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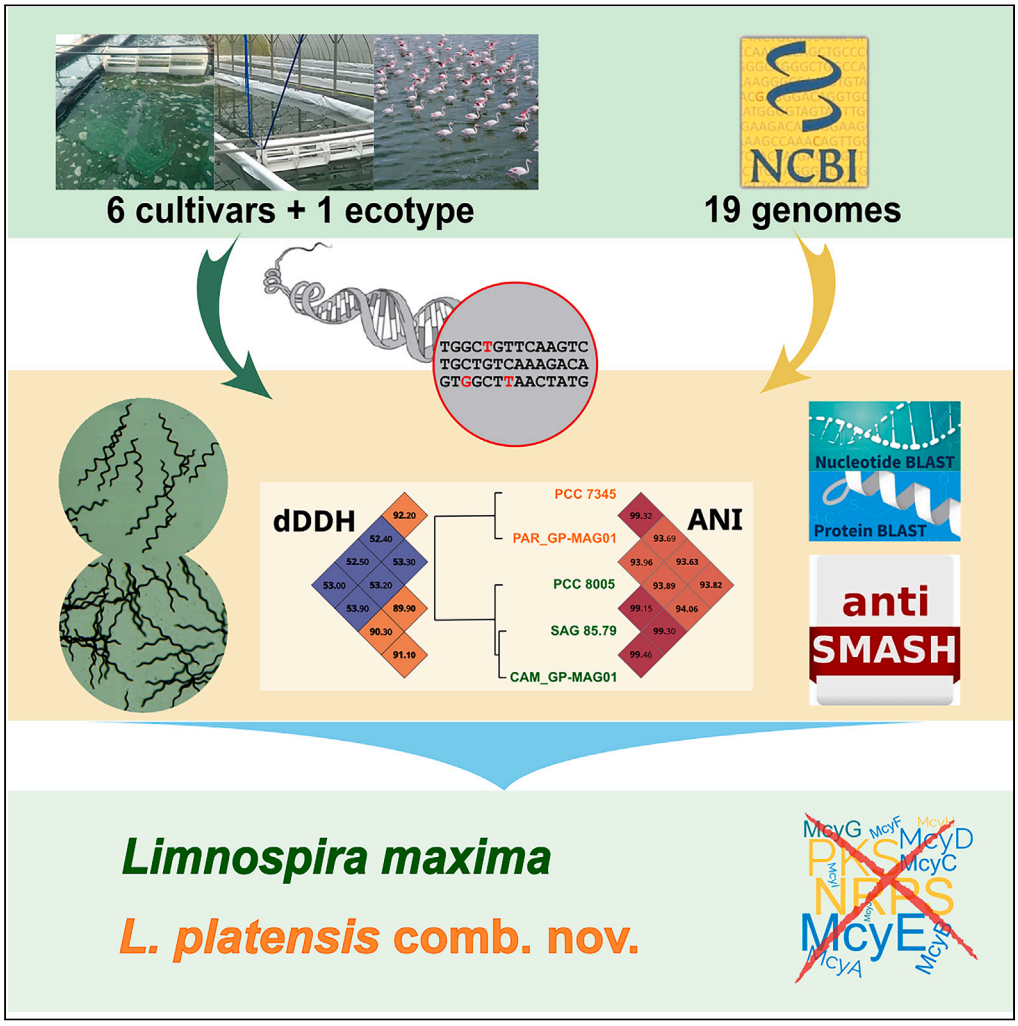


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## Highlights

The genus *Limnospira* comprises two species: *L. maxima* and *L. platensis* comb. nov.

The V3-V4 region of the 16S rRNA gene distinguishes these two *Limnospira* species

The genus *Limnospira* is unable to produce microcystins

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# The genus *Limnospira* contains only two species both unable to produce microcystins: *L. maxima* and *L. platensis* comb. nov.

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## SUMMARY

**Spirulina is the commercial name for edible cyanobacteria of the genus *Limnospira*. The taxonomy of this genus is confusing with four species distributed in two lineages. Furthermore, the species *Limnospira fusiformis* has been cited as toxic by potentially producing microcystins. Taxonomic ambiguity combined with suspected health concerns constitute a major issue for spirulina producers. In a collection of six cultivars and one ecotype, we identified strains of the two lineages through metagenetic and morphological analyses. We demonstrated that the genus *Limnospira* only comprises two distinct species according to genomic comparisons of three genomes obtained in this study and 19 reference genomes. We showed that the V3-V4 region of the 16S rRNA gene is sufficient to identify the genus *Limnospira* and to distinguish the two species. Toxinogenesis investigations on eleven genomes from each *Limnospira* species revealed no genes involved in cyanotoxin synthesis, reflecting the inability of this genus to produce microcystins.**

## INTRODUCTION

Spirulina is the traditional and commercial name for edible filamentous cyanobacteria, which was first assigned to the genera *Spirulina* and *Arthrospira*,[1] and more recently to the genus *Limnospira*.[2] Spirulina is naturally present in alkaline fresh water, rich in carbonates, bicarbonates, nitrates, phosphates, and iron. It grows mainly in the intertropical regions with a wide geographical distribution (Africa, Asia, and Americas) and also in Southern Europe.[3–6] Spirulina has long been used as human food, notably by the Kanembou (Lake Chad) and the Aztecs (Lake Texcoco).[7] Its exceptional nutritional value has led the United Nations Food and Agriculture Organization (FAO) to consider spirulina a food of major interest against malnutrition for the food security and the response to emergency food situations.[8] Spirulina also has strong potential for health, especially in the fields of cardiovascular diseases, viral infections, cancer prevention and therapy, immune response, as well as diabetes and cholesterol control.[9,10]

Many non-edible cyanobacteria are known to produce various cyanotoxins, and "blue-green algae" dietary supplements have long been suspected of containing toxic cyanobacteria and cyanotoxins.[11] In 2018, after a routine check to identify a new strain of spirulina, three French producers growing the *Arthrospira fusiformis* Camargue ecotype were forced to empty their ponds following a warning about this strain's suspected toxicity. Indeed, *Limnospira* (*Arthrospira*) *fusiformis* is cited in inventories of toxic cyanobacteria,[12,13] which refer to the publications of Ballot et al. reporting production of microcystins by Kenyan strains of spirulina classified as *A. fusiformis*.[14,15] Since then, numerous genomic studies on several spirulina strains have consistently shown the absence of toxicity of *Limnospira* sp.[6,16–18] However, none of these strains was identified as *L. fusiformis* in these publications. To reassure the producers and food safety authorities, one or more strains of *L. fusiformis* should be identified to confirm that this species cannot produce cyanotoxins.[19]

Another question for spirulina producers concerns the identification and naming of cultivated spirulina strains. *Arthrospira platensis* was for a long time considered as the only species of spirulina.[20–22] The United States and European authorities consider two species, *Arthrospira maxima* and *A. platensis*.[23,24] The species *A. fusiformis* is sometimes cited as a synonym of *A. platensis* or as a synonym of *A. maxima*.[24,25] When assessing commercially grown taxa in the light of the new genus *Limnospira*, the authors included three species (*L. fusiformis*, *Limnospira indica*, and *Limnospira maxima*) without ruling on the species *A. platensis*.[2] This assignment is compatible with the current "List of

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**Table 1. Cyanobacterial OTUs**

| | | | Samples | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| OTU | Taxa[1] | pid[2] | CAM_GP | ETH_PS | ETH_VR | IND_PP | PAR_GP | PAR_RB | SEN_CD |
| OTU1_TG (clade I) | *Limnospira fusiformis*, *L. maxima* | 100 | 19582 (95.28%) | 9199 (100%) | 25572 (88.76%) | 18618 (99.63%) | | 280 (11.02%) | 14954 (84.73%) |
| OTU1_AA (clade II) | *L. fusiformis*, *L. maxima*, *Arthrospira platensis* | 99.26 | | | 27 (0.09%) | 5 (0.03%) | 31784 (100%) | 2 (0.08%) | 19 (0.11%) |
| OTU6_CA | *Sodalinema* sp. | 100 | 2 (0.01%) | | 3210 (11.14%) | 65 (0.35%) | | 2079 (81.82%) | 2677 (15.17%) |
| OTU26 | *Cyanobium* sp. | 97.50 | 667 (3.25%) | | | | | | |
| OTU48 | *Cyanocohniella* sp. | 96.54 | 295 (1.44%) | | | | | | |
| OTU65 | *Haloleptolyngbya* sp. | 99.26 | | | | | | 180 (0.07%) | |
| OTU310 | *Nodosilinea* sp. | 97.77 | 5 (0.02%) | | | | | | |
| | | Totals | 20551 (100%) | 9199 (100%) | 28809 (100%) | 18688 (100%) | 31784 (100%) | 2541 (100%) | 17650 (100%) |

Reads and proportion of each sample.
[1]"Best hit" taxa from Blastn queries with 100% recovery query and higher percent identity.
[2]Percentage identity between OTU and "best Hit" taxa sequences.

Prokaryotic names with Standing in Nomenclature" (LPSN).[26] Several studies have shown the existence of two or three lineages,[3,6,27–29] but two recent studies proposed that the genus *Limnospira* is monospecific.[30,31] However, the "paracas" strain is perceived by producers as a species different from other cultivated *Limnospira* strains due to its morphotype and its higher productivity. This strain also appears to have a specific sensitivity to a cyanophage that causes "sudden and massive deaths."[32] The phylogenetic markers used in different studies—internally transcribed spacer (ITS), *gvp* gene cluster, 16S rRNA gene, phycocyanin intergenic spacer (*cpcBA*-IGS), and 16S rRNA-ITS sequences—did not allow to determine with certainty the number of species belonging to the genus *Limnospira*. DNA-DNA hybridization (DDH) is considered "the gold standard" for species delineation in prokaryotic taxonomy.[33,34] To our knowledge, no taxonomic study based on this method, either *in vitro* or *in silico*, has been conducted on *Limnospira* and *Arthrospira* genomes. Accordingly, the current study was aimed to determine the number of different bacterial species designated as "spirulina" and to clarify their nomenclature.

In this study, we present the results of 16S rRNA gene amplicon sequencing and metagenomic analysis, complemented with microscopic observations, carried out on seven spirulina culture samples of different origin, including the French "camarguaise" strain. The results of comparisons between 22 genomes, or metagenome-assembled genomes, carried out with the "average orthologous nucleotide identity" (OrthoANI) and "digital DNA-DNA hybridization" (dDDH) methods, allowed us to propose a taxonomic revision of the genus *Limnospira*. We also present an advanced genomic analysis of the identified *L. fusiformis* and other strains, targeting their potential ability to produce toxins. It is discussed if it is still advisable to mention *L. (A.) fusiformis* as a microcystin-producing species.

In the following, we use the genus names *Limnospira* and *Arthrospira* to refer to the spirulina strains, depending on the context. The strains mentioned in this study are named according to the names used in the GenBank database and other cited studies. Taxonomic changes will be proposed in the discussion.

## RESULTS

### 16S rRNA gene amplicon sequencing

The 16S rRNA gene sequences (400–405 bp) resulting from amplicon sequencing of the V3 and V4 hypervariable regions allowed the identification of two operational taxonomic units (OTUs) corresponding to *Limnospira* (*Arthrospira*) and five OTUs corresponding to other cyanobacteria (Table 1).

Concerning OTU1_AA, the alignments obtained with *L. fusiformis* and *L. maxima* on the one hand, and with *A. platensis* on the other hand have the same percentage identity (402/405, 99.26%), but the three nucleotide differences do not concern the same alignment positions.

Examination of the alignment of *Limnospira* OTUs sequences with other sequences from spirulina strains—notably the reference strains of the type species *L. maxima* UTEX 2720, *L. fusiformis* SAG 85.79, *L. indica* PCC 8005, and *A. platensis* PCC 7345—revealed three diagnostic nucleotide sites—namely positions 211, 237, and 241—that allowed us to differentiate between the two clades (Figure 1). OTU1_TG was found in *L. maxima*, *L. fusiformis*, and *L. indica* strains, while OTU1_AA is characteristic for *A. platensis*.

Regardless of its comparably short sequence length, the V3-V4 region of the 16S rRNA gene is sufficient to distinguish between the two clades of *Limnospira* sp., which is in accordance with the phylogenomic trees and comparative genome analyses conducted in this study (see the following).

### Morphology and ultrastructure

The seven spirulina culture samples exhibit a great diversity of *Limnospira* morphotypes, including spiral or helical (Figures 2A, 2B, 3A, and 3B), tightly coiled (Figures 2C and 3C), wavy or weakly spiral (Figures 2D and 3G), and straight trichomes (Figures 3D–3F).

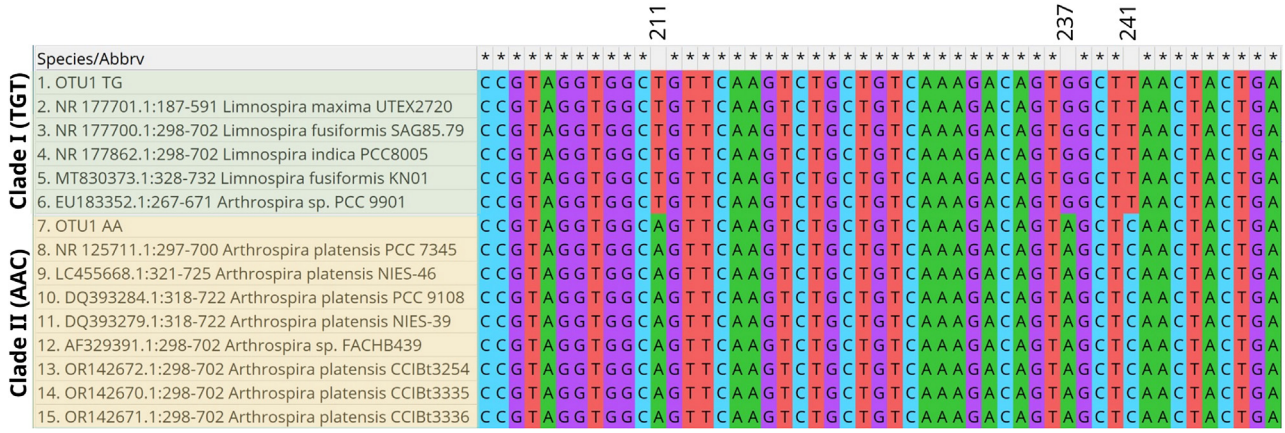


**Figure 1. Alignment position 200 to 250 of the V3-V4 region of the 16S rRNA gene**

Differences between OTU1_TG (clade I) and OTU1_AA (clade II) are located in positions 211, 237, and 241. Complete sequences in Table S1.

The CAM_GP and ETH_PS samples are composed of *Limnospira* spiral trichomes (Figures 2A, 2B, 3A, and 3B) that match the "*fusiformis*" species description.[35]

The IND_PP sample is composed of *Limnospira* tightly coiled trichomes corresponding to the "Lonar" strain description (Figure 3C).[21] Under laboratory conditions, the original monomorphotype (Figure 3C) evolved toward several morphotypes with helical trichomes (Figure 3H), which correspond to a former report of "*Arthrospira maxima*" strains.[5]

The ETH_VR and SEN_CD samples are mainly composed of *Limnospira* straight trichomes and are heavily contaminated by a filamentous cyanobacterium, a putative representative of the genus *Sodalinema* (Figures 3D and 3E). In these samples, some specimens are spiraled and match the "*fusiformis*" species description.[35]

The PAR_RB sample, a mixture of spirulina strains from both clades, was according to the 16S rRNA gene amplicon sequencing analysis also contaminated with a cyanobacterium of the genus *Sodalinema* (Table 1). It is mainly composed of straight *Limnospira* trichomes and some weakly spiraled specimens like the "paracas" strain (Figure 3F).[21,31,36]

The PAR_GP sample is composed of weakly spiraled *Limnospira* trichomes corresponding to the "paracas" strain trichome shape (Figure 3G).

Clade I samples (CAM_GP, ETH_PS, IND_PP, ETH_VR, SEN_CD, and PAR_RB; Table 1) present irregular helical shapes, with a pitch generally smaller than the trichome diameter, and with gradually attenuated trichome and cells diameters at the ends. We have observed a development from these screw-like coiled trichomes toward straight shapes in all our clade I samples in laboratory cultures. The clade II sample, the "paracas" strain (PAR-GP), presents a regular helical shape with a pitch approximately double the diameter. This shape appears wavy in light microscopy (LM) observations (Figure 3G). The morphology of this strain remains stable, without the appearance of straight shapes, in laboratory culture for more than three years.

The presence of a calyptra, a characteristic of the genus *Limnospira*,[2] is observed on most trichome ends in both lineages. However, the calyptra and the attenuation of the diameter of the trichome appear to be more pronounced for the "*fusiformis*"-like strains (Figures 4A–4D).

Transmission electronic microscopy (TEM) observations did not reveal any remarkable differences between the two clades of *Limnospira* (Figure S1). In particular, we observed a thickened cell wall or calyptra at the end of trichomes and thylakoids with an irregular arrangement in PAR_GP samples (clade II, Figures 4E and 4F), as described by Nowicka-Krawczyk et al. for *L. fusiformis* strains belonging to clade I.[2]

## Metagenome assembly and binning of spirulina

Metagenome sequencing, assembly and binning of two spirulina cultures from clade I (ETH_PS and CAM_GP) and one culture from clade II (PAR_GP) resulted in a single cyanobacterial metagenome-assembled genome (MAG) of the genus *Limnospira* per sample (Table 2). All three MAGs have a good quality with a calculated completeness of at least 96% and a contamination rate of less than 1% irrespective of the comparably large number of contigs. Mapping of the Illumina reads on the MAGs resulted in 1600-fold to 2900-fold genome coverage, which is nearly two orders of magnitude greater than required for reliable binning. For future studies, the sequencing depth, which was comparable to a former metagenome study of non-axenic limnic cyanobacteria,[37] could be significantly reduced, thus paving the way for cost-effective metagenomic monitoring of spirulina cultivation. The analysis of the *Limnospira* MAGs showed a comparable G + C content (44.4%–44.7%), coding density (0.83–0.84) and a draft genome size ranging between 4.9 Mbp and 5.4 Mbp (Table 2). Accordingly, metagenome sequencing revealed similar genomic characteristics for *Limnospira* strains of both clades.

## Phylogenomic analyses

Our three newly established MAGs were analyzed with 19 reference genomes of the genus *Limnospira* and ten genomes from closely related cyanobacteria all belonging to clade A of the cyanobacterial species tree.[37,38] The phylogenomic protein tree based on 18,746 variable amino

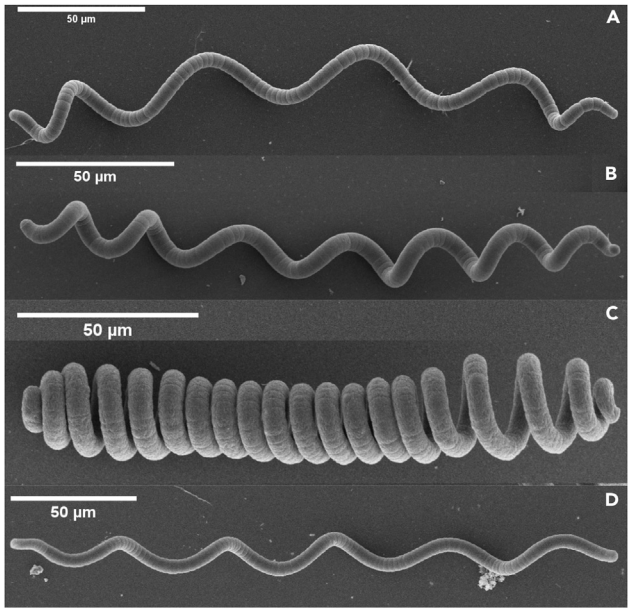


**Figure 2. Scanning electronic microscopy (SEM) pictures of *Limnospira* strains**
CAM_GP (A), ETH_PS (B), IND_PP (C), and PAR_GP (D). Scale bar: 50 µm.

acid positions showed a common branching of all 22 analyzed *Limnospira* genomes in a distinct subtree that is supported by 100 bootstrap proportion (BP; Figure S2). The *Limnospira* subtree is divided in two distinct clades both supported by 100% BP. Clade I comprise two newly established metagenomes (CAM_GP and ETH_PS), several genomes of strains that are designated *L. maxima*, *L. indica*, and *L. fusiformis*, and

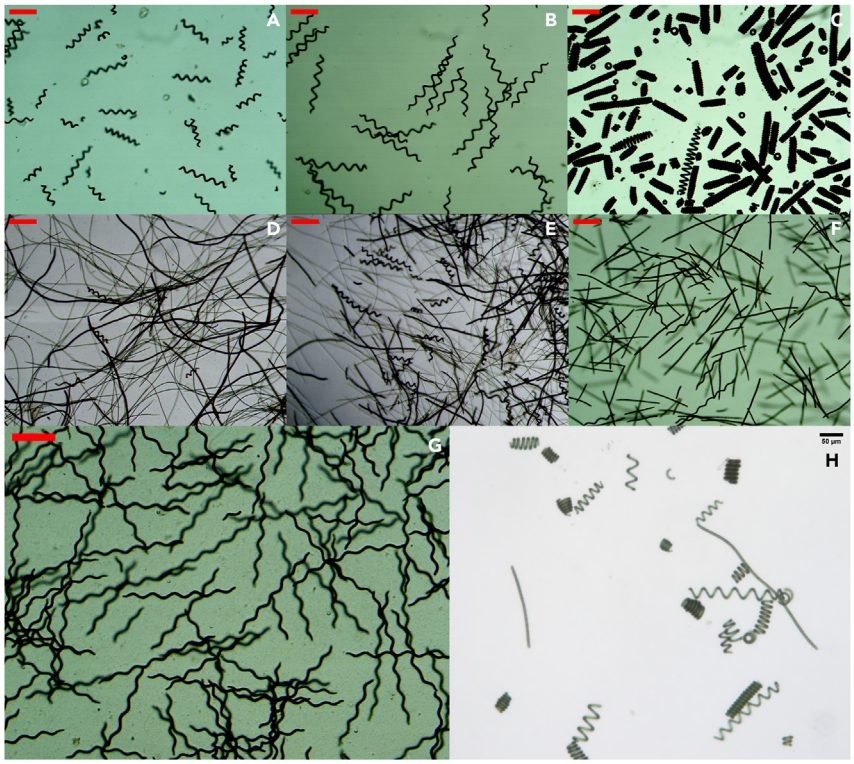


**Figure 3. Light microscopy (LM) views (f-m) of spirulina strains**
CAM_GP (A), ETH_PS (B), IND_PP (C and H), ETH_VR (D), SEN_CD (E), PAR_RB (F), and PAR_GP (G). Scale bar: 50 µm.

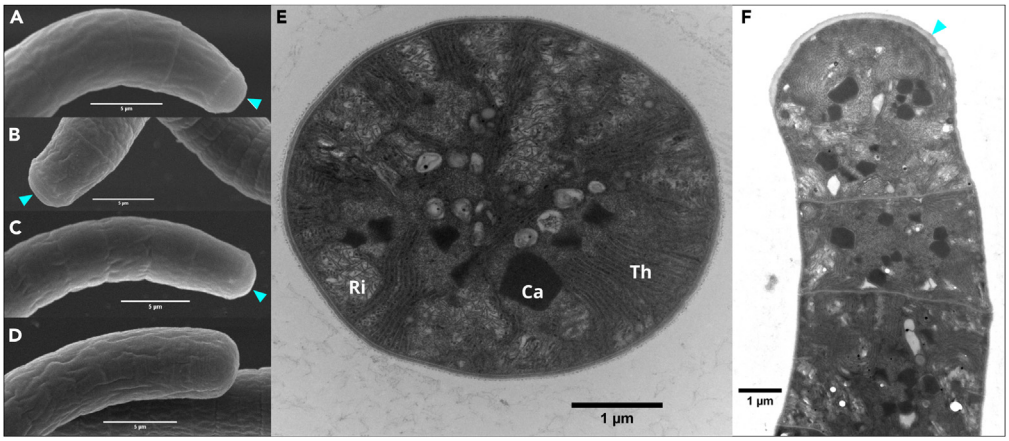


**Figure 4. SEM views (scale bar: 5 µm) of *Limnospira***

CAM_GP (A), ETH_PS (B), PAR_GP (C and D) showing ends of trichomes and TEM views (scale bar: 1µ m) of *Limnospira* - PAR_GP (E and F) showing the ultrastructure including thylakoid membranes (Th), ribosomes (Ri), and carboxysomes (Ca). Blue triangles indicate the calyptra at the end of the trichomes.

it contains also putative "*Arthrospira*" genomes that were apparently misclassified (strain C1) or unclassified (strains O9.13F, TJSD091, and TJSD092). Clade II comprises genomes of different *L. platensis* strains including the new MAG from the PAR_GP culture. However, the internal branching pattern especially of clade I remained unresolved and the position of the comparably fast evolving *Arthrospira* sp. O9.13F genome might be influenced by long-branch attraction.[39]

With the aim of a better phylogenetic resolution, we reanalyzed the relationships of the 22 *Limnospira* strains with a maximum likelihood (ML) tree based on 32,918 variable nucleotide positions (Figure 5). The subanalysis showed a distinct branching pattern of the eleven *A. platensis* strains in clade II, comprising a common branch (98% BP) with three subtrees ([1] PCC 7345 – NIES-39, [2] Paraca, PAR_GP, and [3] YZ – FACHB-971) and *A. platensis* FACHB-439 in a basal position. In contrast, the close relationship of the strains in clade 1 with a largely unresolved topology was confirmed by the nucleotide tree. A notable finding was the common branching of *Arthrospira* sp. O9.13F with *L. indica* PCC 8005 and our *L. maxima* ETH_PS MAG, which is supported by 100% BP regardless of its accelerated evolutionary rate. Our phylogenomic analyses, as well as a previous phylogenetic analyze based on 16S-ITS (Roussel et al., 2023), showed that the supposed *Arthrospira* strains C1, O9.13F, TJSD091, and TJSD092 are located in clade I. However, our phylogenetic analyses showed a very close relationship of all strains of clade I, but provided no evidence for the presence of distinct lineages corresponding to the three previously proposed species (*L. maxima*, *L. fusiformis*, and *L. indica*).

## Average Nucleotide Identity

The 231 pairwise orthologous average nucleotide identity (OrthoANI) comparisons among 22 *Limnospira* and *Arthrospira* strains are presented in Figure 6.

The *Limnospira* OrthoANI analysis is consistent with the results of the phylogenomic analyses (Figures 5 and S2) and allows to distinguish between the two clades (Figure 6). Clade I include all strains designated as *L. maxima*, *L. fusiformis*, and *L. indica*. Clade II includes only strains of the *A. platensis* lineage. The intra-clade OrthoANI values are greater than 98.7% with an average of 99.3% for clade I and 99.4% for clade II. The OrthoANI values between strains of different clades are ranging between 93.5% and 94.7% with an average of 93.9%. All values are below the ANI threshold of 95% (or 96%), which is generally accepted as the intra-species boundary for prokaryotes.[40] Accordingly, all taxa of clade I belong to the same bacterial species, while the taxa of clade II represent a closely related second species of the genus *Limnospira*.

**Table 2. *Limnospira* MAGs and their characteristics**

| Sample-MAG | Clade | Taxon[1] | Completeness (%) | Contamination (%) | Mean contig length | N50 (contigs) | Coding density | # contigs | Genome size | G + C (%) | Coverage |
|---|---|---|---|---|---|---|---|---|---|---|---|
| ETH_PS-MAG01 | I | *Limnospira fusiformis* | 97.09 | 00.44 | 6052 | 16808 | 0.83 | 881 | 5,332,277 | 44.67 | 1613 |
| CAM_GP-MAG01 | I | *Limnospira fusiformis* | 96.00 | 00.66 | 13773 | 20213 | 0.84 | 356 | 4,903,374 | 44.51 | 2912 |
| PAR_GP-MAG01 | II | « *Limnospira platensis* » | 98.14 | 00.00 | 7946 | 33073 | 0.83 | 678 | 5,387,508 | 44.43 | 1958 |

[1]According GTDB-Tk classification.

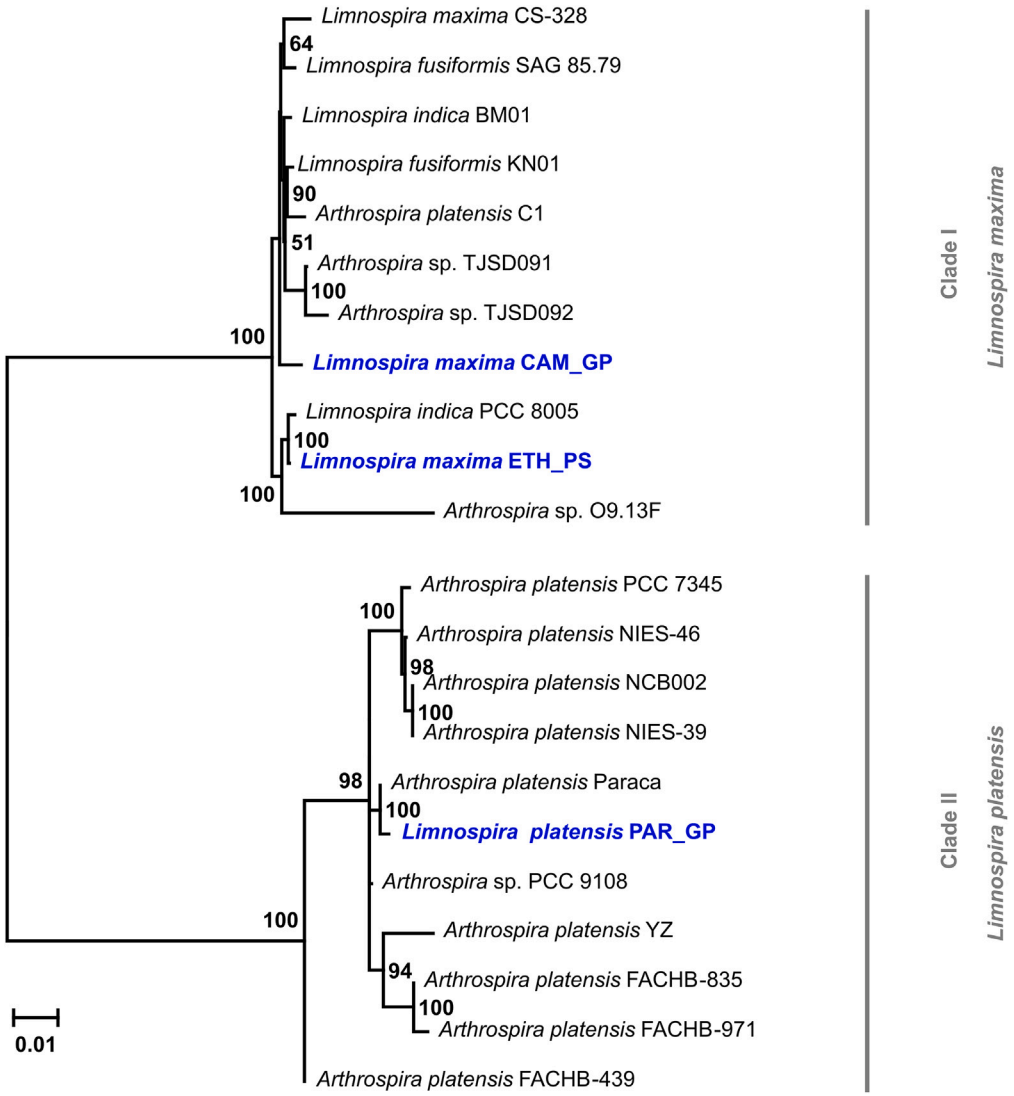


**Figure 5. Phylogenomic maximum likelihood tree of three newly established *Limnospira* Metagenome-Assembled Genomes (MAGs) from spirulina cultures (CAM_GP, ETH_PS, and PAR_GP) and 19 cyanobacterial reference genomes from the genus Limnospira**

The RaxML tree was constructed from 32,918 variable nucleotide positions based on an amino acid alignment of 92 housekeeping genes under the GTR4Γ model using 100 bootstrap replicates. Bootstrap values >50% are shown at the nodes. MAGs from the current study are highlighted in blue and bold. Genome assembly accession numbers are listed in key resources table. The bar represents relative number of changes per amino acid position.

## Digital DNA-DNA hybridization

The 231 pairwise genome comparisons using the dDDH among 22 *Limnospira* and *Arthrospira* strains are presented in Figure 7.

In agreement with the phylogenomic trees (Figures 5 and S2) and the OrthoANI analysis (Figure 6), the dDDH analysis allowed to distinguish between clade I and II (Figure 7). The intra-clade dDDH values are ranging between 85.4% and 99.4%. The inter-clade dDDH values are much lower at 49.6%–56.6%. They are clearly below the threshold of 70% for the delineation of two bacterial species,[34,41] which confirms the conclusion of our OrthoANI analysis that clade I and clade II represent two different species of the genus *Limnospira*. In addition, the high dDDH values for all strains except O9.13F from clade I (≥89.9%) reflect their very close relationship. *Arthrospira* O9.13F has the most divergent genome of clade I (dDDH: 85.4%–89.3%), but nevertheless definitely belongs to the same species. This conclusion is independently supported by the placement of strain O9.13F in the phylogenomic tree (Figure 5). The contradictory position within a 16S-ITS tree in a recent study reflects the rather limited phylogenetic resolution of the 16S rRNA gene.[31]

## Toxinogenesis

Blastn searches with the microcystin gene clusters of *Microcystis aeruginosa* K-139, *Planktothrix agardhii* NIVA-CY 126/8, and *Anabaena* sp. 90 (see method details section) revealed no hits in the three *Limnospira* MAGs established in the current study and 19 reference genomes of

| | | Clade I | | | | | | | | | | | Clade II | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Strain | *L. maxima* CS-328 | *L. fusiformis* SAG85.79 | *L. indica* BM01 | *L. fusiformis* KN01 | *A. platensis* C1 | *Arthrospira* sp. TJSD091 | *Arthrospira* sp. TJSD092 | ***L. maxima* CAM_GP** | *L. indica* PCC8005 | ***L. maxima* ETH_PS** | *Arthrospira* sp. O9.13F | *A. platensis* PCC 7345 | *A. platensis* NIES-46 | *A. platensis* NCB002 | *A. platensis* NIES-39 | *A. platensis* paraca | ***L. platensis* PAR_GP** | *Arthrospira* sp. PCC 9108 | *A. platensis* YZ | *A. platensis* FACHB-835 | *A. platensis* FACHB-971 | *A. platensis* FACHB-439 |
| Clade I | *L. maxima* CS-328 | | 99.42 | 99.42 | 99.43 | 99.35 | 99.38 | 99.37 | 99.42 | 99.18 | 99.21 | 98.73 | 93.67 | 93.63 | 93.61 | 93.60 | 93.77 | 93.88 | 93.72 | 93.99 | 94.18 | 94.12 | 94.45 |
| | *L. fusiformis* SAG85.79 | 99.42 | | 99.40 | 99.44 | 99.39 | 99.31 | 99.31 | 99.46 | 99.15 | 99.25 | 98.72 | 93.63 | 93.65 | 93.59 | 93.57 | 93.70 | 93.89 | 93.67 | 93.93 | 94.11 | 94.10 | 94.41 |
| | *L. indica* BM01 | 99.42 | 99.40 | | 99.52 | 99.40 | 99.45 | 99.39 | 99.46 | 99.33 | 99.31 | 98.81 | 93.67 | 93.71 | 93.65 | 93.62 | 93.71 | 93.94 | 93.75 | 94.02 | 94.08 | 94.05 | 94.42 |
| | *L. fusiformis* KN01 | 99.43 | 99.44 | 99.52 | | 99.43 | 99.45 | 99.46 | 99.54 | 99.30 | 99.37 | 98.84 | 93.75 | 93.69 | 93.77 | 93.72 | 93.89 | 93.92 | 93.76 | 94.15 | 94.15 | 94.13 | 94.48 |
| | *A. platensis* C1 | 99.35 | 99.39 | 99.40 | 99.43 | | 99.34 | 99.33 | 99.43 | 99.16 | 99.23 | 98.70 | 93.64 | 93.66 | 93.53 | 93.54 | 93.69 | 93.84 | 93.59 | 93.98 | 94.10 | 94.08 | 94.38 |
| | *Arthrospira* sp. TJSD091 | 99.38 | 99.31 | 99.45 | 99.45 | 99.34 | | 99.78 | 99.44 | 99.19 | 99.23 | 98.71 | 93.73 | 93.75 | 93.66 | 93.65 | 93.80 | 93.93 | 93.77 | 94.09 | 94.17 | 94.10 | 94.54 |
| | *Arthrospira* sp. TJSD092 | 99.37 | 99.31 | 99.39 | 99.46 | 99.33 | 99.78 | | 99.44 | 99.17 | 99.21 | 98.76 | 93.70 | 93.73 | 93.73 | 93.65 | 93.83 | 93.92 | 93.70 | 94.09 | 94.12 | 94.16 | 94.53 |
| | ***L. maxima* CAM_GP** | 99.42 | 99.46 | 99.46 | 99.54 | 99.43 | 99.44 | 99.44 | | 99.30 | 99.34 | 98.87 | 93.82 | 93.87 | 93.85 | 93.86 | 93.93 | 94.06 | 93.96 | 94.28 | 94.31 | 94.31 | 94.67 |
| | *L. indica* PCC8005 | 99.18 | 99.15 | 99.33 | 99.30 | 99.16 | 99.19 | 99.17 | 99.30 | | 99.69 | 98.99 | 93.69 | 93.72 | 93.67 | 93.59 | 93.69 | 93.96 | 93.70 | 94.03 | 94.08 | 94.04 | 94.40 |
| | ***L. maxima* ETH_PS** | 99.21 | 99.25 | 99.31 | 99.37 | 99.23 | 99.23 | 99.21 | 99.34 | 99.69 | | 98.99 | 93.83 | 93.78 | 93.82 | 93.79 | 93.92 | 94.08 | 93.95 | 94.21 | 94.27 | 94.18 | 94.60 |
| | *Arthrospira* sp. O9.13F | 98.73 | 98.72 | 98.81 | 98.84 | 98.70 | 98.71 | 98.76 | 98.87 | 98.99 | 98.99 | | 93.62 | 93.59 | 93.62 | 93.59 | 93.74 | 93.80 | 93.66 | 93.91 | 94.00 | 93.99 | 94.26 |
| Clade II | *A. platensis* PCC 7345 | 93.67 | 93.63 | 93.67 | 93.75 | 93.64 | 93.73 | 93.70 | 93.82 | 93.69 | 93.83 | 93.62 | | 99.61 | 99.62 | 99.61 | 99.49 | 99.32 | 99.53 | 99.28 | 99.32 | 99.26 | 99.01 |
| | *A. platensis* NIES-46 | 93.63 | 93.65 | 93.71 | 93.69 | 93.66 | 93.75 | 93.73 | 93.87 | 93.72 | 93.78 | 93.59 | 99.61 | | 99.77 | 99.76 | 99.52 | 99.28 | 99.54 | 99.30 | 99.33 | 99.32 | 99.04 |
| | *A. platensis* NCB002 | 93.61 | 93.59 | 93.65 | 93.77 | 93.53 | 93.66 | 93.73 | 93.85 | 93.67 | 93.82 | 93.62 | 99.62 | 99.77 | | 99.86 | 99.58 | 99.26 | 99.58 | 99.32 | 99.35 | 99.27 | 99.09 |
| | *A. platensis* NIES-39 | 93.60 | 93.57 | 93.62 | 93.72 | 93.54 | 93.65 | 93.65 | 93.86 | 93.59 | 93.79 | 93.59 | 99.61 | 99.76 | 99.86 | | 99.54 | 99.27 | 99.56 | 99.27 | 99.34 | 99.32 | 99.10 |
| | *A. platensis* paraca | 93.77 | 93.70 | 93.71 | 93.89 | 93.69 | 93.80 | 93.83 | 93.93 | 93.69 | 93.92 | 93.74 | 99.49 | 99.52 | 99.58 | 99.54 | | 99.71 | 99.64 | 99.42 | 99.48 | 99.44 | 99.17 |
| | ***L. platensis* PAR_GP** | 93.88 | 93.89 | 93.94 | 93.92 | 93.84 | 93.93 | 93.92 | 94.06 | 93.96 | 94.08 | 93.80 | 99.32 | 99.28 | 99.26 | 99.27 | 99.71 | | 99.52 | 99.33 | 99.37 | 99.33 | 99.02 |
| | *Arthrospira* sp. PCC 9108 | 93.72 | 93.67 | 93.75 | 93.76 | 93.59 | 93.77 | 93.70 | 93.96 | 93.70 | 93.95 | 93.66 | 99.53 | 99.54 | 99.58 | 99.56 | 99.64 | 99.52 | | 99.56 | 99.67 | 99.60 | 99.36 |
| | *A. platensis* YZ | 93.99 | 93.93 | 94.02 | 94.15 | 93.98 | 94.09 | 94.09 | 94.28 | 94.03 | 94.21 | 93.91 | 99.28 | 99.30 | 99.32 | 99.27 | 99.42 | 99.33 | 99.56 | | 99.48 | 99.43 | 99.21 |
| | *A. platensis* FACHB-835 | 94.18 | 94.11 | 94.08 | 94.15 | 94.10 | 94.17 | 94.12 | 94.31 | 94.08 | 94.27 | 94.00 | 99.32 | 99.33 | 99.35 | 99.34 | 99.48 | 99.37 | 99.67 | 99.48 | | 99.86 | 99.28 |
| | *A. platensis* FACHB-971 | 94.12 | 94.10 | 94.05 | 94.13 | 94.08 | 94.10 | 94.16 | 94.31 | 94.04 | 94.18 | 93.99 | 99.26 | 99.32 | 99.27 | 99.32 | 99.44 | 99.33 | 99.60 | 99.43 | 99.86 | | 99.25 |
| | *A. platensis* FACHB-439 | 94.45 | 94.41 | 94.42 | 94.48 | 94.38 | 94.54 | 94.53 | 94.67 | 94.40 | 94.60 | 94.26 | 99.01 | 99.04 | 99.09 | 99.10 | 99.17 | 99.02 | 99.36 | 99.21 | 99.28 | 99.25 | |

**Figure 6. Pairwise OrthoANI among *Limnospira* and *Arthrospira* strains**

*Limnospira* and *Arthrospira* (see key resources table), which documents the absence of very closely related sequences. Analogous blastp and tblastn analyses with Myc proteins for microcystin biosynthesis (see method details section) did not result in the detection of any enzymes related to non-ribosomal peptide synthases or polyketide synthases. Accordingly, our homology searches revealed no indication for the presence of genes related to the biosynthesis of this cyanotoxin. In order to confirm this conclusion, our three newly established MAGs and 19 reference genomes of the genus *Limnospira* (*Arthrospira*) were analyzed with the antiSMASH 7.1.0 software. However, neither non-ribosomal peptide synthase (NRPS) nor polyketide synthase (PKS) clusters could be detected in any of the analyzed genomes. This result allowed us to conclude that none of the analyzed 22 strains of the genus *Limnospira* (*Arthrospira*) has the genetic capacity to produce microcystins or other cyanotoxins whose biosynthesis gene clusters are known.

## DISCUSSION

### Taxonomy

There is a consensus on the existence of two distinct lineages in the genus *Limnospira*—using chronologically ITS region, *gvp* gene cluster, 16S rRNA gene, *cpcBA*-IGS, ANI, *is*DDH, and 16S-ITS[3,6,27,29–31]—which should be distinguished according to the criteria of prokaryotic taxonomy to solve the dilemma of the four species described under the "Botanical Code" (ICN, *L. maxima*, *L. fusiformis*, *L. indica*, "*L.* [*A.*] *platensis*"). However, a recent publication, based on the analysis of the 16S rRNA gene, 16S-ITS sequences, ANI and morphological characteristics, concluded that the genus *Limnospira* is monospecific with two distinct clades.[31] The inter-clade ANI value reported in the aforementioned publication (93.4%) should lead to the conclusion that the genus *Limnospira* contains two species when using the ANI threshold of 95 or 96% for the differentiation between species.[40,42] Values generated by the original ANI and OrthoANI are comparable,

| | | Clade I | | | | | | | | | | | Clade II | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Strain | *L. maxima* CS-328 | *L. fusiformis* SAG85.79 | *L. indica* BM01 | *L. fusiformis* KN01 | *A. platensis* C1 | *Arthrospira* sp. TJSD091 | *Arthrospira* sp. TJSD092 | ***L. maxima* CAM_GP** | *L. indica* PCC8005 | ***L. maxima* ETH_PS** | *Arthrospira* sp. O9.13F | *A. platensis* PCC 7345 | *A. platensis* NIES-46 | *A. platensis* NCB002 | *A. platensis* NIES-39 | *A. platensis* paraca | ***L. platensis* PAR_GP** | *Arthrospira* sp. PCC 9108 | *A. platensis* YZ | *A. platensis* FACHB-835 | *A. platensis* FACHB-971 | *A. platensis* FACHB-439 |
| Clade I | *L. maxima* CS-328 | | 92.9 | 93.5 | 92.9 | 93.0 | 92.4 | 92.6 | 91.5 | 90.4 | 91.1 | 86.0 | 52.5 | 51.9 | 49.9 | 49.9 | 50.9 | 53.3 | 50.4 | 52.0 | 54.3 | 54.0 | 56.2 |
| | *L. fusiformis* SAG85.79 | 92.9 | | 92.7 | 92.3 | 92.4 | 91.7 | 91.7 | 91.1 | 89.9 | 90.9 | 85.5 | 52.5 | 51.7 | 49.7 | 49.7 | 50.8 | 53.2 | 50.2 | 51.9 | 54.1 | 53.8 | 55.9 |
| | *L. indica* BM01 | 93.5 | 92.7 | | 94.0 | 93.0 | 93.0 | 93.1 | 92.1 | 95.2 | 92.6 | 86.8 | 52.7 | 52.1 | 50.3 | 50.3 | 51.1 | 53.4 | 50.7 | 52.3 | 54.4 | 54.1 | 56.0 |
| | *L. fusiformis* KN01 | 92.9 | 92.3 | 94.0 | | 92.3 | 93.0 | 92.6 | 93.8 | 91.3 | 92.7 | 87.3 | 52.4 | 51.9 | 50.9 | 50.9 | 51.8 | 53.2 | 51.3 | 52.8 | 54.2 | 53.9 | 55.4 |
| | *A. platensis* C1 | 93.0 | 92.4 | 93.0 | 92.3 | | 92.1 | 92.5 | 90.7 | 90.0 | 90.7 | 85.4 | 52.3 | 51.6 | 49.7 | 49.8 | 50.6 | 53.0 | 50.3 | 51.8 | 54.0 | 53.8 | 55.7 |
| | *Arthrospira* sp. TJSD091 | 92.4 | 91.7 | 93.0 | 93.0 | 92.1 | | 96.9 | 92.0 | 90.2 | 91.2 | 86.1 | 52.6 | 52.0 | 50.2 | 50.3 | 51.3 | 53.3 | 50.6 | 52.3 | 54.2 | 53.9 | 55.7 |
| | *Arthrospira* sp. TJSD092 | 92.6 | 91.7 | 93.1 | 92.6 | 92.5 | 96.9 | | 90.8 | 90.2 | 90.8 | 85.5 | 52.6 | 51.9 | 49.8 | 49.9 | 50.9 | 53.2 | 50.2 | 51.9 | 54.2 | 53.8 | 56.0 |
| | ***L. maxima* CAM_GP** | 91.5 | 91.1 | 92.1 | 93.8 | 90.7 | 92.0 | 90.8 | | 90.3 | 92.9 | 87.6 | 53.0 | 52.5 | 51.1 | 51.1 | 51.9 | 53.9 | 51.5 | 53.0 | 54.9 | 54.6 | 55.9 |
| | *L. indica* PCC8005 | 90.4 | 89.9 | 95.2 | 91.3 | 90.0 | 90.2 | 90.2 | 90.3 | | 95.4 | 87.8 | 52.4 | 51.9 | 49.9 | 50.0 | 50.8 | 53.3 | 50.3 | 51.9 | 54.0 | 53.8 | 55.7 |
| | ***L. maxima* ETH_PS** | 91.1 | 90.9 | 92.6 | 92.7 | 90.7 | 91.2 | 90.8 | 92.9 | 95.4 | | 89.3 | 53.6 | 53.0 | 51.6 | 51.7 | 52.4 | 54.4 | 52.0 | 53.5 | 55.4 | 55.1 | 56.6 |
| | *Arthrospira* sp. O9.13F | 86.0 | 85.5 | 86.8 | 87.3 | 85.4 | 86.1 | 85.5 | 87.6 | 87.8 | 89.3 | | 52.3 | 51.7 | 49.6 | 49.7 | 50.5 | 53.0 | 50.1 | 51.5 | 54.0 | 53.7 | 55.0 |
| Clade II | *A. platensis* PCC 7345 | 52.5 | 52.5 | 52.7 | 52.4 | 52.3 | 52.6 | 52.6 | 53.0 | 52.4 | 53.6 | 52.3 | | 94.5 | 92.7 | 93.0 | 92.3 | 92.2 | 92.3 | 90.4 | 91.4 | 91.6 | 89.2 |
| | *A. platensis* NIES-46 | 51.9 | 51.7 | 52.1 | 51.9 | 51.6 | 52.0 | 51.9 | 52.5 | 51.9 | 53.0 | 51.7 | 94.5 | | 95.1 | 95.2 | 92.8 | 91.6 | 92.5 | 90.6 | 92.8 | 92.6 | 90.3 |
| | *A. platensis* NCB002 | 49.9 | 49.7 | 50.3 | 50.9 | 49.7 | 50.2 | 49.8 | 51.1 | 49.9 | 51.6 | 49.6 | 92.7 | 95.1 | | 99.4 | 96.4 | 89.5 | 94.0 | 91.4 | 89.8 | 89.0 | 87.9 |
| | *A. platensis* NIES-39 | 49.9 | 49.7 | 50.3 | 50.9 | 49.8 | 50.3 | 49.9 | 51.1 | 50.0 | 51.7 | 49.7 | 93.0 | 95.2 | 99.4 | | 96.8 | 89.6 | 94.1 | 91.6 | 90.0 | 89.2 | 88.0 |
| | *A. platensis* paraca | 50.9 | 50.8 | 51.1 | 51.8 | 50.6 | 51.3 | 50.9 | 51.9 | 50.8 | 52.4 | 50.5 | 92.3 | 92.8 | 96.4 | 96.8 | | 94.0 | 96.1 | 93.4 | 92.0 | 91.2 | 89.7 |
| | ***L. platensis* PAR_GP** | 53.3 | 53.2 | 53.4 | 53.2 | 53.0 | 53.3 | 53.2 | 53.9 | 53.3 | 54.4 | 53.0 | 92.2 | 91.6 | 89.5 | 89.6 | 94.0 | | 91.9 | 90.3 | 92.1 | 92.4 | 89.8 |
| | *Arthrospira* sp. PCC 9108 | 50.4 | 50.2 | 50.7 | 51.3 | 50.3 | 50.6 | 50.2 | 51.5 | 50.3 | 52.0 | 50.1 | 92.3 | 92.5 | 94.0 | 94.1 | 96.1 | 91.9 | | 95.7 | 93.5 | 92.5 | 91.2 |
| | *A. platensis* YZ | 52.0 | 51.9 | 52.3 | 52.8 | 51.8 | 52.3 | 51.9 | 53.0 | 51.9 | 53.5 | 51.5 | 90.4 | 90.6 | 91.4 | 91.6 | 93.4 | 90.3 | 95.7 | | 91.9 | 91.1 | 90.4 |
| | *A. platensis* FACHB-835 | 54.3 | 54.1 | 54.4 | 54.2 | 54.0 | 54.2 | 54.2 | 54.9 | 54.0 | 55.4 | 54.0 | 91.4 | 92.8 | 89.8 | 90.0 | 92.0 | 92.1 | 93.5 | 91.9 | | 97.9 | 93.0 |
| | *A. platensis* FACHB-971 | 54.0 | 53.8 | 54.1 | 53.9 | 53.8 | 53.9 | 53.8 | 54.6 | 53.8 | 55.1 | 53.7 | 91.6 | 92.6 | 89.0 | 89.2 | 91.2 | 92.4 | 92.5 | 91.1 | 97.9 | | 92.7 |
| | *A. platensis* FACHB-439 | 56.2 | 55.9 | 56.0 | 55.4 | 55.7 | 55.7 | 56.0 | 55.9 | 55.7 | 56.6 | 55.0 | 89.2 | 90.3 | 87.9 | 88.0 | 89.7 | 89.8 | 91.2 | 90.4 | 93.0 | 92.7 | |

**Figure 7. Pairwise dDDH among *Limnospira* and *Arthrospira* strains**

and the cut-off for species delineation is 95%~96% for both methods.[43] The OrthoANI values for *Limnospira* genomes compared in this study clearly support the presence of two species based on inter-clade OrthoANI values ranging between 93.5% and 94.6% and an intra-clade OrthoANI above 98.7% for clade I and above 99.0% for clade II. These results are comparable to those obtained for other genera of cyanobacteria. Species delineation in the cyanobacterial genera *Raphidiopsis* and *Microcystis* could be assessed with species boundaries occurring at about 96% ANI at the genomic level.[44] ANI analysis on three *Microcoleus* species revealed inter-species ANI values between 86% and 93% and intra-species ANI values of more than 98%.[45]

DDH has been used by taxonomists since the 1960s as the most important criterion for the delineation of prokaryotic species.[46] However, the determination of wet-lab DDH values is laborious and depends on DNA isolated from purified bacterial isolates, which formerly excluded non-axenic cyanobacteria from this crucial tool in bacterial taxonomy. With the aid of established algorithms, it is possible to calculate *in silico* digital DDH (dDDH) values that are very close to the wet-lab DDH values and have the same delineation threshold between species.[34,47] A value below 70% DDH (or dDDH) is the generally accepted threshold for delineating species[41,48] and a value of 79–80% has been proposed for subspecies delineation.[49] Furthermore, progress in metagenome sequencing and binning paved the way to analyze the MAGs of non-axenic cyanobacteria with dDDH. The dDDH values, established in this study, are ranging between 85.4% and 95.2% for intra-clade comparisons and between 49.6% and 56.6% for inter-clade comparisons. These values clearly documented that the two clades of *Limnospira* correspond to two different species. Furthermore, the intra-species comparison within the same clade showed that none of the two species comprises further subspecies.

The separation of spirulina into two clades is confirmed by the different methods used in this study, which give consistent results on the affiliation of the different strains to one or the other clade. In particular, the sequences of the hypervariable regions V3 and V4 of the 16S rRNA gene allow an unequivocally identification of the genus *Limnospira* and are sufficient to distinguish its two species in agreement with genome-based methods (Figure 1).

The phylogenomic tree (Figure 5) documented that clade I includes the type species of the genus *Limnospira* Nowicka-Krawczyk et al. 2019, which is designated *L. fusiformis* (Woronichin 1934) Nowicka-Krawczyk et al. 2019 (reference strain: SAG 85.79) and *L. indica* (Desikachary and Jeeji Bai 1992) Nowicka-Krawczyk et al. 2019 (reference strain: PCC 8005).[2] In addition, three diagnostic nucleotide positions in the V3-V4 region of the 16S rRNA gene showed that *L. maxima* (Setchell and Gardner 1917) Nowicka-Krawczyk et al. 2019 (reference strain: UTEX LB 2720) also belongs to clade 1 (Figure 1), and a comparison of the complete 16S rRNA genes revealed an identity of 100% with *L. fusiformis* SAG 85.79 and *L. indica* PCC 8005.[31] Our OrthoANI comparison (Figure 6) and dDDH analysis (Figure 7) provided clear evidence that all strains of clade I belong to the same species. Accordingly, the current study showed that the species names *Limnospira fusiformis* (Woronichin 1934) Nowicka-Krawczyk et al. 2019 and *Limnospira indica* (Desikachary and Jeeji Bai 1992) Nowicka-Krawczyk et al. 2019 are heterotypic synonyms of the type species *Limnospira maxima* (Setchell and Gardner 1917) Nowicka-Krawczyk et al. 2019. Taxa of clade II includes the strain PCC 7345 of the species *Arthrospira platensis* Gomont 1892, which was recently proposed to be considered as the reference strain,[31] as well as other strains assigned to the same species (Figure 5). This species was not transferred to the genus *Limnospira* given the benthic character in the description of *A. platensis* by Gomont in 1892.[2] A recent study showed that *A. platensis* can present benthic and planktonic forms and suggests that this species should therefore be placed in the genus *Limnospira*.[50] The 16S rRNA gene sequences published in the aforementioned study fall into our *Limnospira* clade II. Therefore, we propose the second species of the genus *Limnospira* to be named *L. platensis*. The type strain of this species is *Limnospira platensis* PCC 7345. A list of more than 130 spirulina strains and their correct species identification is provided in the supplementary material (Table S2).

Spirulina exhibits a diversity and morphological plasticity that does not allow a formal identification of one or the other species.[3,5,21,31] Nevertheless, we observed that the shape of the trichomes of *L. platensis* strain paracas (PAR_GP) is distinguished by the regularity of the pitch and the diameter of the helix. This regularity of coiling is also visible in the images of *L. platensis* PCC 7345 available in the photo library of the Pasteur Institute collection (phototheque.pasteur.fr), as well as in the images of other strains of *L. platensis*.[50] However, this distinguishing feature must be confirmed by further studies on strains of the two *Limnospira* species, whose identification will ideally be based on (meta-) genome sequencing.

## Taxonomic considerations on the genus *Limnospira* (Nowicka-Krawczyk et al. 2019)

Biological material or DNA was neither available for the first described *Arthrospira maxima* species (Setchell and Gardner 1917) nor for the first described *Arthrospira platensis* species (Gomont 1892).

The original description of the genus *Limnospira*[2] is given with the following amendments including data that describes the type specie (*L. maxima*) and his heterotypic synonyms (*L. fusiformis* and *L. indica*).

*Class:* Cyanophyceae.

*Order:* Oscillatoriales

*Family:* Microcoleaceae

*Type species*: *Limnospira maxima* (Setchell and Gardner 1917) Nowicka-Krawczyk et al. 2019.

*Morphology:* Trichomes solitary, isopolar, cylindrical, unbranched, more or less tapering toward the ends, not or slightly constricted at cross-walls, blue green or dark green. Trichomes are usually spirally coiled and can present different shapes—screw-like coiled, tightly coiled, weakly spiraled—with a tendency to loosening the coils until straight shape. Spiral diameter and pitch are usually irregular. Cells are always shorter (2–7 μm) than wide (6–12(16) μm). Terminal cells rounded or subcapitate with thickened outer cell wall or calyptra.

*Ultrastructure:* Thylakoids have an irregular arrangement. Aerotopes are facultatively present.

*Ecology:* Planktic in freshwater, brackish and alkaline-saline waters. Usually photoautotrophic and able of mixotrophy.[51,52]

*Etymology:* Límni- [Gr., λίμνη], lake, lagoon; -speíra [Gr., σπείρα], spiral; the generic name refers to the morphology of trichomes and typical habitat, i.e., lakes.

### *Limnospira maxima* (Setchell and Gardner 1917) Nowicka-Krawczyk et al. 2019. Basonym: *Arthrospira maxima* (Setchell and Gardner 1917)

Heterotypic synonym: *Limnospira fusiformis* (Woronichin 1934) Nowicka-Krawczyk et al. 2019; *Limnospira indica* (Desikachary and Jeeji Bai 1992) Nowicka-Krawczyk et al. 2019.

Type strain: PCC 8005. Deposited in Pasteur Cyanobacteria Collection (France, as "*Arthrospira* sp."), in DSMZ-German Collection of Microorganisms and Cell Cultures (Germany) and in SCK CEN (Belgium).

### *Limnospira platensis* (Gomont 1892) comb. nov.

Basonyms: *Arthrospira platensis* (Gomont 1892), *Spirulina platensis* (Geitler 1925).

Type strain: PCC 7345. Deposited in Pasteur Cyanobacteria Collection (France, as "*Arthrospira* sp.") and in The University of Texas Culture Collection of Algae (USA, = UTEX LB 1926 as "*Spirulina platensis*").

The description of *Limonospira platensis* is identical to that of *Limnospira* genus except for the following modifications.

*Morphology:* Regular helical shape with constant diameter and pitch, the latter greater than the diameter. The shape is stable without tendency to loosening the coils.

*Ecology:* Planktic, and rarely benthic.

### *Limnospira* toxinogenesis

Examination of the three *Limnospira* sp. MAGs from this study and 19 reference genomes showed that the genus *Limnospira* do not contain any genes that are involved in the biosynthesis of microcystin. The results of Ballot et al. who reported about microcystins in Kenyan strains of *L. fusiformis* have never been reproduced by other studies focusing on the detection of toxins in *Limnospira* cultures.[14,15,53,54] The hypothesis that the "*Arthrospira fusiformis*" cultures examined by Ballot et al. were contaminated by toxin-producing cyanobacteria of other genera cannot be ruled out. The protocol for isolating *A. fusiformis* has not been described, the isolates were not deposited in a public culture collection, and Lakes Sonachi and Bogoria, from which the studied "*A. fusiformis*" isolates originated, contain the microcystin-producing picocyanobacteria *Synechococcus* sp. and *Synechocystis* sp.[14,15] Moreover, ten genes (*mcyA* to *mcyJ*), which are located in a 55 kb gene cluster in the genome of *Microcystis aeruginosa* PCC 7806SL and *Anabaena* sp. 90, are required for the biosynthesis of microcystin. None of these genes or corresponding proteins could be found in the 22 genomes studied, including four strains previously identified as *L. (A.) fusiformis* (SAG 85.79, KN01, CAM_GP, and ETH_PS). Therefore, the assumption that certain strains of *Limnospira* can produce microcystins does not appear to be a credible hypothesis. Consequently, we propose that *Limnospira* sp., in particular *L. maxima* (previously *L. fusiformis*), should no longer be included in the lists of toxic cyanobacteria.

### Limitations of the study

The Kenyan strains of "*Arthrospira fusiformis*" studied by Ballot et al. in 2005 are no longer available. We would have liked to include them in our study to check their supposed toxicity.

The observation of a morphological difference between the two *Limnospira* species was carried out with a single strain of *Limnospira platensis*. Although this observation is confirmed by images available on the web and in a publication,[50] it is necessary to complete the observations on a larger number of strains.

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests can be directed to Dr. Felice Mastroleo (felice.mastroleo@sckcen.be).

### Materials availability statement

This study did not generate new unique reagents.

### Data and code availability

- The genetic data used in this article are available in NCBI GenBank. Metagenome data are deposited in the following BioProjects: PRJNA1085564 (*Limnospira maxima* CAM_GP), PRJNA1085565 (*Limnospira maxima* ETH_PS), and PRJNA1085629 (*Limnospira platensis* PAR_GP). Fastq files of the 16S rRNA amplicon were submitted to NCBI's Short Read Archive and are publicly available under accession number PRJNA1095467.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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## AUTHOR CONTRIBUTIONS

Conceptualization, P.-E.P., J.P., V.P., and F.M.; methodology, P.-E.P., P.M., and H.B.; validation, V.P., J.P., and F.M.; formal analysis, P.-E.P., P.M., and H.B.; investigation, P.-E.P.; resources, V.P.; data curation, P.-E.P. and P.M.; writing – original draft preparation, P.-E.P.; writing – review and editing, F.M., J.P., and P.-E.P.; visualization, P.-E.P. and J.P.; supervision, V.P., J.P., and F.M.; project administration, P.-E.P., V.P., and F.M.; funding acquisition, V.P. and F.M. All authors have read and agreed to the published version of the manuscript.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

## STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE

## SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2024.110845.

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## STAR★METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Biological samples | | |
| Spirulina cultures | This paper | Table 1 |
| Deposited data | | |
| Genome sequences of 19 *Limnospira* and *Arthrospira* reference strains | NCBI GenBank | Accession numbers in Table 2 |
| Meta Assembled Genomes (MAGs) | This paper | NCBI Bioproject: PRJNA1085564, PRJNA1085565, PRJNA1085629 |
| V3-V4 region of the 16S rRNA gene sequences | This paper | NCBI Bioproject: PRJNA1095467 |
| Software and algorithms | | |
| OCToPUS pipeline v.1.0 | Mysara et al.[55] | https://github.com/M-Mysara/OCToPUS |
| Ribosomal Database Project (RDP v.18) | Wang et al.[56] | https://rdp.cme.msu.edu/ |
| MEGA 11 | Tamura et al.[57] | https://www.megasoftware.net/ |
| MEGAHIT | Li et al.[58] | https://github.com/voutcn/megahit |
| MaxBin 2.0 | Wu et al.[59] | https://sourceforge.net/projects/maxbin2/ |
| MetaBAT | Kang et al.[60] | https://bitbucket.org/berkeleylab/metabat/src/master/ |
| Concoct | Alneberg et al.[59] | https://github.com/BinPro/CONCOCT |
| DAS Tool | Sieber et al.[61] | https://github.com/cmks/DAS_Tool |
| CheckM | Parks et al.[62] | https://ecogenomics.github.io/CheckM/ |
| DFAST | Tanizawa et al.[63] | https://github.com/nigyta/dfast_core/ |
| InterProScan | Jones et al.[64] | https://github.com/ebi-pf-team/interproscan |
| SignalP | Almagro Armenteros et al.[65] | https://services.healthtech.dtu.dk/services/SignalP-5.0/ |
| Genome Taxonomy Database Toolkit GTDB-Tk v2.1.0 | Parks et al.[66] | https://github.com/ecogenomics/gtdbtk. |
| RAxML v8.2.10 | Stamataki.[67] | https://github.com/stamatak/standard-RAxML. |
| Orthologous Average Nucleotide Identity Tool v0.93.1. | Lee et al.[43] | https://www.ezbiocloud.net/tools/orthoani |
| Genome-to-Genome Distance Calculator v3.0 | Meier-Kolthoff et al.[47] | https://ggdc.dsmz.de/ggdc.php/ |
| BLAST+ | Camacho et al.[68] | https://blast.ncbi.nlm.nih.gov/Blast.cgi |
| antiSMASH 7.1.0 | Blin et al.[69] | https://antismash.secondarymetabolites.org |

### EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

#### Spirulina strains

Sixteen samples of different spirulina cultures, from producers of the "Fédération des Spiruliniers de France (FSF, www.spirulinersdefrance.fr)", constituted the initial collection of spirulina strains. A survey of producers made it possible to identify the initial suppliers of the strains used, and to reduce our sampling to seven strains originating from France, Ethiopia, India, Peru and Senegal. These spirulina strains and their uses in this study are listed below.

| Strain ID | Putative origin | Provider | 16S rRNA (V3-V4) | Microscopy | Genome assembly |
|---|---|---|---|---|---|
| CAM_GP | France (Camargue) | spirulinasolution.fr | ● | ● | ● |
| ETH_PS | Ethiopia | spirulinepourquoipas.com | ● | ● | ● |
| ETH_VR | Ethiopia | spirulinedugarlaban.com | ● | ● | – |
| IND_PP | India (Lonar) | laspiraleverte.com | ● | ● | – |
| PAR_GP | Peru (Paracas) | spirulinasolution.fr | ● | ● | ● |
| PAR_RB | Peru (Paracas) | algosud.com | ● | ● | – |
| SEN_CD | Senegal | spirulinedeslandes.com | ● | ● | – |

Optical microscopy observations, unless otherwise stated, and DNA extractions for amplicon sequencing were carried out on the samples received from the producers; electron microscopy observations and metagenomics were carried out on samples sub-cultivated in the laboratory. Spirulina samples were cultivated in cell culture flask with vented cap (Greiner Bio-One, Belgium), in modified Zarrouk medium[70] at room temperature and following day/night regime illumination. Flasks were continuously mixed using an orbital shaker at 120 rpm (Edmund Bülher, Germany).

## METHOD DETAILS

### Microscopy

Optical LM observations were carried out on living and cultivated samples using a Zeiss Axiolab microscope, an Axiocam 305 camera and Zeiss Zen 3.6 software.

For scanning electronic microscopy (SEM) and TEM, the specimens were fixed in cold (4°C) and saline (10 g NaCl per liter) solution of 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.2.

For SEM observations, one milliliter drop of this cell suspension were deposed on poly-L-lysine coated slides. After settling for 2 h, the slides were rinsed in Milli-Q water and dehydrated in a graded ethanol series: 30%, 50%, 70%, 90% and 100% (30 min for each immersion step). The last immersion in ethanol 100% was repeated twice. The samples were critical point dried in an Emitech K850 instrument, mounted on aluminum stubs with the use of double-sided carbon adhesive and coated with gold/palladium in a Quorum Technologies SC7640 sputter coater. The samples were examined under a Hitachi S-3400-N scanning electron microscope operated at an accelerating voltage of 10 kV.

For TEM observations, specimens' pellets obtained after centrifugation, were post-fixed in 2% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.2) for 1 h at room temperature. The postfixed specimens were processed in graded ethyl alcohol, propylene oxide, Spurr resin and polymerized for 24 h at 60°C. Ultrathin sections (60–90 nm) were cut on an ultramicrotome Power tome PC-RMC Boeckeler, placed on 300 and 200 mesh copper grids and stained with uranyl acetate followed by lead citrate. The samples were examined under a transmission electron microscope Hitachi H-7650 at an accelerating voltage of 80 kV.

### DNA isolation

DNA from 5 mL spirulina culture samples was isolated using the protocol adapted for filamentous cyanobacteria of the genus *Limnospira* (*Arthrospira*) developed by Morin et al.[71] Briefly, cells were collected by centrifugation (10 min, 3500 rpm) and resuspended into 0.5 mL of 0.15 M NaCl and 0.1 M EDTA. Following three freeze-thaw cycles, the cells were collected by centrifugation (10 min, 8000 rpm), resuspended in 0.5 mL TE Buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and transferred into fresh tubes for enzymatic cell wall lysis using 100 µL of 50 mg/mL lysozyme, for 30 min at 37°C. Proteins were digested with 5 µL of 50 mg/mL proteinase K in 2% SDS final concentration, for 1 h at 37°C. Polysaccharides, proteins and cell wall debris were thereafter removed by selective precipitation: 150 µL of 5 M NaCl was added to the tubes, followed by 0.1 volume of a 10% cetyltrimethylammonium bromide (CTAB) stock solution and incubated at 65°C for 10 min. Nucleic acids purification was achieved by extraction in 1 volume of chloroform:isoamyl alcohol. The tubes were placed on ice for 30 min to allow precipitation of CTAB complexes, before being centrifuged (10 min, 8000 rpm). The supernatant was transferred into a fresh tube, gently mixed with 0.6 volume of isopropanol until DNA precipitated. The DNA pellets were recovered by centrifugation, (10 min, 4°C, 15000 rpm) and washed with 1 mL of ice-cold 70% ethanol to remove any residual salt. After a final centrifugation (5 min, 4°C, 15000 rpm), the supernatant was discarded, and the pellets were dried before being resuspended in 100 µL TE Buffer. The samples were treated with 1 µL of RNAse (10 mg/mL) for 1 h at 37°C and then kept at −80°C until further use.

### 16S rRNA gene amplicon sequencing

High-throughput paired-end amplicon sequencing of the V3-V4 hypervariable region of the 16S rRNA gene was conducted on BaseClear's Illumina MiSeq (V3 chemistry) platform according to the manufacturer's guidelines. Sequence data of the 16S rRNA gene amplicons were processed using OCToPUS pipeline v.1.0,[55] and analyzed as previously described,[72] generating the OTUs. The OTUs were classified using Ribosomal Database Project (RDP v.18) dataset[56] and their purity were assessed using the oligotyping approach[73] and classified using Blastn algorithm choosing the rRNA/ITS database. The sequence alignments and their examination were carried out with MEGA 11.[57]

### Metagenome sequencing and binning

High-throughput short-read metagenome sequencing was conducted as previously described.[37] The NEBNext Ultra II FS DNA Library Prep Kit (New England Biolabs) was used for the construction of the Illumina libraries. Sequencing was performed on the Illumina NovaSeq 6000 system using the v3 chemistry with 600 cycles. The fastq-mcf tool of ea-utils v1.04.803 was used for quality control and adapter clipping.

We used our previously established metagenome assembly and binning pipeline for the analysis of short-read Illumina sequences.[37] In short, processed sequence data were assembled with MEGAHIT,[58] metagenomic binning was conducted with three methods, namely MaxBin 2.0,[59] MetaBAT,[60] Concoct,[74] and subsequently analyzed with DAS Tool[61] to retrieve the most reliable MAGs. The quality of the MAGs was investigated with CheckM[62] and coverage values were calculated per bbmap. Finally, genomes were annotated by DFAST,[63]

InterProScan[64] and SignalP.[65] The initial taxonomic classification of the cyanobacterial MAGs was conducted with the Genome Taxonomy Database Toolkit GTDB-Tk v2.1.0 on the GTDB reference data version r207.[66]

## Phylogenomic analyses

Genome-based phylogenomic analyses of cyanobacteria were conducted as previously reported.[75] Briefly, the phylogenomic trees were reconstructed from concatenated amino acid or nucleotide alignments of 92 housekeeping genes (Up-to-Date-Bacterial Core Gene [UBCG] dataset.[76] The alignments were manually refined with the edit-option of the MUST package[77] and the application of G-blocks;[78] The ML trees were calculated with RAxML v8.2.10 and inferred under a GTR+4Γ model using 100 bootstrap replicates.[67]

## Genome comparison

ANI was calculated using the OrthoANI algorithm with OAT (Orthologous ANI Tool) version 0.93.1. OrthoANI is highly correlated with ANI and show approximately 0.1% higher values than the original ANI.[43]

DDDH was calculated using Genome-to-Genome Distance Calculator (GGDC 3.0, formula 2) with the platform available under https://tygs.dsmz.de.[34,47] The *Limnospira* and *Arthrospira* genomes used for the OrthoANI and the dDDH analysis are those published in GenBank as available on 09/30/2023, complemented with the later released assembly of the reference strain PCC 7345 of *A. platensis* (see key resources table for details).

## Toxinogenesis analysis

The search for nucleotide and protein sequences for the synthesis of microcystins, was carried out based on the current knowledge of cyanotoxins and their metabolic synthesis pathways.[79,80] In summary, microcystins are secondary metabolites, cyclic heptapeptides whose biosynthesis depends on ten genes (*mcyA-J*), grouped in a cluster of approximately 55kbp, which encode a set of multifunctional enzymes: non-ribosomal peptide synthetases (NRPSs), polyketide synthetases (PKSs), tailoring enzymes and a transporter enzyme. All sequences were retrieved from GenBank.

*Genes involved in microcystin biosynthesis*

| Sequence description | Organism | Length (bp) | Accession |
|---|---|---|---|
| *mcyD, mcyE, mcyF, mcyG, mcyH, mcyI, mcyJ, dnaN* genes | *Microcystis aeruginosa* K-139 | 36,468 | AB032549 |
| complete gene cluster: *mcyA* to *mcyJ* | *Planktothrix agardhii* NIVA-CYA 126/8 | 55,575 | AJ441056 |
| complete gene cluster: *mcyA* to *mcyJ* | *Anabaena* sp. 90 | 57,817 | AJ536156 |

*Proteins involved in microcystin biosynthesis by Microcystis aeruginosa* PCC 7806SL

| Protein | Name | Length (aa) | Accession |
|---|---|---|---|
| McyA | non-ribosomal peptide synthetase | 2,795 | ARI82521 |
| McyB | non-ribosomal peptide synthetase | 2,126 | ARI82522 |
| McyC | non-ribosomal peptide synthetase | 1,291 | ARI82523 |
| McyD | type I polyketide synthase | 3,906 | ARI82520 |
| McyE | hybrid non-ribosomal peptide synthetase/type I polyketide synthase | 3,487 | ARI82519 |
| McyF | aspartate/glutamate racemase family protein | 251 | ARI82518 |
| McyG | type I polyketide synthase | 2,631 | ARI82517 |
| McyH | ATP-binding cassette domain-containing protein | 585 | ARI82516 |
| McyI | phosphoglycerate dehydrogenase | 337 | ARI82515 |
| McyJ | methyltransferase domain-containing protein | 311 | ARI82514 |

Nucleotide sequence was searched using the blastn algorithm and protein sequence was investigated with tblastn and blastp.[68] We also used the software 'antibiotics and secondary metabolite analysis shell - antiSMASH 7.1.0'[69] to detect possible NRPS and PKS in the *Limnospira* genomes.

## QUANTIFICATION AND STATISTICAL ANALYSIS

Phylogenomic analysis under ML (Figures 5 and S2) was conducted with RAxML (Randomized Axelerated ML) version 8.2.10.[67] Number of bootstrap replicates ($n$ = 100) are mentioned in figure legend. Bootstrap values are show at the phylogenomic tree nodes.

## ADDITIONAL RESOURCES

No additional resources.