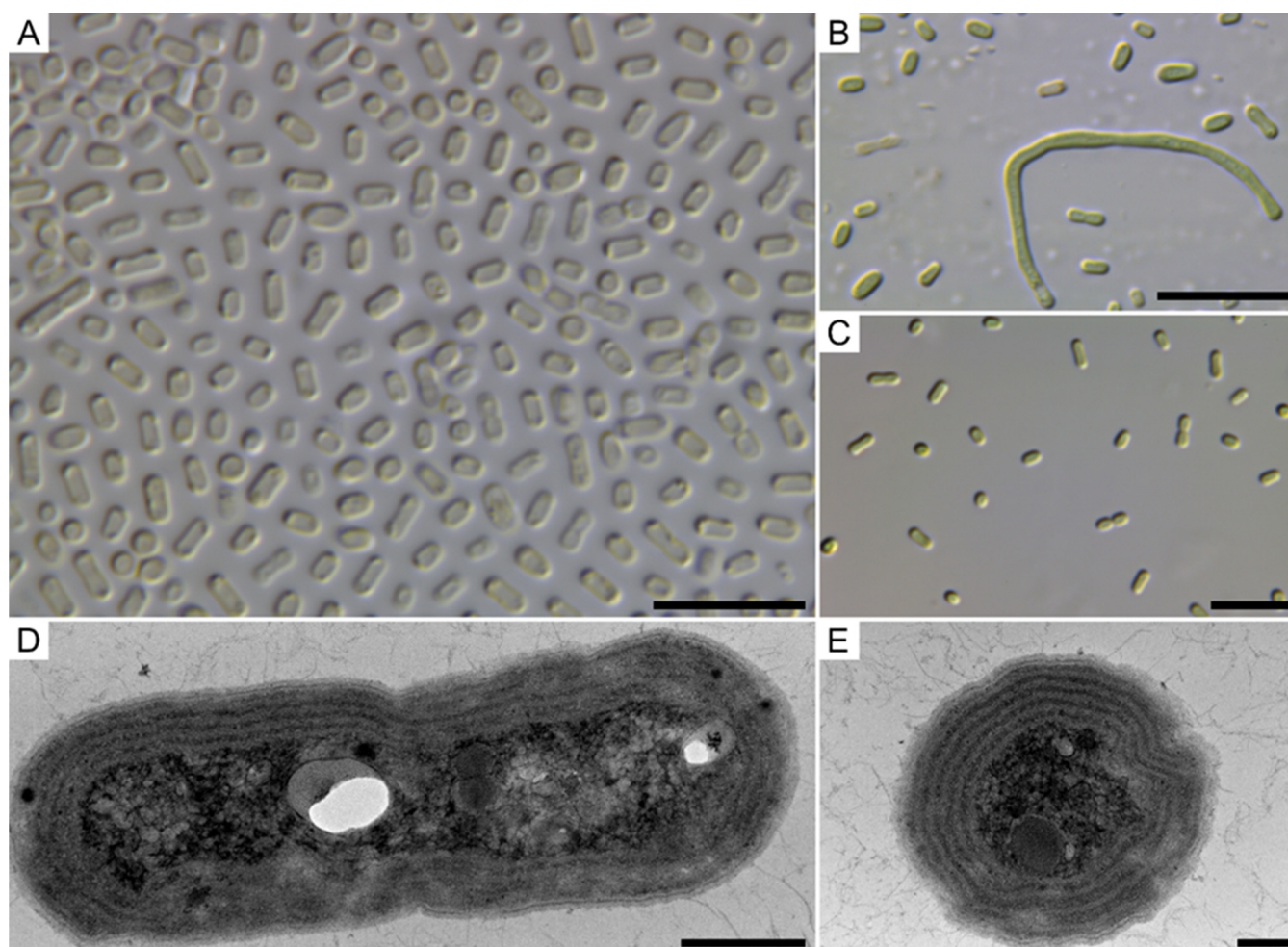

Figure 1. Morphology of *Pseudocalidococcus azoricus* BACA0444 under light microscope and TEM. (**A**) Different cell morphologies in DIC; (**B**) Normal and elongated cells in DIC; (**C**) Cells with incomplete binary fission; (**D**) Transversal cut showing four parietal thylakoids; (**E**) Longitudinal cut showing five parietal thylakoids in the cell. Scale bars 10 μm (**A**–**C**), 500 nm (**D**), and 200 nm (**E**).

Holotype: Dried material preserved in a permanently inactive state at Herbário Ruy Telles Palhinha, University of Azores, Portugal, under the AZB 4202 code.

Type locality: Lagoa Comprida, Lajes, Flores Island (Azores archipelago, Portugal); 39°26′26.052″ N 31°13′19.0128″ W, collected by the MONITAIA team project.

Habitat: Aquatic in freshwater lakes and atmophytic.

Etymology: *Pseudocalidococcus*: Pseudo (fake)—calidum (hot)—coccus: fake thermal cyanobacteria, as it is positioned inside a supposedly thermal family; masculine gender; *azoricus*: isolated from the Azores archipelago.

Reference strain: BACA0444 (Bank of Algae and Cyanobacteria of the Azores, Azores, Portugal), isolated by Rita Cordeiro.

Gene Sequences: GenBank accession number OM732240 for the 16S rRNA and 16S–23S rRNA ITS genes and GenBank accession number GCA_031729055 for the genome assembly.

3.1. Morphological Analysis

The four strains studied in this work have very similar morphological characteristics (Table 2) despite originating from different ecosystems in the Azores; namely, from a small lake in Furnas village (São Miguel Island), Lake Empadadas Norte (São Miguel Island), Lake Comprida (Flores Island), and an atmophytic site in Ribeira Grande (São Miguel Island). This represents a large geographical distance of separated populations from where the strains of *P. azoricus* were isolated.

Table 2. Cell dimensions in the four studied strains, with the minimum, maximum, and arithmetic mean of the length and width in micrometers. The *P. azoricus* cell dimensions correspond to the combined values of the four strains.

	Length			**Width**			**Ratio (Length/Width)**		
	Min	**Max**	**Mean**	**Min**	**Max**	**Mean**	**Min**	**Max**	**Mean**
BACA0433	1.8	6.5	3.0	0.8	1.4	1.1	1.7	6.3	2.6
BACA0444	1.6	4.0	2.6	1.0	1.6	1.3	1.1	3.4	2.0
BACA0446	1.8	5.1	3.0	1.0	1.9	1.4	1.2	4.0	2.2
BACA0781	2.0	4.6	3.0	1.1	2.0	1.5	1.3	3.4	2.0
P. azoricus	1.6	6.5	2.9	0.8	2.0	1.4	1.1	6.3	2.2

The four strains presented the same morphological cell characteristics, but not all presented the elongated cells, as seen in Figure 1B, with up to five parietally arranged thylakoids (Figure 1E). An attempt was made to compare the morphological characteristics of our strains with the strains that fall within the *Pseudocalidococcus* phylogenetic clade, but no description was found in the literature.

3.2. 16S rRNA Phylogeny and 16S–23S ITS Secondary Structures

The four Azorean strains were grouped together with other *Synechococcus* sp. strains (EO68, CHAB TP201738, IPPAS B-1202, PCC 6312, and PCC 6603) in the 16S rRNA phylogenetic analysis (Figure 2), near *Thermosynechococcus*, with strong support (100 ML, 1 BI), suggesting the position of *Pseudocalidococcus* in the Thermosynechococcaceae. Furthermore, the *Synechococcus* sp. strains positioned in the cluster of *Pseudocalidococcus azoricus* are genetically closely related and must all belong to the genus *Pseudocalidococcus*. Therefore, *Pseudocalidococcus* has a wide geographical distribution, being present at least in the United States of America (a freshwater pond in California, PCC 6603 strain) and Kazakhstan (Issyk Lake, IPPAS B-1202 strain), besides the Azores archipelago.

The secondary structure of the D1-D1′ and Box-B helix of the 16S–23S rRNA ITS is shown in Figure 3. As expected and reinforcing the distance of the *Pseudocalidococcus* genus to *Thermosynechococcus*, a large difference was observed between the folded structures in both the sequence and folding. A marked difference can be seen in the formation of the different lateral bulges in both genera in the D1-D1′ helix and in the Box-B helix in the mid-internal loop of *Pseudocalidococcus*, in contrast with its absence in *Thermosynechococcus*.
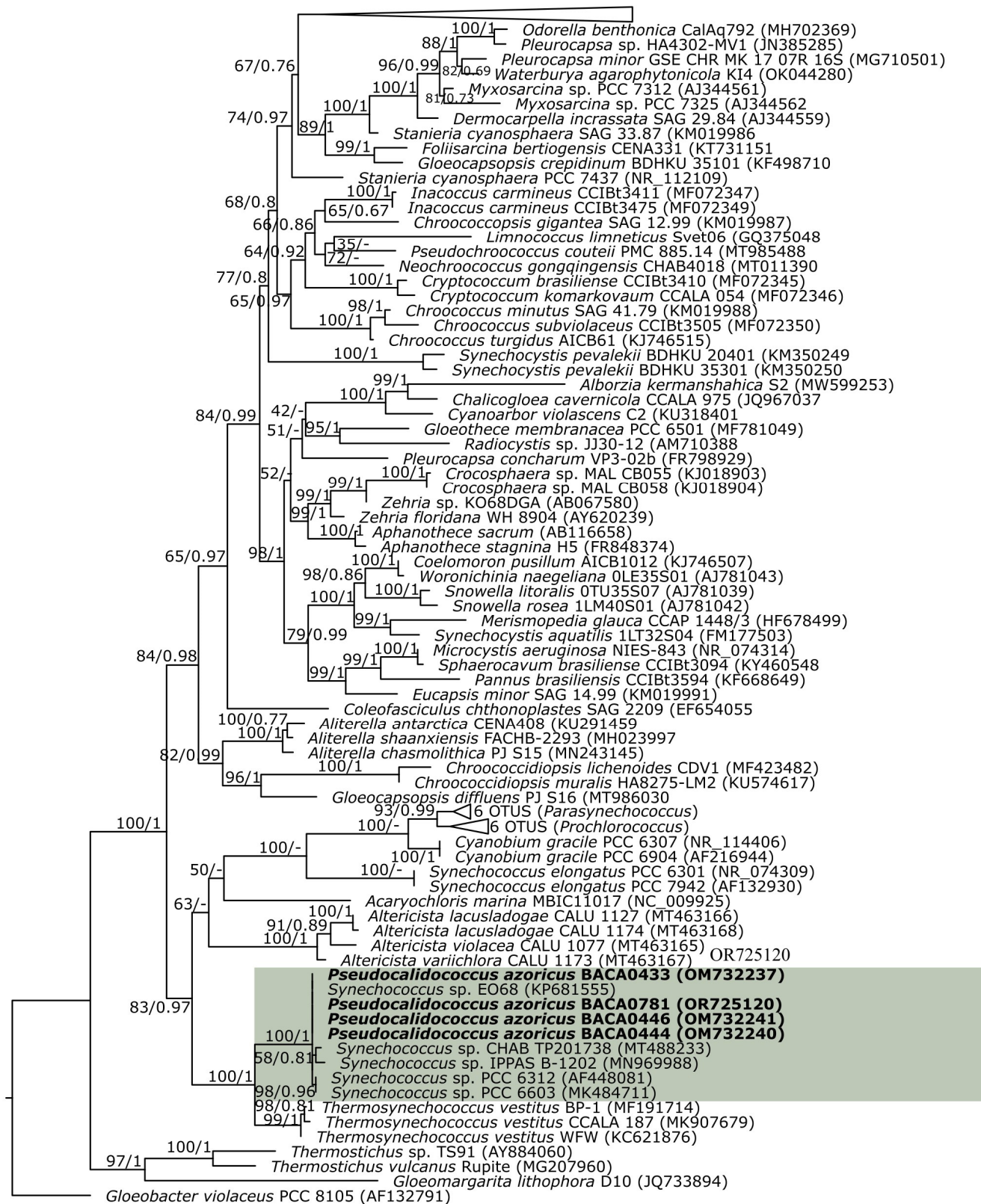
Figure 2. 16S rRNA partial maximum likelihood (ML) phylogenetic tree. Bootstrap values for the maximum likelihood and posterior probabilities for the Bayesian inference are indicated on the tree. The studied strains are in bold font. The new genus is in the green shade.
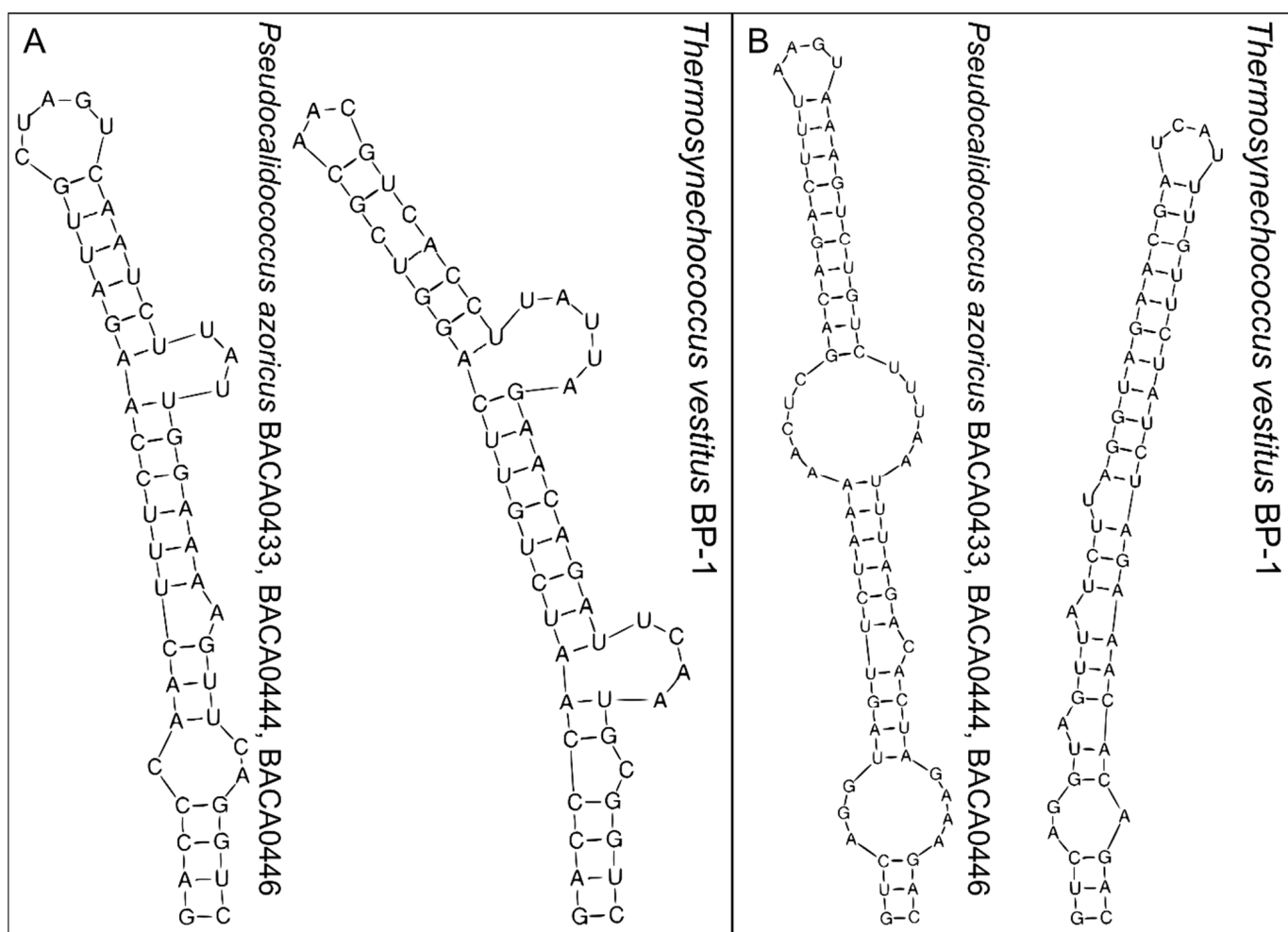
Figure 3. The 16S–23S rRNA ITS secondary structures of the D1-D1′ (**A**) and Box-B (**B**) region of *Pseudocalidococcus azoricus* BACA0444 and the closest phylogenetic species of *Thermosynechococcus vestitus* BP-1.

3.3. Genomic Analysis

The produced genome is of high quality, with 34 contigs, a size of 3,463,985 base pairs (Figure 4), and a GC content of 48.7%. Quality control showed a 99.53% completeness and a 0.12% contamination according to CheckM v1.2.2, a 99.8% completeness and a 0.0% contamination according to CheckM2 v1.0.2, and a 98.4% completeness according to BUSCO v5.5.0. Assembly data and annotation statistics to the closest phylogenetic genera are presented in Table 3.

The phylogenomic analysis placed the new genus in the same position as the 16S rRNA phylogenetic analysis, confirming its similarity with *Thermosynechococcus*, with a good bootstrap support of 100 (Figure 5). The ANI and AAI analysis supported the gene separation against *Thermosynechococcus*, with low values (below 70%). The ANI, AAI, and DDH supported the presence of at least two species in the genus, following the genomic analysis of the available data.
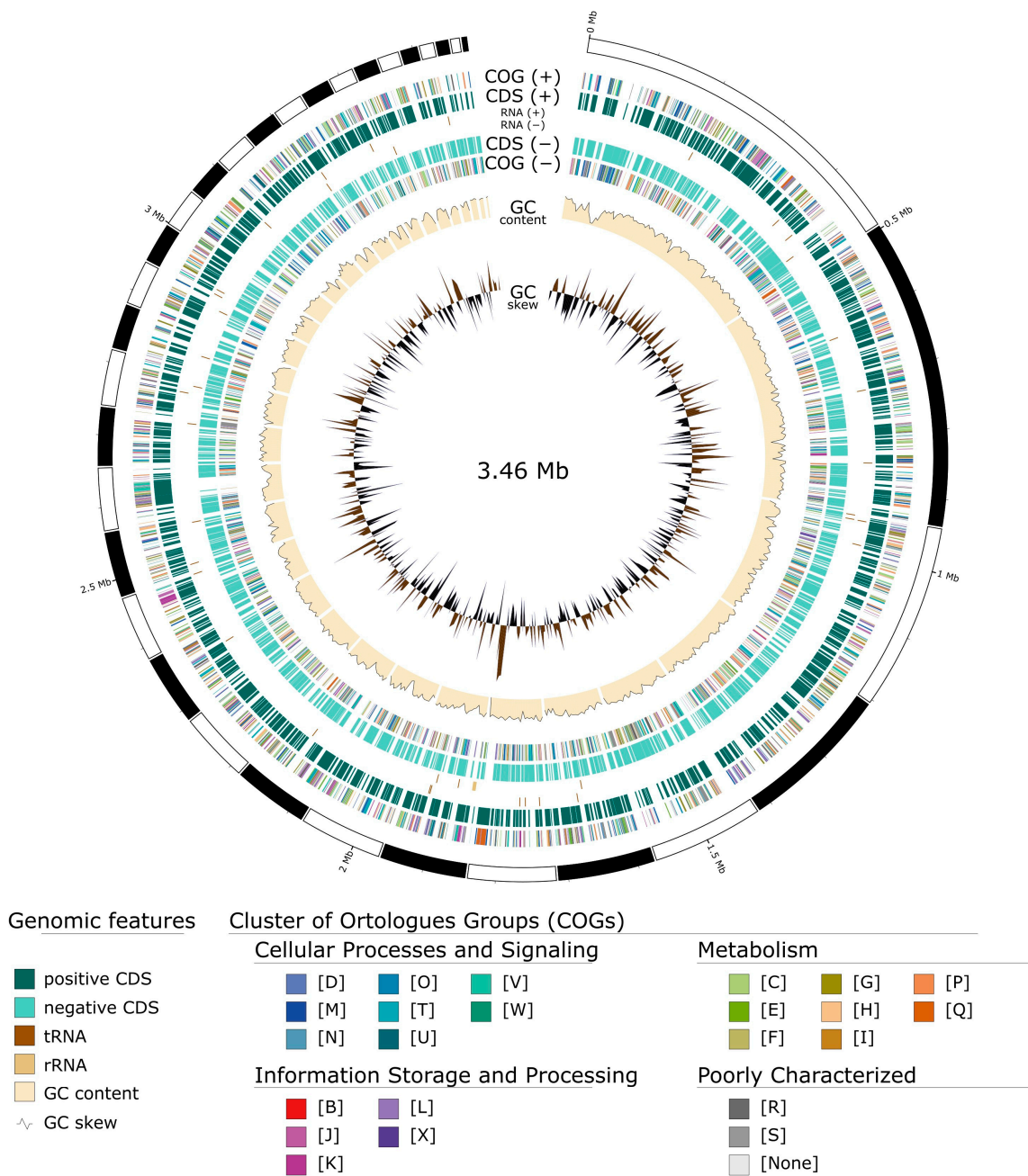
Figure 4. Circular genome representation of *Pseudocalidococcus azoricus* BACA0444 using GenoVi [56].

Table 3. Genome data from *Pseudocalidococcus azoricus* and the closest phylogenetic genera. Statistical data of assembly were retrieved from CheckM v1.2.2 and Bakta v1.8.2.

Species	*P. azoricus*	*Pseudocalido-coccus* sp.	*T. vestitus*	*T. vestitus*	*Thermosynecho-coccus* sp.	*A. marina*	*S. elongatus*
Strain	BACA0444	PCC 6312	E542	BP-1	HN-54	MBIC11017	PCC 7942
Origin	Azores, Portugal	California, USA	Ganzi, China	Beppu, Japan	Hunan, China	Republic of Palau	California, USA
Habitat	Freshwater	Freshwater	Thermal	Thermal	Thermal	Marine	Freshwater
GenBank accession	GCA_031729055	GCA_000316685	GCA_003555505	GCA_000011345	GCA_023650955	GCA_000018105	GCA_000012525
Number of contigs	34	2	1	1	1	10	2
Completeness	99.53	99.29	100.0	99.76	100.0	99.53	100.0
Contamination	0.12	0.0	0.12	0.12	0.12	5.07	0.0
N50	125,609	3,697,276	2,650,294	2,593,857	2,705,963	6,503,724	2,695,903

Table 3. *Cont.*

| Species	*P. azoricus*	*Pseudocalido- coccus* sp.	*T. vestitus*	*T. vestitus*	*Thermosynecho- coccus* sp.	*A. marina*	*S. elongatus*
Genome size (bp)	3,463,985	3,720,499	2,650,294	2,593,857	2,705,963	8,361,599	2,742,269
G + C content (%)	48.7	48.5	53.3	53.9	53.1	47.0	55.4
Coding density (%)	86.7	87.4	92.7	90.3	91.8	84.3	89.5
No. of rRNA genes	3	3	3	3	3	6	6
No. of tRNA genes	40	41	42	42	43	76	45
No. of protein- coding genes	3386	3699	2541	2514	2610	7760	2720
No. of pseudogenes	15	4	1	0	2	14	0
No. of hypotheticals genes	356	132	41	81	102	424	63
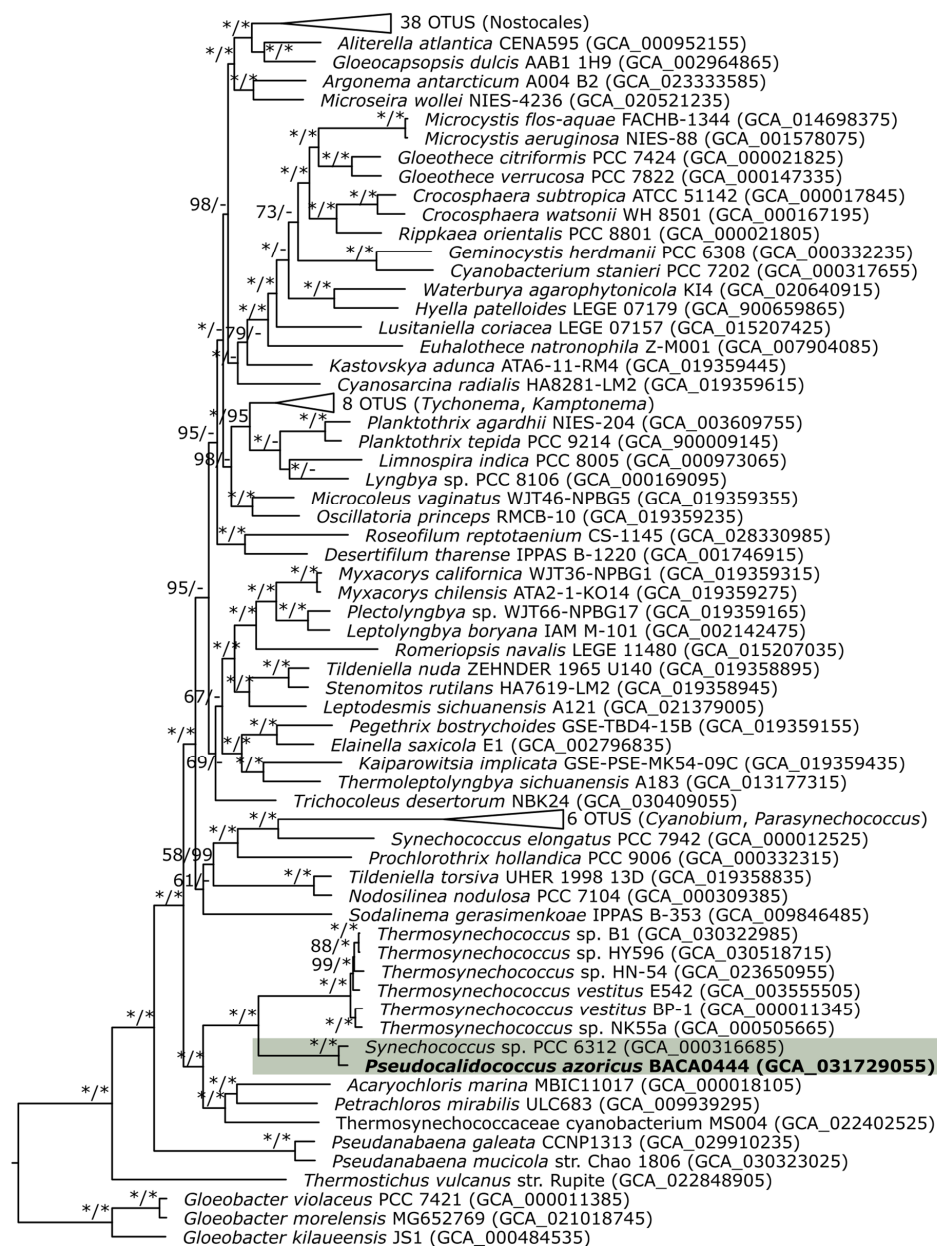
Figure 5. Amino acid maximum likelihood phylogenomic tree with 114 OTUS of 217 concatenated BUSCO genes. Bootstrap values for the maximum likelihood based on amino acids and nucleotides are indicated on the tree. The new genus is in the green shade. * 100% bootstrap.

4. Discussion

The new genus *Pseudocalidococcus* is phylogenetically closely related to *Thermosynechococcus*. However, it is noteworthy that *Thermosynechococcus* is recognized as a genus strictly associated with thermal environments [11,20], and *Pseudocalidococcus* strains have been isolated, not only from freshwater lakes, but also from an atmophytic habitat on a rock wall. This habitat distribution suggests that *Pseudocalidococcus* is primarily thermotolerant rather than thermophilic. Genetically, the 16S rRNA pairwise distance (Table 4) is slightly above (94.8%) the recommended minimum threshold values of 94.5% for the 16S rRNA [57]. However, the combination of the 16S rRNA phylogenetic distance (Figure 1), the genomic analysis, the ANI and AAI values, and the phylogenomic analysis strongly support the creation of the new genus, *Pseudocalidococcus*.

Table 4. The 16S pairwise distance similarity, the 16S rRNA ITS pairwise distance similarity, and the ANI, AAI, and DDH (identities/HSP length) percentages.

	1	2	3	4	5	6
1. *Pseudocalidococcus azoricus* BACA0444						
2. *Pseudocalidococcus* sp. PCC 6312	99.8 | 93.4 | 90.0 | 92.0 | 39.5					
3. *Thermosynechococcus vestitus* E542	94.8 | 77.5 | 69.4 | 66.4 | 22.9	94.6 | 76.9 | 69.4 | 66.3 | 22.8				
4. *Thermosynechococcus elongatus* BP-1	94.8 | 80.6 | 69.5 | 66.4 | 19.5	94.8 | 80.5 | 69.5 | 60.9 | 21.9	99.7 | 96.5 | 87.6 | 91.6 | 33.3			
5. *Thermosynechococcus* sp. HN-54	94.9 | 79.6 | 69.5 | 66.3 | 21.6	94.8 | 78.9 | 69.4 | 66.3 | 23.0	99.4 | 91.5 | 88.2 | 93.1 | 34.5	99.3 | 95.1 | 86.5 | 90.9 | 30.9		
6. *Acaryochloris marina* MBIC11017	90.0 | 70.5 | 67.1 | 61.8 | 24.1	90.1 | 71.2 | 67.2 | 61.7 | 25.8	90.7 | 72.3 | 67.1 | 61.9 | 25.8	90.6 | 74.6 | 67.1 | 61.7 | 29.3	90.7 | 72.3 | 67.2 | 61.8 | 30.1	
7. *Synechococcus elongatus* PCC 7942	90.6 | 67.3 | 66.5 | 60.7 | 34.4	90.5 | 65.3 | 66.5 | 60.7 | 39.4	90.4 | 66.2 | 67.2 | 61.3 | 20.3	90.5 | 68.8 | 67.3 | 61.3 | 28.8	90.5 | 64.9 | 67.2 | 61.3 | 20.3	90.2 | 64.9 | 66.1 | 60.2 | 24.4

Compared to *Synechococcus*, *Pseudocalidococcus* is morphologically very similar. *Pseudocalidococcus* present the same type of involution/elongated cells when in culture, as described for the type species *Synechococcus elongatus* [58,59]. However, phylogenetically, it is distantly placed from *S. elongatus* PCC 6301, the currently accepted reference strain. The phylogenetic distance provides strong support for recognizing the difference between these genera.

Pseudocalidococcus azoricus falls within the general morphological cell description of *Synechococcus nidulans* [59]. However, this can be very problematic, as the validity of the latter is questionable. *Synechococcus nidulans* is a comb., cited in Bourrelly (1970) [60], with the basionym of *Lauterbornia nidulans* (Richter) Pringsheim [60], and *L. nidulans* with the basionym of *Aphanothece nidulans* Richter [61]. Thus, *Aphanothece nidulans* and *Synechococcus nidulans*, both currently considered valid, have the same holotype, which is not taxonomically acceptable. In the same year, Komárek (1970) [58] describes *Synechococcus leopoliensis* comb. nov., arguing that *Aphanothece nidulans* and *Lauterbornia nidulans* are different taxa, including the latter as a synonym of *Synechococcus leopoliensis* [58]. To increase the complexity of the subject, the strain used by Pringsheim (1968) [61] for *Lauterbornia* description [61] was Kratz-Allen/Bloom 625, which are synonyms of PCC 6301, CCAP 1405/1, and SAG 1402-1 [58,62]. PCC 6301 is the reference strain of *Synechococcus* [63], and the currently accepted neotype of *Synechococcus elongatus* [62,64].

Therefore, *Synechococcus nidulans* is a nom. inval., as it has no valid holotype (it is invalid under the International Code of Nomenclature for algae, fungi, and plants), and only *Aphanothece nidulans* is still valid, as previously confirmed [58]. Under these terms, *Lauterbornia nidulans* should be considered as a synonym for *Synechococcus elongatus*, as they are all based on the same strain, with morphological differences probably re-

lated to culture conditions and/or long-term maintenance [65]. This approach allows the separation of *Synechococcus leopoliensis*, which should be regarded as a synonym of *Romeria leopoliensis*, as suggested by Komárek and Anagnostidis (2005) [66]. Considering that *Synechococcus nidulans* is a nom. inval., we disregard this taxon for the proposal of the new species. However, as there are many reports of this taxon in the literature, its validity should be reassessed as soon as possible.

In the *Pseudocalidococcus* clade, genomic data are only available for the strains *P. azoricus* BACA0444 and *Pseudocalidococcus* sp. PCC 6312. The 16S rRNA similarity of these two strains is quite high, with only a 0.2% difference, indicating that these strains belong to the same species, following the criteria of 98.7% for species delimitation in bacteria [67,68]. However, the ANI and AAI values (90.0% and 92.0%, respectively) are well below the recommended 95% threshold for species separation [69,70], supporting the possible separation of these strains in two distinct species. This hypothesis was also supported by the DDH analysis, with a value of 39.5%, considered a good support value for species distinction [71,72]. These contradictory results can be problematic, as the 16S rRNA similarity has been used as a reference for species delimitation [21]. The 98.7% recommended threshold value [67,68] is widely used, and this value is based on DNA–DNA hybridization and the correlation that exists between the 98.7% 16S rRNA values and the 70% DNA–DNA hybridization value, which is the gold standard for microbial species delimitation [71,72]. This pattern was also observed in the *Thermosynechococcus* strains, as the 16S rRNA similarity (recommended 98.7%) does not match with the ANI and DDH similarities (suggested as 95% and 70%, respectively); instead, a difference of only 0.4–0.7% 16S rRNA similarity corresponds to a more than 10% divergence in the ANI values, and much lower than 70% in the DDH.

To our knowledge, no generic value is accepted for genera distinction using genomic ANI or AAI criteria. Based on the 16S rRNA, a 94.5% similarity is suggested as the threshold for genera separation [57]. In the Cyanophyceae, these values are often not followed, e.g., in Nostocales phylogenetic studies, as different values are applied, resulting in some confusion [73]. The sole use of general genetic threshold values from bacterial broad studies, which normally do not even include cyanobacteria data due to the lack of available genomes, must be avoided. Future cyanobacteria taxonomic studies should adopt a heuristic approach, integrating traditional markers (morphological and amplicons) and genomic data. The 16S rRNA analysis must be complemented with a deep genomic approach, including phylogenomic, ANI, AAI, DDH, and other criteria that might support the new taxa (e.g., GC content, coding density, and number of genes), and through the use of a pangenome analysis [24,26].

5. Conclusions

This work provided a concise description of a new coccoid cyanobacteria, *Pseudocalidococcus azoricus* gen. sp. nov., using a polyphasic approach. This approach allowed the separation of what would appear to be a *Synechococcus nidulans* strain to a new and well-defined genus that probably has a global distribution. With the predictable future increase in genomic data, this study provides a new perspective on the values that should be applied in cyanobacteria taxonomy. The growing accessibility of genomic data and the increase in available software or pipelines, such as KABOOM, that facilitate the recovery and use of genomes or metagenomes should be considered in new taxa descriptions, as they bring important insights when discussing closely related taxa with few differentiating morphological characteristics. Our results reinforce the need for deeper studies in cyanobacteria taxonomy, with larger datasets to clarify if the minimum values suggested for species and genera delimitation can be blindly applied. Using such criteria in cyanobacteria may be too conservative and undermine the knowledge of cyanobacterial diversity.

Data Availability Statement: KABOOM is written in python and is freely available at GitHub (https://github.com/rubenluz/KABOOM.git, accessed on 31 August 2023).

Conflicts of Interest: The authors declare no conflict of interest.

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