Comparative Genomic Analysis of Cyanobacteria as Amphibian Food Sources: Insights into High-Temperature Tolerance Potential

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ABSTRACT

Hot springs represent one of the most accessible yet extreme environments on the Earth's surface. We isolated and characterized two novel cyanobacteria strains of genus *Leptolyngbya* , *L*. sp. Akita and *L*. sp. Seranma from geothermal hot-springs in Japan. These strains showed distinct morphological features; *L*. sp. Akita exhibited linear filamentous structures and blue-green when cultured under white light, while *L*. sp. Seranma exhibited coiled structures and brown color under the same conditions. Whole-genome sequencing and comprehensive genomic analyses of their genomes identified acquisition of gene clusters related to metabolic processes which may associated with the strain specific phenotypes. In addition, intestinal metagenomic analysis of tadpoles (*Buergeria buergeri* and *B. japonica*) sympatrically living

with these strains in the hot-springs suggested that the tadpoles utilized these *Leptolyngbya* as temporal or regular foods in high temperature environments. These findings provide insights into the ecological significance of these cyanobacteria in extreme environments and their potential applications in biotechnology and ecological conservation of primary consumers including amphibians.

Keywords: Ecosystem; Adaptive evolution; Hot-spring; Climate change; Microbiome; Symbiosis

1. INTRODUCTION

Cyanobacteria are essential microorganisms that act as primary producers in aquatic ecosystems, playing a pivotal role in sustaining the dynamics of the food web. In extreme environments, where nutrient and biomass availability is constrained, the environmental adaptation and establishment of cyanobacteria are critical events that influence local ecosystems. Temperature stands out as one of the most fundamental environmental factors to which all organisms, including cyanobacteria, must adapt during their evolution and the expansion of their distribution ranges.

In the context of climate change, the adaptation and formation of ecosystems in extreme temperature conditions, particularly high temperatures, have garnered increasing attention. Notably, novel strains of cyanobacteria that have adapted to extreme high-temperature environments have been identified within multiple families, including *Oculatellaceae* (Tang et al., 2021), *Leptolyngbyaceae* (Tang et al., 2022), *Trichocoleusaceae* (Tang et al., 2023), *Thermosynechococcaceae* (Arnold et al., 2024), and *Geminocystaceae* (Chen et al., 2025). The evolutionary adaptations of cyanobacteria to high-temperature environments not only underscore their resilience but also highlight their potential as bioindicators of climate change impacts on aquatic ecosystems.

As these microorganisms flourish in extreme conditions, they significantly contribute to primary productivity, which is vital for the survival of various aquatic species that depend on them as a food source. Furthermore, they are increasingly recognized as producers of bioactive nutrient compounds, including proteins, carbohydrates, essential fatty acids, vitamins, minerals, and other functional bioactive compounds, which have potential applications in food or supplements (Brito et al., 2020; Farag et al., 2016; Pestana et al., 2020; Wu et al., 2016). Additionally, the metabolic pathways of thermophilic cyanobacteria have evolved to enable the synthesis of unique compounds that can be harnessed for biotechnological applications, such as biofuels and pharmaceuticals, thereby broadening their significance beyond ecological contributions (Brito et al., 2020). As climate change continues to reshape aquatic environments, understanding these adaptations not only informs us about the resilience of cyanobacteria but also emphasizes their potential role in sustainable practices aimed at mitigating the impacts of rising temperatures on ecosystems (Farag et al., 2016).

In a unique ecosystem adapted to high-temperature environments, we recently discovered two homologous cases of ecological symbiosis between cyanobacteria and anuran amphibians of the genus *Buergeria* in natural hot springs in Japan. One of the *Buergeria* species, *B. japonica*, known as the "hot-spring frog," has tadpoles that thrive in the highest recorded water temperature (46.1℃) in natural hot springs on Kuchinoshima Island, Kagoshima Prefecture (Komaki et al., 2016). Another species, *B. buergeri*, has also been reported to inhabit a river where a natural hot spring occurs in Kawara-no-yukko, Yuzawa City, Akita Prefecture (Takayanagi, 2019). Interestingly, both populations of *Buergeria* species coexist with microbial mats and utilize these mats as their food source.

Amphibians are crucial vertebrates that serve as Omnivore during tadpole stage and Consumers after

metamorphosis within the trophic level. Moreover, Nutrient regeneration, stemming from both the herbivorous and carnivorous feeding activities of tadpoles, positively influenced the production rates of benthic algae, phytoplankton, and herbivorous benthic chironomid larvae (Iwai et al., 2012). Consequently, both local ecosystems, initiated by cyanobacteria and expanded by sympatric amphibians, represent intrinsic cases for understanding ecological evolutionary adaptation and ecosystem formation.

The main goal of this research is to better understand how cyanobacteria adapt to extreme temperatures and the effects of these adaptations on ecosystem stability and dynamics. By looking at the symbiotic connections between cyanobacteria and amphibians, especially the genus *Buergeria*, we seek to clarify how these relationships improve nutrient cycling and productivity in harsh environments. In this study, we aimed to clarify the evolutionary genomic adaptation of cyanobacteria and its connections with amphibians living in hot springs. To achieve this, we first isolated two novel strains of cyanobacteria from these extreme environments. Following this, we determined the whole genome sequences of the isolated strains and conducted comprehensive comparative genomic analyses to identify the specific genes responsible for their remarkable high-temperature tolerance. Additionally, we performed metagenomic analyses of the intestinal microbiota of the tadpoles to confirm their dietary reliance on these cyanobacterial sources. This multifaceted approach not only enhances our understanding of the ecological dynamics at play but also sheds light on the evolutionary adaptations that enable both cyanobacteria and amphibians to thrive in extreme thermal conditions.

2. MATERIALS AND METHODS

2.1 Sample collection, isolation, and growth conditions

Cyanobacteria were isolated from microbial mats collected at two geothermal hot spring sites in Japan: one from “Kawara-no-yukko” (hot-spring in Yakunaigawa river), Akinomiya Akita Pref. in August 2022 (hereafter referred to as Akita) and the other from Seranma hot-spring, Kuchinoshima Island, Kagoshima Pref. in June 2023 (hereafter referred to as Seranma). At both sites, microbial mats were found in small, warm water streams originating from the hot springs and eaten by tadpoles of frogs which belong to genus *Buergeria* (*B. buergeri* in “Kawara-no-yukko” and *B. japonica* in Seranma hot-spring). We collected the mats using sterile forceps and spoons and were immediately transferred into 50 mL Falcon tubes filled with the local hot spring water. During transport to the laboratory, the samples were kept cool (approximately 10 ℃) and dark condition to maintain microbial viability. In the laboratory, the samples were gently rinsed with sterile distilled water to remove loosely attached sediments and non-target debris. Small fragments of the mat were then inoculated into 200 mL Erlenmeyer flasks containing 50 mL of sterile BG11 liquid medium. These flasks were incubated at 34°C in a growth chamber (PHCbi, MLR-352, Japan) equipped with 37 W fluorescent lamps (FL40SSENW37HF3, Panasonic, Japan), with the light intensity set to 50 μmol photons/m^2/s, to enrich phototrophic cyanobacteria. After several days of cultivation, cultures displaying characteristic green-brown pigmentation were subcultured

two to three times in fresh BG11 medium to reduce microbial diversity and further enrich cyanobacterial populations. Following enrichment, aliquots of the liquid cultures were spread onto BG11 agar plates and incubated at 40 °C under continuous illumination to obtain single cyanobacterial colonies. After several days of incubation, filamentous, aggregated colonies appeared on the plates. To eliminate bacterial contamination and obtain axenic cultures, streaking was repeated multiple times on fresh BG11 agar plates. Colonies exhibiting consistent morphology were subsequently transferred to liquid BG11 medium for further purification.

Complementary chromatic acclimation experiments were performed using a multi-cultivator system (MC-1000, Photon Systems Instruments, Czech Republic) equipped with programmable LED light modules. Cyanobacterial cultures were incubated at 40°C under continuous illumination of white, red, or green LED light, each set to a photosynthetic photon flux density (PPFD) of 60□μmol photons□m□^{2}□s□^{1}. The peak emission wavelengths of the red and green LEDs were approximately 660 nm and 520 nm, respectively.

2.2 Genomic DNA extraction and sequencing of two cyanobacteria isolates

Genomic DNA of two purified cyanobacterial cultures was extracted for whole-genome sequencing using the DNeasy PowerBiofilm Kit (Qiagen), and libraries for Oxford Nanopore Technology (ONT) sequencer were constructed using a ligation library preparation kit (SQK-LSK114, ONT). All these procedures were followed manufacturer instructions and the quality and molecular weight of the genomic DNA were measured using Qubit fluorometric quantification system (Thermo Fisher Scientific, Inc.). Sequencing was conducted using MinION sequencer (MinION Mk1B, ONT) and Flongle flow cell (FLO-FLG114, ONT). The raw sequence data were basecalled using the super accuracy model with duplex basecalling, utilizing Dorado version 0.9.5 and duplex tools version 0.3.3.

2.3 Genome assembly, assessment of completeness, gene prediction, and annotation

Sequenced reads more than 10 q-score were assembled with Flye (v2.9.4-b1799) (Kolmogorov et al., 2019) with "--nano-hq" parameter. Assessments of genome completeness were performed in each using Benchmarking Universal Single-Copy Orthologs (BUSCO (version: 5.7.0)) gene set (cyanobacteria_odb10) (Manni et al., 2021). From these two assembled genome sequences and published genomic FASTA files of other *Leptolyngbya* spp. (Table S1), gene prediction and annotation were performed using prokaryotic gene annotation pipeline, PGAP (version: 2024-07-18.build7555) (Zhao et al., 2012). Estimation of functional domains in each gene was performed using InterProScan (version: 5.59-91.0) (Jones et al., 2014), and Pfam IDs obtained from InterProScan were translated to GO terms using pfam2go in ragp (version: 0.3.5.9000) package (Dragićević et al., 2021).

2.4 Molecular phylogenetic analyses using 16S ribosomal RNA (rRNA) and Unicore gene set

Firstly, to infer the phylogenetic placement of our isolated cultured strains in phylum Cyanobacteriota, we analyzed 16S rRNA sequences of strain Akita and Seranma using Cydrasil (Roush et al., 2021). We then conducted genus level phylogenetic analyses with 16S rRNAs and Unicore (universal and core) gene (single-copy core genes, common to most members of a clade) of *Leptolyngbya* spp. To refine gene annotation of *Leptolyngbya* spp. genomes, we conducted PGAP annotation pipeline for all genomic data. In the 16S rRNA analyses, to exclude truncated copy of 16S rRNA, we selected copies 1,400 bp or more in the length in each strain. We could not find 16S rRNA sequence 1,400 bp or more in *L*. sp. JSC-1, the sequence deposited in NCBI was used (accession ID: FJ788926). After multiple alignment of 16S rRNA sequences using MAFFT (version: 7.525, parameters: --auto) (Katoh & Standley, 2013), a phylogenetic tree was constructed using IQ-TREE (version: 2.4.0, parameters: -m MFP -B 1000) (Minh et al., 2020). In the analyses of Unicore gene set, single-copy genes in more than 80% of all strains of *Leptolyngbya* spp. were extracted. Using the pipeline included in Unicore package (Kim et al., 2024), this gene set was concatenated, aligned, and used for the phylogenetic tree construction. These phylogenetic trees with bootstrap values were visualized as unrooted trees using iTOL (web server; https://itol.embl.de/) (Letunic & Bork, 2024).

2.5 Synteny identification and comparative genome analysis using predicted gene models

We conducted synteny plots between *L*. sp. Akita and *L*. sp. Seranma, *L*. sp. and their phylogenetically closest strains using ReCCO (version: 1.0-beta, download link of script; https://www.genome.jp/ftp/tools/ReCCO/ReCCO_Readme.html), and the plots were visualized using DiGAlign (version: 2.0, web server; https://www.genome.jp/digalign/) (Nishimura et al., 2024) with default parameters and small modifications for proper execution.

For comparative genomic analyses, reciprocal BLASTP (version: 2.15.0) (Altschup et al., 1990) searches were performed between predicted genes of strain Akita, strain Seranma, and *L*. sp. JSC-1 and predicted genes of each strain of *Leptolyngbya* spp.. We classified these genes of strain Akita, Seranma, and *L*. sp. JSC-1 into three categories: the gene was reciprocal best hit (RBH), not the reciprocal but best hit (BH), and no homologous genes (NH). We used e-value $\leq$ 1.0e-10 in this BLASTP searches. After classifying genes into these three categories, hierarchical clustering based on Gower's distance and Ward’s method were performed using state matrices of strain Akita, strain Seranma, and *L*. sp. JSC-1 was formed by classified category vectors corresponding to other *Leptolyngbya* strains, respectively. To obtain significantly enriched Gene Ontology (GO) terms in each gene cluster, genes were divided into some clusters using appropriate thresholds from the results of hierarchical clustering, and Fisher's exact probability tests with multiplicity adjustment using Benjamini-Hochberg method were performed in each gene cluster.

2.7 Metagenomic analysis of tadpole intestinal microbiota using 16S rRNA

To confirm ecological significance of the cyanobacteria in extreme environment, we verify tadpole feeding by metagenomic analysis of tadpole intestinal microbiota using 16S rRNA. We obtained tadpoles of the Japanese bell-ring frog, *B. buergeri* from both "Kawara-no-yukko" in August 2022 and tadpoles of the Ryukyu bell-ring frog, *B. japonica* from Seranma hot-spring in May 2022. Three *B. buergeri* tadpoles from different water temperatures of 26 °C, 28 °C, and 37 °C, and nine *B. japonica* tadpoles from the same water temperature of about 40 °C were collected and preserved in 70% ethanol after deep anesthetization using MS-222 and kept in a -30 °C freezer until DNA extraction. All sample collection was approved by Hiroshima University Animal Research Committee, approval number G14-2. Total DNA including intestinal microbial metagenomic DNA and the DNA of tadpole was extracted from tadpole intestine using DNAs-ici!-F (Rizo Inc., Tsukuba, Japan) according to the manufacturer instructions and dissolved in 50 μL of nuclease-free water. We amplified full length 16S rRNA gene using 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'). Polymerase chain reaction (PCR) amplification was conducted in 25 μL reaction with 0.5 U KOD FX Neo (Toyobo Co., Ltd. Osaka, Japan), 1 x PCR Buffer for KOD FX Neo, 0.4 mM each dNTP, and 0.2 μM each primer and 1 μL total DNA samples with the following cycle condition: initial denaturation at 94 °C for 3 min, then 35 cycles of 10 sec denaturation at 98 ˚C, 30 sec annealing at 55 °C, and 2 min elongation at 68 °C. The PCR products were then removed the remaining primers by polyethylene glycol precipitation and dissolved in 5 μL nuclease-free water. After measurement of concentration using Qubit Fluorometer (Thermo Fisher Scientific) and Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific), each precipitated sample were used for barcoded library construction for Nanopore sequencing following native barcoding protocol (SQK-LSK109 with EXP-NBD104 and EXP-NBD114, NBE_9065_v109_revAP_14Aug2019). Following manufacturer instructions, sequencing was conducted using MinION sequencer (MinION Mk1B, ONT) and Flongle flow cell (FLO-FLG001, ONT). MinKNOW (version: 22.03.6) was used for basecalling and demultiplexing with Super-accuate basecalling model, and the resultant FASTQ files of each sample were and analyzed EPI2MElabs platform (version: 5.2.3) with wf-metagenomics nextflow workflow (version: 2.13.0) and pre-built "core-nt" Kraken2/Bracken database (downloaded 4/27/2025, https://benlangmead.github.io/aws-indexes/k2). Kruskal-Wallis tests, glm tests, Welch's t-tests, and Wilcoxon tests with Benjamini-Hochberg method were performed between read abundances estimated by Bracken of each genus using ALDEx2 (version: 1.36.0) (Fernandes et al., 2013) packages.

2.8 Assessment of heat tolerance in *Xenopus tropicalis* tadpoles fed cyanobacteria

To examine the effects of the *Leptolyngbya* strains isolated in this study on the heat tolerance of tadpoles, we used a model amphibian species, *Xenopus tropicalis* tadpoles because breeding season of *B. buergeri* and *B. japonica* were limited (May to July). The tadpoles were fed dry powders of *Spirulina*, *Leptolyngbya* sp. Akita, or *Synechococcus*, and reared at 25□°C, 30□°C, and 35□°C. To ensure

reproducibility, we conducted multiple trials for each condition (Table S17). In every condition, 10–23 tadpoles were used, and survival rates were recorded every 24 hours. Welch's t-tests were performed with Benjamini-Hochberg method for daily survival rates, and for two parameters (mortality rates and inflection points) estimated by fitting the time series of survival rates using scipy.optimize.curve_fit function. The survival rate over time, as modeled using the following sigmoidal function:

Here, and represented the mortality rates and inflection points in each trial, respectively.

3. RESULTS

3.1 Morphological evaluation and whole-genome sequencing of the isolated cyanobacterium strains

After repeated subcultures of microbial mats collected from hot-springs in Kawara-no-yukko and Seranma, fine green and brown cyanobacterium strains were obtained. Both strains exhibited filamentous and nonheterocystous structures under a light microscope (Fig. 1A and 1B). Considering their hot-spring inhabitation and the molecular phylogenetic relationships (see next section), we considered both isolated strains as members of the genus *Leptolyngbya*, and designated them as *Leptolygbya* sp. Akita and *L.* sp. Seranma, named after their respective collection sites. *L.* sp. Akita showed a relatively linear filamentous structure, while *L.* sp. Seranma tended to have a partially coiled filamentous structure. *L.* sp. Seranma also exhibits complementary chromatic acclimation, appearing brown under white light and green under red light (Fig. 1C), similar to *L.* sp. JSC-1 (Gan et al., 2014).

We obtained 405,522,732 bp and 933,140,049 bp of raw sequence data of *L.* sp. Akita and *L.* sp. Seranma (Table 1). Their resultant genome assemblies based on these data consists of three circular and a linear contigs for Akita (6,829,225 bp in total) and two circular and two linear for Seranma (8,049,769 bp in total) (Table 1). The BUSCO completeness values of these genomes were 95.5% and 99.6%, while that only 1.4% and 0.3 % of the missing BUSCO genes, respectively.

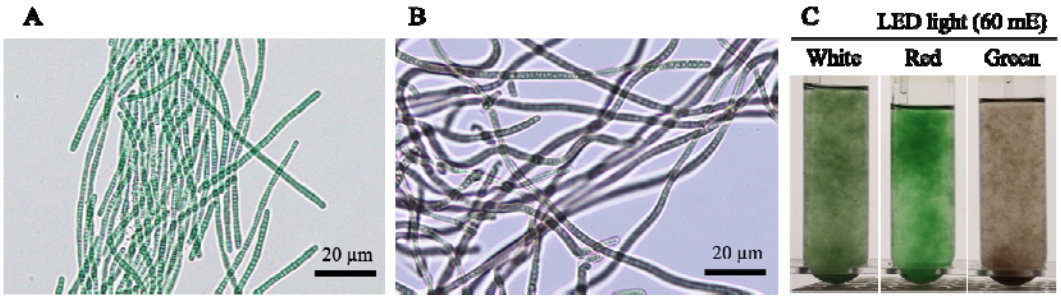

Fig. 1 Photographs of novel isolated strains. Microphotographs of (A) *L.* sp. Akita, (B) *L.* sp. Seranma, and (C) Complementary chromatic acclimation responses of *L.* sp. Seranma under different light qualities. Cultures were grown under continuous illumination of white, red, or green LED light using the MC-1000 multi-cultivator system. Photographs were taken 8 days after inoculation to visualize morphological and pigmentary changes associated with photoacclimation.

Table 1. Metrics of the draft genomes of strains Akita and Seranma with BUSCO completeness.

Strain	*Leptolyngbya* sp. Akita	*Leptolyngbya* sp. Seranma
Total number of reads	98,144	163,820
Total length of reads (bp)	405,522,732	933,140,049
N50 of reads (bp)	17,945	6,502
> Q20 (%)	65.18	81.28
> Q30 (%)	35.04	67.18
Total number of contigs	4	4
Total assembly size (bp)	6,829,225	8,049,769
GC content (%)	46.75	50.77
Assembly size and state of contigs (bp)	contig_1: LC, 277,981 contig_3: CC, 6,300,885 contig_4: CC, 10,498 contig_5: CC, 239,861	contig_1: LC, 1,313,761 contig_2: CC, 6,476,496 contig_3: LC, 226,323 contig_4: CC, 33,189
BUSCO completeness (%, cyanobacteria_odb10, 773 genes from 141 genomes)	Complete: 95.5 (Single-copy: 95.2, Duplicated: 0.3) Fragmented: 3.1 Missing: 1.4	Complete: 99.6 (Single-copy: 98.6, Duplicated: 1.0) Fragmented: 0.1 Missing: 0.3
Total number of estimated genes from PGAP (genes)	6,307	6,752

Linear and circular contigs were indicated as LC and CC, respectively.

3.2 Phylogenetic relationships in cyanobacteria and genus *Leptolyngbya*

The phylogenetic tree of 16S rRNA generated by Cydrasil showed that both isolated cultured strains were in a branch containing most of *Leptolyngbya* sp. (Fig. S1). We then inferred the evolutionary relationships of the two strains and known strains of *Leptolyngbya* (except for the strains with extremely low BUSCO completeness) using 16S rRNA and the Unicore data set of *Leptolyngbya* spp. Based on the PGAP annotation, 75 copies of 16S rRNA were identified in *Leptolyngbya* spp. genomes and 48 of these (including a manually added sequence of *L*. sp. JSC-1) were extracted as valid 16S rRNA and used for the phylogenetic analysis. In the phylogenetic tree of 16S rRNA, *L*. sp. Akita was placed in a cluster containing *L*. *boryana*, *L*. sp. FACHB-161, *L*. sp. FACHB-238, *L*. sp. FACHB-239, *L*. sp. FACHB-402, and *L*. sp. GGD, while all copies of *L*. sp. Seranma were placed in a cluster containing *L*. sp. JSC-1 (Fig. 2A). However, evolutionary relationships of the strains within clusters were not clear.

Using Unicore pipeline, 408 proteins were selected as Unicore gene set, and the multiple sequence alignment resulted 100,760 aa (including gaps) in total. The phylogenetic tree of Unicore gene set showed basically similar clustering to that of 16S rRNA and clear evolutionary relationships of strains within the clusters. In the Unicore tree, *L*. sp. GGD was the closest strain of *L*. sp. Akita and *L*. sp. JSC-1 was the closest strain of *L*. sp. Seranma (Fig. 2B).

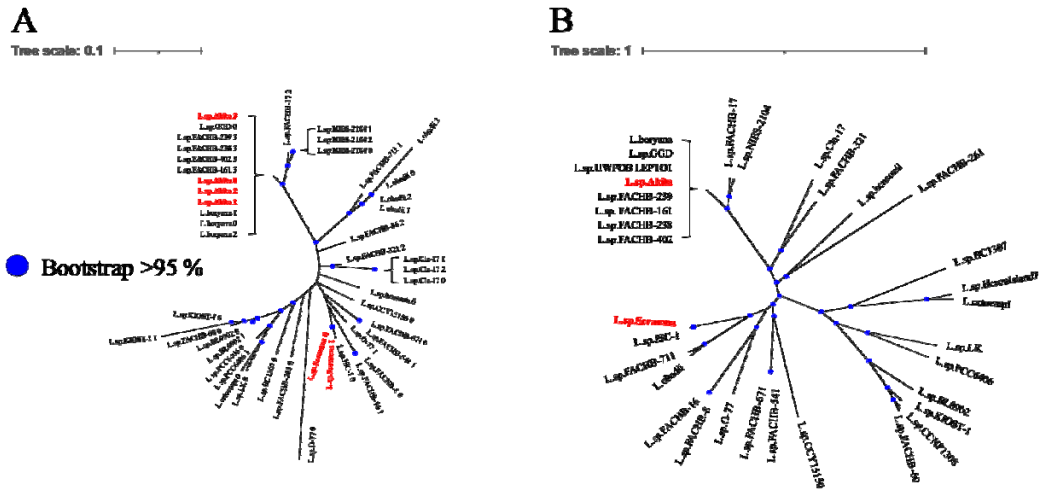

Fig. 2 Unrooted phylogenetic trees and highlighted internal nodes with bootstrap values >95 % in *Leptolyngbya* spp.. (A) 16S rRNA and (B) Unicore gene set

3.3 Synteny conservation patterns between *Leptolyngbya* spp.

We conducted synteny analyses in the combination of the closest strains: *L*. sp. Akita - *L. boryana* and *L*. sp. Seranma - *L*. sp. JSC-1. The resultant synteny plots showed macro synteny contig_2 of *L*. sp. Akita and the chromosome of *L. boryana* (NZ_CP130144.1) with 95~100% identity and two large genomic inversions approximately 0.7~2 Mb (Fig. 3A). Interestingly, *L. boryana* had few homologous genomic regions with contig_1 (approximately 0.3 Mb, 5' end in Fig. 3A) in *L*. sp. Akita. *L*. sp. Seranma and *L*. sp. JSC-1 also showed macro synteny over the whole length of contig_2 in *L*. sp. Seranma and the scaffold in *L*. sp. JSC-1 (NZ_KL662191.1) with an identity of 95~100%, except for three genomic inversions approximately 0.4 Mb and four genomic transpositions approximately 40~80 kb (Fig. 3B). In addition, the other scaffold in *L*. sp. JSC-1 (NZ_KL662192.1) showed high homology with contig_1 in *L*. sp. Seranma.

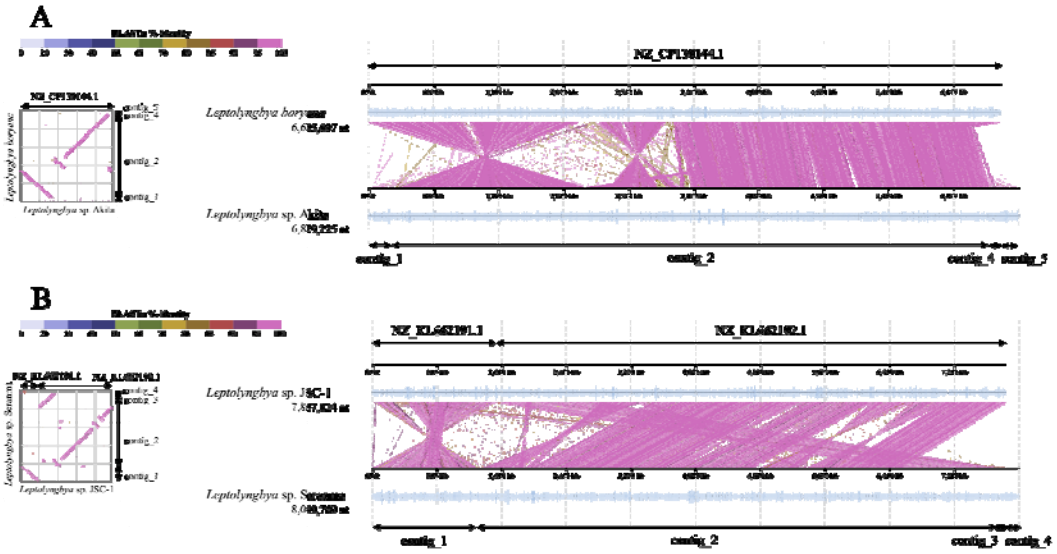

Fig. 3 Synteny plots and dot plots between pairs of *Leptolyngbya* strains. (A) *L*. sp. Akita and *L. boryana* (B) *L*. sp. Seranma and *L*. sp. JSC-1

3.4 Strain- or strain-group-specific gene enrichment and their functional estimation based on comparative genomic analysis.

Based on PGAP annotation pipeline, 6,307, 6,752, and 6,354 genes were predicted in the genome sequences of *L*. sp. Akita, *L*. sp. Seranma, and *L*. sp. JSC-1, respectively (see Table S1 for predicted genes in including other *Leptolyngbya* strains). Reciprocal BLASTP searches were performed between predicted genes of *L*. sp. Akita (Table S2) (or *L*. sp. Seranma (Table S3) or *L*. sp. JSC-1 (Table S4)) and predicted genes of each strain of *Leptolyngbya* spp., and hierarchically clusterings were performed using

BLASTP hit category matrices of *L*. sp. Akita, *L*. sp. Seranma, and *L*. sp. JSC-1. From dendrograms obtained by hierarchical clustering, all predicted genes of *L*. sp. Akita, *L*. sp. Seranma, and *L*. sp. JSC-1 strains were divided into 15, 13, and 12 gene clusters by selecting appropriate threshold values, respectively (Table S5, Fig. S2). Enrichment analyses were performed for the strain-specific gene clusters which contains genes specifically accumulated in the target strains and found few homologs in the other strains. These clusters necessarily accumulated the highest number of NH genes, and GO terms, "nitrogen compound metabolic process" and "transition metal ion binding" were identified as significantly enriched terms (q-value (false discovery rate) <0.05 and odds ratio >1) in gene cluster #0 of *L*. sp. Akita (Table 2A, Table S9). In addition, "DNA transposition" and "transposase activity" were identified as significantly enriched terms in gene cluster #0 of *L*. sp. Seranma (Table 2B, Table S10). On the other hand, no significantly enriched GO terms were identified in gene cluster #2 of *L*. sp. JSC-1 (Table S11). Instead, "phycobilisome" and "photosynthesis"-related GO terms were identified as significantly enriched terms in gene cluster #2 of *L*. sp. Seranma (Table 2C, Table S10), and gene cluster #0 of *L*. sp. JSC-1 (Table 2D, Table S11). The genes included in these clusters tend to be orthologs because they were only found in synteny blocks of these closely related strains (*L*. sp. Seranma and *L*. sp. JSC-1), but may not be in the other strains.

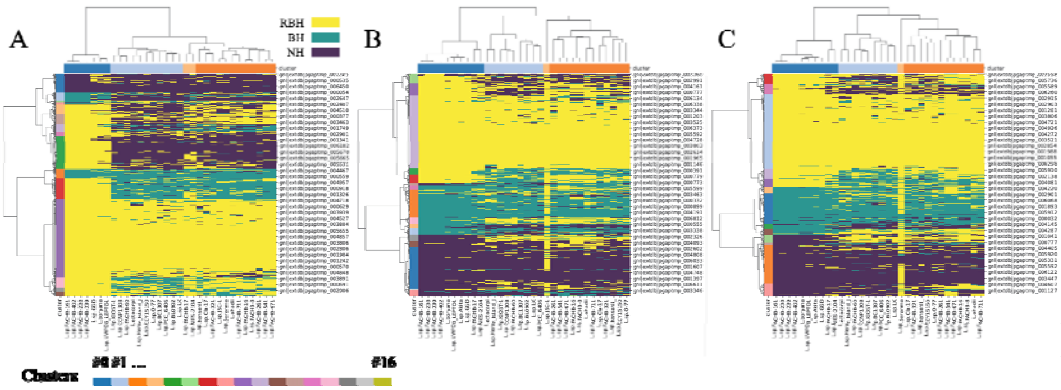

Fig. 4 Matrices showed reciprocal BLASTP search between genes of the target strain and genes of the other *Leptolyngbya* strains with dendrograms. The target strains were (A) *L*. sp. Akita, (B) *L*. sp. Seranma, and (C) *L*. sp. JSC-1, respectively. Genes or strains were sorted and colored by their cluster. See Table S9-S11 for enriched GO terms in each cluster.

Table 2. Enriched statistically significant GO terms.

(A) GO terms in cluster #0 of *L*. sp. Akita	Odds ratio	p-value	q-value
nitrogen compound metabolic process	3.929595	0.002765	0.024888
transition metal ion binding	9.431028	3.14E-05	0.000848

(B) GO terms in cluster #0 of *L*. sp. Seranma	Odds ratio	p-value	q-value
DNA transposition	3.012703	0.000855	0.007693
transposase activity	3.012703	0.000855	0.007693

(C) GO terms in cluster #2 of *L*. sp. Seranma	Odds ratio	p-value	q-value
ADP binding	5.244827	0.000105	0.001732
calcium ion binding	3.337617	6.06E-08	1.79E-06
chlorophyll binding	5.403762	0.003696	0.039071
electron transporter, transferring electrons within the cyclic electron transport pathway of photosynthesis activity	5.67395	0.001406	0.018914
phosphorelay sensor kinase activity	3.139509	1.35E-10	5.00E-09
phosphorelay signal transduction system	3.215825	6.79E-14	1.01E-11
photosynthesis	2.770283	3.02E-05	0.00056
photosynthesis, light reaction	5.268667	2.44E-05	0.000516
photosynthetic electron transport chain	4.863385	0.005365	0.048788
photosynthetic electron transport in photosystem II	5.67395	0.001406	0.018914
photosystem	5.403762	0.003696	0.039071
phycobilisome	3.868602	3.94E-06	9.72E-05
protein binding	3.011933	4.93E-12	2.61E-10
protein kinase activity	2.786315	0.005871	0.048788
protein-phycocyanobilin linkage	4.364577	0.004156	0.041006
regulation of DNA-templated transcription	1.713048	0.003155	0.038908
signal transduction	3.221473	5.30E-12	2.61E-10

(D) GO terms in cluster #0 of *L*. sp. JSC-1	Odds ratio	p-value	q-value
ADP binding	5.004463	0.000666	0.01147
DNA-binding transcription factor activity	2.185412	0.001194	0.016823
calcium ion binding	4.72987	2.06E-15	3.20E-13
phosphorelay sensor kinase activity	2.428381	5.21E-06	0.000161
phosphorelay signal transduction system	2.991173	3.18E-12	2.47E-10
photosynthesis, light reaction	4.677374	0.000261	0.005782
phycobilisome	3.011945	0.000847	0.013136
protein binding	2.452584	1.04E-07	5.36E-06
regulation of DNA-templated transcription	2.05933	4.35E-05	0.001124
sequence-specific DNA binding	3.373379	0.000521	0.010101

signal transduction	2.507906	2.89E-07	1.12E-05

3.5 Comparison of intestinal microbiota of *B. buergeri* and *B. japonica* tadpoles

Intestinal metagenomic analysis of *B. buergeri* and *B. japonica* tadpoles detected 351 genera with non-zero reads from by using Kraken2/Bracken pipeline. *Leptolyngbya* was detected in the intestine of *B. buergeri* at a maximum of 1.3%, and was not detected in all tadpoles derived from 37℃. On the other hand, *Leptolyngbya* was detected in all intestines of *B. japonica*, accounting for a maximum of approximately 21% (Fig. 5). Then, we performed a statistical analysis using ALDEx2 to test which genera showed significant differences in the variances or abundances among tadpole groups. The result of Kruskal-Wallis tests showed that there was no significant abundance (q-value >0.05) of any genera between *B. buergeri* tadpoles derived from different temperatures (26 °C, 28 °C, 37 °C) and those of *B. japonica* tadpoles derived from 40 ℃. On the other hand, the result of glm tests showed statistical significant variance (q-value <0.05) of *Leptolyngbya* and *Limnothrix* (Table S12). The results of Welch's t-test showed that there was no significant abundance in any comparative pairs of tadpole groups ("all *B. buergeri* tadpoles and all *B. japonica* tadpoles", "*B. buergeri* tadpoles derived from 26 °C and *B. buergeri* tadpoles derived from 28 °C", "*B. buergeri* tadpoles derived from 26 °C and *B. buergeri* tadpoles derived from 37 ℃", and "*B. buergeri* tadpoles derived from 28 °C and *B. buergeri* tadpoles derived from 37 °C"). In contrast, the results of Wilcoxon tests on these pairs of tadpole groups showed significant abundance of *Azospira*, *Cephaleuros*, *Citrobacter*, *Enterobacter*, *Escherichia*, *Leptolyngbya*, *Mycobacterium*, *Oryzias*, *Pseudophryne*, *Rhizobium*, *Streptococcus*, *Tribolium*, and *Trichoderma* (Table S13-S16), except for the vertebrate genera possibly derived from host or contamination. *Leptolyngbya* was detected as a unique significantly abundant genus only in the pair of "all *B. buergeri* tadpoles and all *B. japonica* tadpoles".

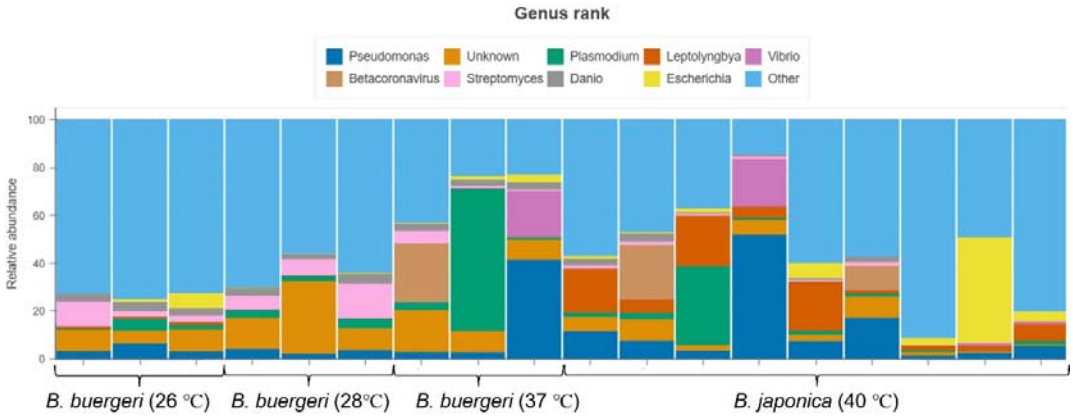

Fig. 5 Read abundances of *B. buergeri* and *B. japonica* estimated from Kraken2/Bracken pipeline. Barplot of the 9 most abundant taxa at the genus rank in all the samples. Any remaining taxa have been collapsed under the "Others" category to facilitate the visualization. The y-axis indicates the relative abundance of each taxon in percentages for each sample. See Table S12-S16 for

statistically significant taxa in each test.

3.6 Effects of temperatures and foods on survival dynamics in *X. tropicalis* tadpoles

In simple comparative analysis of survival rates, survival rates declined with increasing temperature, being lower at 30□°C than at 25□°C, and further reduced at 35□°C compared to 30□°C. This trend was consistent across all time points and was observed with both *Spirulina* and *L.* sp. Akita as food sources. When we compare survival rates between two different food sources, lower survival rates were repeatedly observed in *Spirulina*- than *L.* sp. Akita-fed experiments at 35□°C on day1 and day2 (Fig. 6C). However, no significant differences were found on any time points (Table S18, q-value >0.05).

Throughout the entire period, survival rate curves followed a sigmoidal pattern over time (either exhibiting a sharp decline toward zero or a gradual decline with exponential decay between time points). Therefore, to model these changes in survival rates over time, we used a sigmoid function characterized by two parameters: the negative growth rate (i.e., mortality rate) and the inflection point. The dynamics of these time series suggested a scenario in which continuous exposure to high temperatures led to a gradual decline in the health of tadpoles and sudden death after a lethal time threshold. The mortality rate was higher, and the inflection point occurred earlier at 35□°C than at the other temperature conditions (Fig. 6D). Additionally, although no significant differences were found on these parameters (Table S20, q-value >0.05), the estimated parameters of the survival rate curves tended to shift when *L.* sp. Akita was fed instead of *Spirulina* at 35□°C (Fig. 6D).

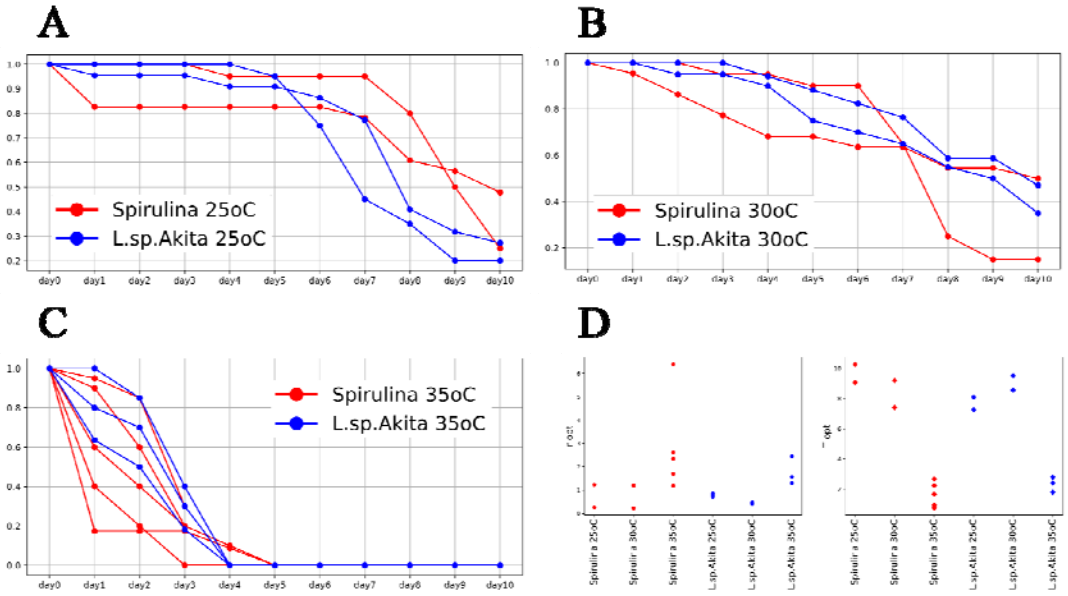

Fig. 6 Time series of survival rates and estimated parameters. Survival rates in each trial at (A) 25□, (B) 30□, and (C) 35□. Estimated parameters (D): mortality rate ( ) and inflection point ( ) in each trial.

4. DISCUSSION

4.1 Evolutionary relationships of two novel strains of genus *Leptolyngbya*

We isolated two novel strains of *Leptolyngbya* from "Kawara-no-yukko" and "Seranma", both had filamentous, nonheterocystous structures and hot-spring habitats. Results of whole genome sequencing showed that draft genomes of both strains have a single circular chromosome of about 6 Mb and several shorter circular or linear contigs than the chromosome.

Synteny plots showed that *L*. *boryana* had few homologous genomic regions across a contig with the length of approximately 280 kb in the draft genome of *L*. sp. Akita, so this suggested that these genomic regions might be a newly gained region in *L*. sp. Akita or a closely related strain group of *Leptolyngbya*. Although there are few similar reports in other strains of *Leptolyngbya*, some strains of *Leptolyngbya* also might have genomic regions in addition to their chromosomes. This was consistent with the fact that a single contig of *L*. sp. Seranma and a single scaffold of *L*. sp. JSC-1 were almost homologous in addition to their chromosomes.

Phylogenetic trees of 16S rRNA and Unicore gene set showed that *L*. sp. Akita and *L*. sp. Seranma were closely related to the strain group of *L*. *boryana* and that of *L*. sp. JSC-1, respectively, and these trends were also observed in the BLAST-based gene composition analysis. In particular, the gene composition analysis revealed that GO terms of "nitrogen compound metabolic process" and "transition metal ion binding" were enriched in the gene cluster, which was specific to the strain group of *L*. sp. Akita. All of the genes of *L*. sp. Akita, which had these GO terms in this gene clusters, were "NHLP leader peptide family RiPP (ribosomally synthesized and posttranslationally modified peptides) precursor". Interestingly, these genes formed a cluster on the genome of *L*. sp. Akita, and similar gene clusters were found on the genomes of *L*. *boryana* and *L*. sp. GGD. This suggested that the gene cluster of "NHLP leader peptide family RiPP precursor", same as the biosynthetic gene cluster (BGC) in cyanobacteria (Yi et al., 2024), was a characteristic genetic element that could explain the strain group of *L*. sp. Akita. The gene composition analysis also revealed that GO terms of "DNA transposition" and "transposase activity" were enriched in the gene cluster, which was specific to the strain group of *L*. sp. Seranma. All of these genes are transposases, so it suggests that these strain-specific transposases may drive genomic evolution in *L*. sp. Seranma for adaptation to its habitat. This result is consistent with studies reporting correlations between the ability of adaptation to a wide range of environments in *Leptolyngbya* and diversity in the distribution and density of microsatellites in their genomes (Yao et al., 2021). On the other hand, while no GO terms were enriched in the strain-group-specific gene clusters of *L*. sp. JSC-1, "phycobilisome" (PBS) or "photosynthesis"-related GO terms were enriched in the gene clusters, which tended to be BH (not reciprocal but best hit (i.e., simply homologous)) in the other strains except for *L*. sp. Seranma, *L*. sp. JSC-1, and *L*. sp. 7M. Among these genes, C-phycoerythrin subunit alpha (CpeA) and C-phycoerythrin subunit beta (CpeB) were unique to the strain group containing *L*. sp Seranma, *L*. sp. JSC-1, *L*. ectocarpi, *L*. sp. Heron Island J, and *L*. sp. 'hensonii', and the BH genes in the other strains were phycocyanin

subunit alpha or phycocyanin subunit beta. A mechanism that a part of the C-phycoerythrin family proteins composing PBS were replaced with CpeA and CpeB under far-red light had been reported in chromatic acclimation of *L*. sp. JSC-1 (Gan et al., 2014). Interestingly, not only CpeA and CpeB, allophycocyanin subunit alpha-B (ApcD), allophycocyanin subunit beta (ApcB), photosystem I core protein PsaA, and photosystem I core protein PsaB were also included in the strain-specific gene cluster, so the different sequences and structures of these genes from homologs in other strains, with no reports of chromatic acclimation, might play an important role in chromatic acclimation. In general, these results were consistent with the phylogenetic relationships of the newly isolated strains inferred by 16S and Unicore, and also indicated that our gene composition analysis could identify the characteristic genes in the targeted strain group.

4.2 Ecological relationships of cyanobacteria and sympatric animals and utilization as functional food resources

Finally, we focused on the high-temperature tolerance of *B. buergeri* and *B. japonica* tadpoles that sympatrically lived in their habitats, hot-springs of “Kawahara-no-Yukko” and “Seranma”, and compared their food habits using intestinal metagenomic analysis. The results of glm tests suggested that genus *Letolyngbya* and *Limnothrix* were the characteristic parameters that could explain the difference between food habitats of *B. buergeri* and *B. japonica* tadpoles on Generalized Linear Model (GLM). Similarly, Wilcoxon tests supported genus *Leptolyngbya* as the parameter that could indicate the difference in food habitats between *B. buergeri* and *B. japonica* tadpoles. Considering that reads of the genus *Letolyngbya* tend to be more frequently detected in *B. japonica* tadpoles and that a few reads of the genus *Limnothrix* were only detected in *B. buergeri* tadpoles derived from 27 ℃, it was suggested that *Leptolyngbya* could more strongly explain the difference between *B. buergeri* and *B. japonica*, especially more frequently used food resources. Unfortunately, in the other genuses that showed statistical significance between *B. buergeri* tadpoles derived from each temperature, the number of reads was smaller than that of genus *Leptolyngbya*, and the differences in food habits of *B. buergeri* under each temperature environment were unclear. In addition, it was also important to note that parametric hypothesis tests detected no statistical significance, whereas nonparametric hypothesis tests did. These results suggested that the difference couldn’t be detected in their food habitats of tadpoles if we assumed the read abundances of genus *Leptolyngbya* to follow a normal distribution. In other words, each tadpole might use *Leptolyngbya* as temporary food rather than regular food. *B. buergeri* and *B. japonica* tadpoles collected from these hot-springs didn’t show clear high-temperature tolerance in the rearing experiments, so the high-temperature tolerance of these tadpoles might have high phenotypic plasticity (data not shown), and these temporary food resources might cause the plasticity, especially in *B. japonica*. Overall, bacterial mats containing *Leptolyngbya* might allow amphibians that preferred low-temperature environments to obtain the new adaptive trait, high-temperature tolerance, but it was difficult to prove that the amount of

Leptolyngbya as their food directly gave them high-temperature tolerance. Thus, we plan to perform a feeding experiment on tadpoles using isolated and cultured *Leptolyngbya* to verify the mechanisms in the future.

4.3 Insights and limitations from cyanobacteria-feeding experiments on tadpole heat tolerance

In the feeding experiments with *X. tropicalis* tadpoles, we did not detect significant differences in survival rates on each observation day between the 25□°C and 35□°C trials, nor in the mortality rates or the inflection points in survival rate curves. However, in *X. tropicalis* tadpoles, one trial in which *Spirulina* was fed exhibited a dramatic decline in the survival rate on day1, while no such trend was observed in any trials in which *L*. sp. Akita was fed. This suggests that *L*. sp. Akita may contribute to the stability of heat tolerance rather than to the enhancement of it compared to *Spirulina*. Furthermore, the small number of trials (n = 2 in some cases) made it difficult to detect significant differences in survival rates or estimated parameters, even when using median-based tests such as Wilcoxon tests (data not shown). In addition, we assumed that the survival rate followed a binomial distribution and constructed a generalized linear model using the survival rate as the response variable, and time point, food type (i.e., whether *L*. sp Akita was provided), and temperature as explanatory variables. Variable selection using L1-regularized cross-validation showed that the regression coefficient for food type was the smallest among these explanatory variables (data not shown). To better assess changes in survival rates of tadpoles derived from food resources, additional trials are needed.

Another challenge is that the sigmoidal patterns in survival rate curves were observed even under non-high temperatures (e.g., 25□°C). This suggests that pure cyanobacterial powder may be nutritionally limited. In this regard, feeding cyanobacterial powder as part of compound foods, similar to approaches used in broiler studies, could more clearly reveal its effects on their survival rates under high temperatures. Indeed, in natural environments, frog tadpoles feed on a variety of resources, including submerged leaves, mosses, and microbial mats that also contain cyanobacteria. Thus, using compound foods in experiments may be a more ecologically realistic and appropriate approach.

It is also possible that a combination of host and food matching may influence their heat tolerance. For example, feeding *L*. sp. Akita to *B. buergeri* tadpoles or *L*. sp. Seranma to *B. japonica* tadpoles may yield stronger heat tolerance than *L*. sp. Akita to *X. tropicalis* tadpoles. Intestinal metagenomic analysis suggests that *Leptolyngbya* may be an important food resource, particularly in *B. japonica* tadpoles. Therefore, to show clearer heat tolerance derived from food resources, it might be better to use *B. buergeri* tadpoles and *L*. sp. Akita, or *B. japonica* tadpoles and *L*. sp. Seranma, with their close geographical and ecological relationships. In the future, we aim to clarify how feeding *Leptolyngbya* affects heat tolerance in tadpoles, with the ongoing experiments in which *L*. sp. Akita and *L*. sp. Seranma are fed to *B. buergeri* and *B. japonica* tadpoles, respectively.

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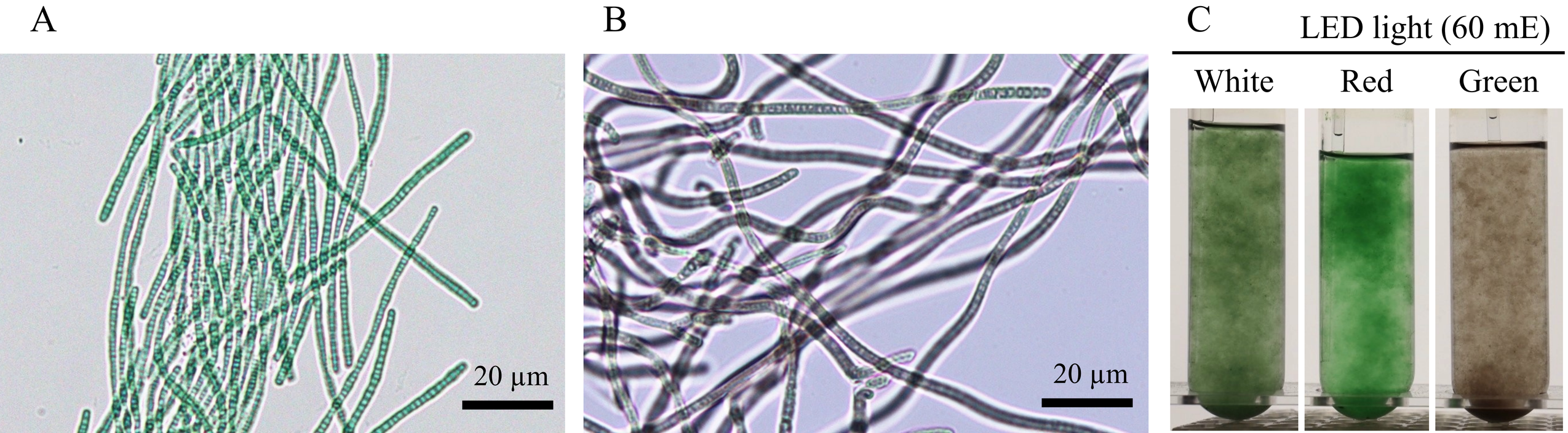
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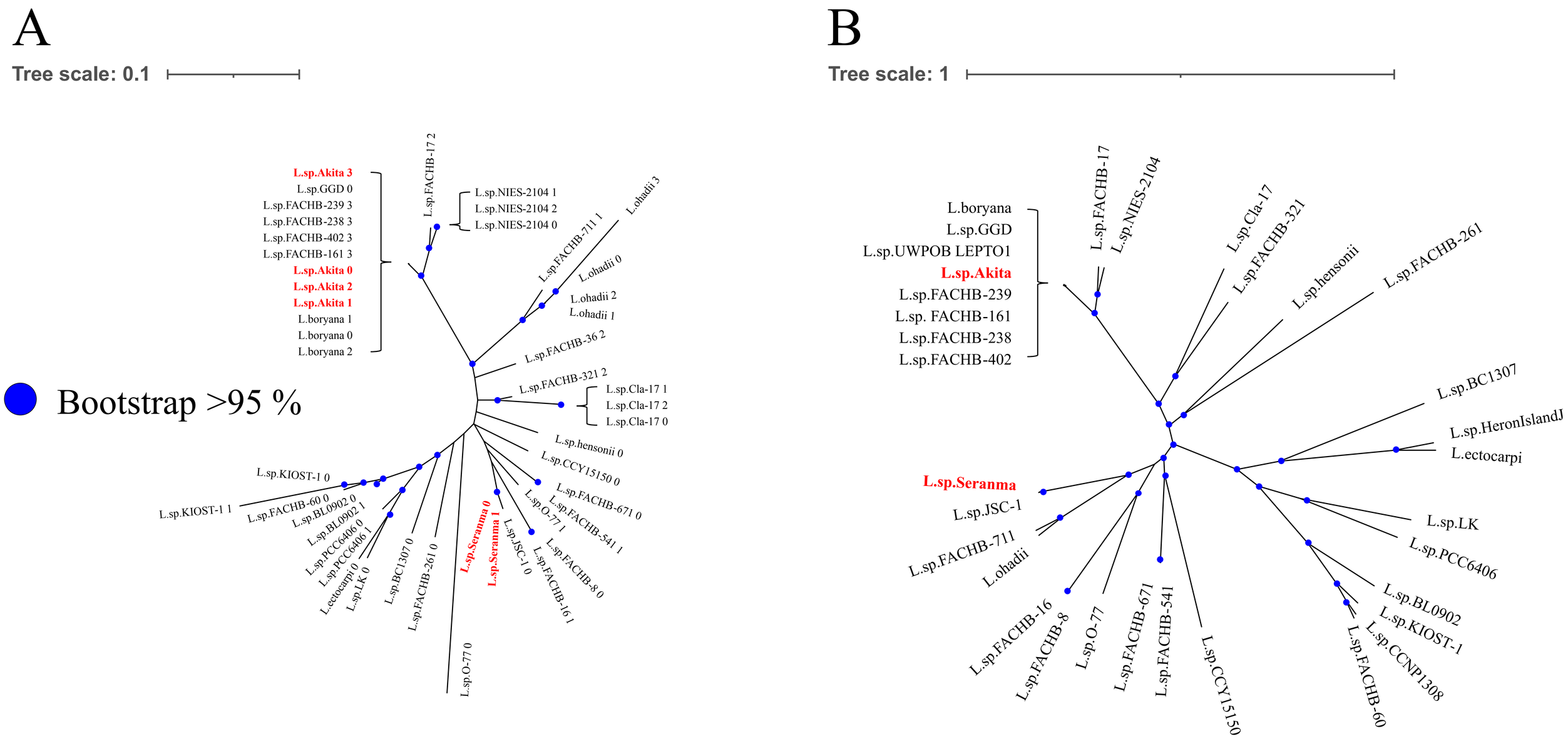
20 µm
B
20 µm
C
LED light (60 mE)
White
Red
Green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Tree scale: 0.1
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L.sp.GGD 0
L.sp.FACHB-239 3
L.sp.FACHB-238 3
L.sp.FACHB-402 3
L.sp.FACHB-161 3
L.sp.Akita 0
L.sp.Akita 2
L.sp.Akita 1
L.boryana 1
L.boryana 0
L.boryana 2
L.sp.FACHB-17 2
L.sp.NIES-2104 1
L.sp.NIES-2104 2
L.sp.NIES-2104 0
L.sp.FACHB-711 1
L.ohadii 3
L.ohadii 0
L.ohadii 2
L.ohadii 1
L.sp.FACHB-36 2
L.sp.FACHB-321 2
L.sp.Cla-17 1
L.sp.Cla-17 2
L.sp.Cla-17 0
L.sp.hensonii 0
L.sp.CCY15150 0
L.sp.FACHB-671 0
L.sp.FACHB-541 1
L.sp.O-77 1
L.sp.FACHB-8 0
L.sp.FACHB-16 1
L.sp.JSC-1 0
L.sp.Seranma 1
L.sp.Seranma 0
L.sp.FACHB-261 0
L.sp.BC1307 0
L.sp.LK 0
L.ectocarpi 0
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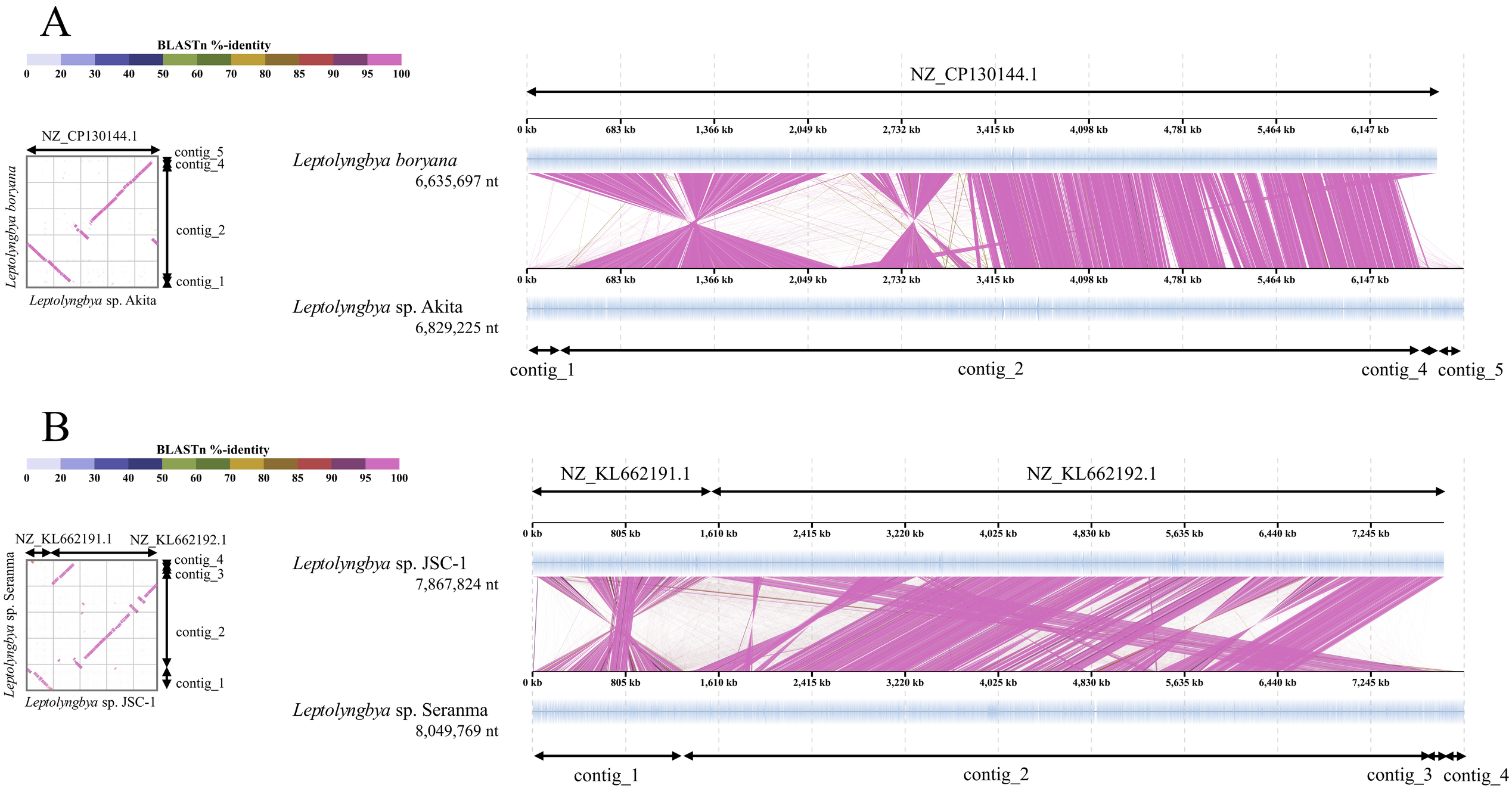
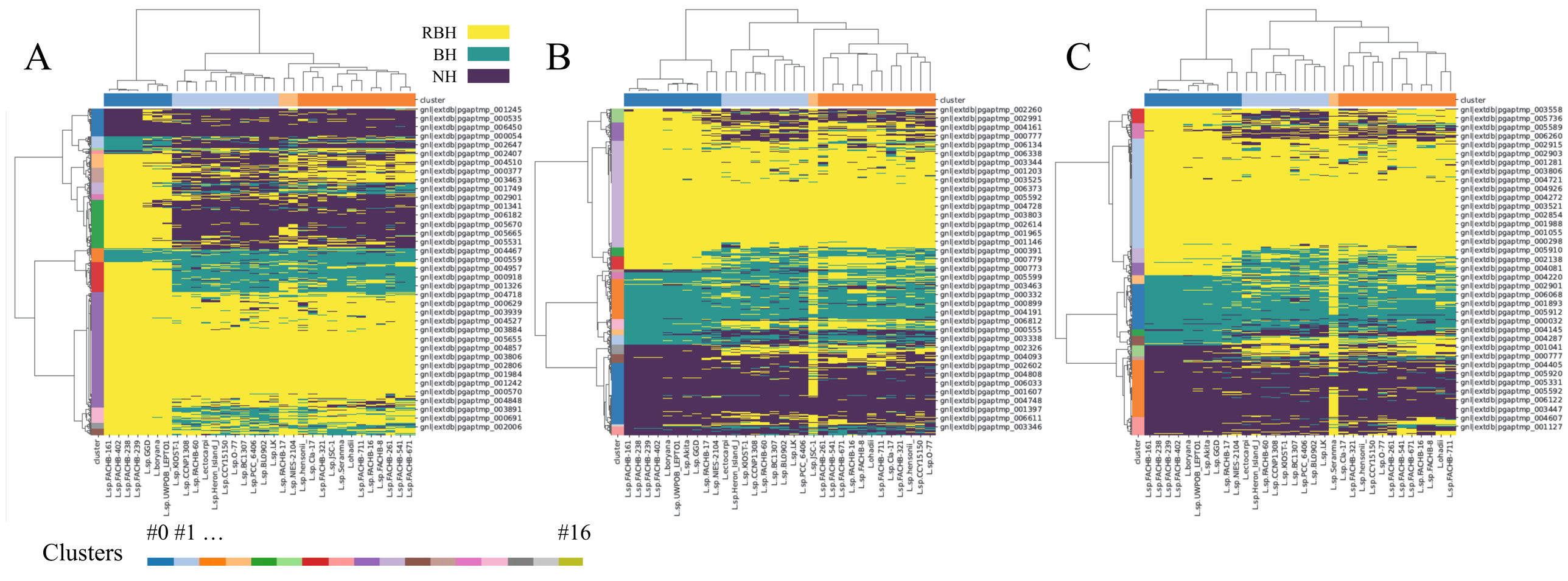
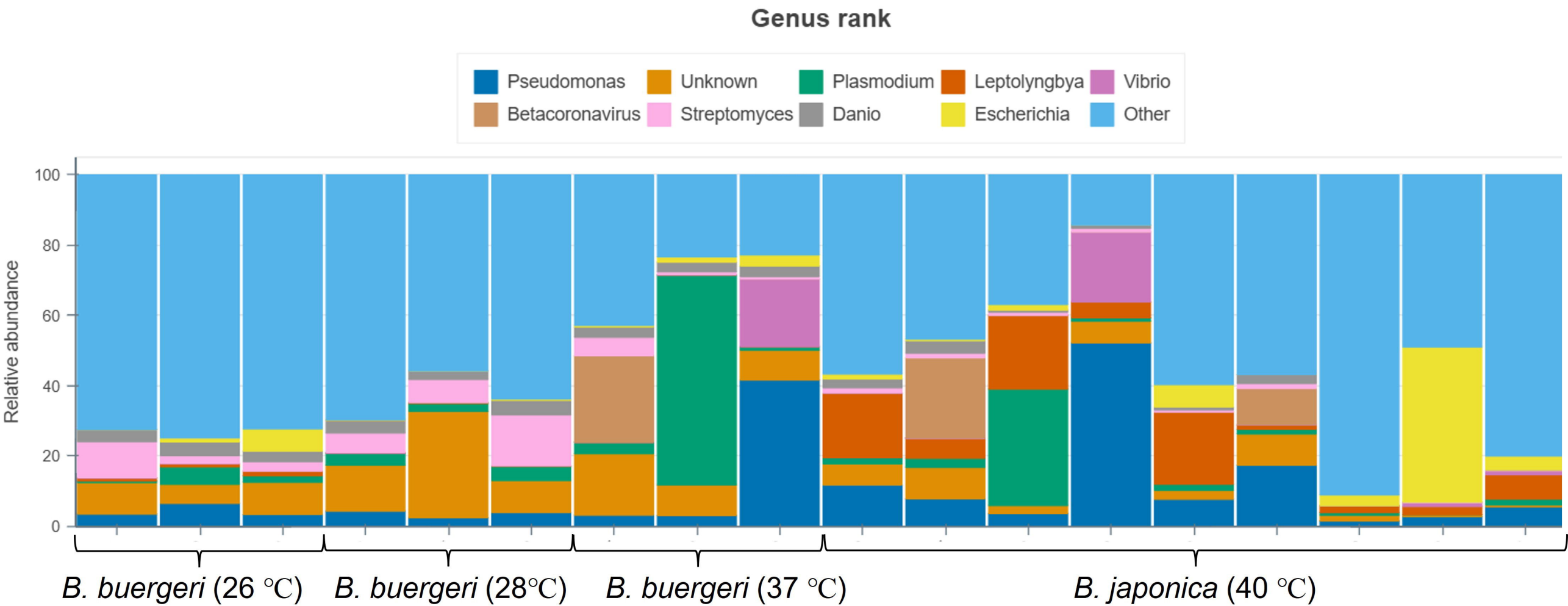
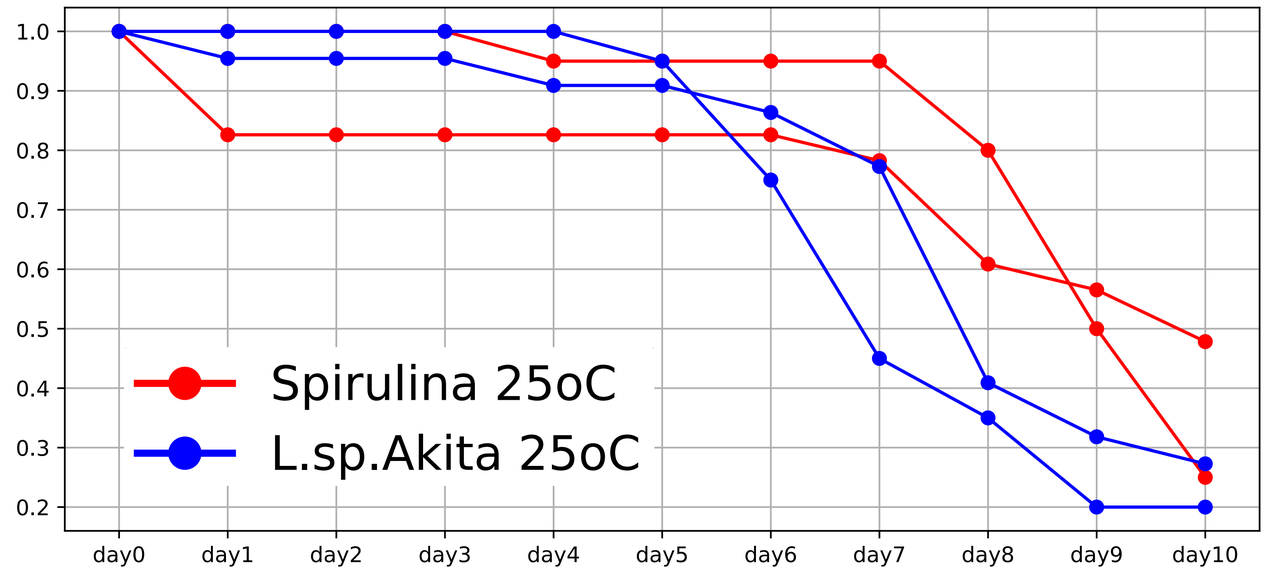
L.sp.PCC6406 0
L.sp.BL0902 1
L.sp.BL0902 0
L.sp.FACHB-60 0
L.sp.KIOST-1 1
L.sp.KIOST-1 0
L.sp.O-77 0
Bootstrap >95 %
B
Tree scale: 1
L.boryana
L.sp.GGD
L.sp.UWPOB LEPTO1
L.sp.Akita
L.sp.FACHB-239
L.sp. FACHB-161
L.sp.FACHB-238
L.sp.FACHB-402
L.sp.FACHB-17
L.sp.NIES-2104
L.sp.Cla-17
L.sp.FACHB-321
L.sp.hensonii
L.sp.FACHB-261
L.sp.BC1307
L.sp.HeronIslandJ
L.ectocarpi
L.sp.LK
L.sp.PCC6406
L.sp.BL0902
L.sp.KIOST-1
L.sp.CCNP1308
L.sp.FACHB-60
L.sp.CCY15150
L.sp.FACHB-541
L.sp.FACHB-671
L.sp.O-77
L.sp.FACHB-8
L.sp.FACHB-16
L.ohadii
L.sp.FACHB-711
L.sp.JSC-1
L.sp.Seranma


A
BLASTn %-identity
0 20 30 40 50 60 70 80 85 90 95 100
NZ_CP130144.1
contig_5
contig_4
contig_2
contig_1
Leptolyngbya boryana
Leptolyngbya sp. Akita
Leptolyngbya boryana
6,635,697 nt
Leptolyngbya sp. Akita
6,829,225 nt
NZ_CP130144.1
0 kb 683 kb 1,366 kb 2,049 kb 2,732 kb 3,415 kb 4,098 kb 4,781 kb 5,464 kb 6,147 kb
contig_1
contig_2
contig_4
contig_5
B
BLASTn %-identity
0 20 30 40 50 60 70 80 85 90 95 100
NZ_KL662191.1
NZ_KL662192.1
contig_4
contig_3
contig_2
contig_1
Leptolyngbya sp. Seranma
Leptolyngbya sp. JSC-1
Leptolyngbya sp. JSC-1
7,867,824 nt
Leptolyngbya sp. Seranma
8,049,769 nt
NZ_KL662191.1
NZ_KL662192.1
0 kb 805 kb 1,610 kb 2,415 kb 3,220 kb 4,025 kb 4,830 kb 5,635 kb 6,440 kb 7,245 kb
contig_1
contig_2
contig_3
contig_4

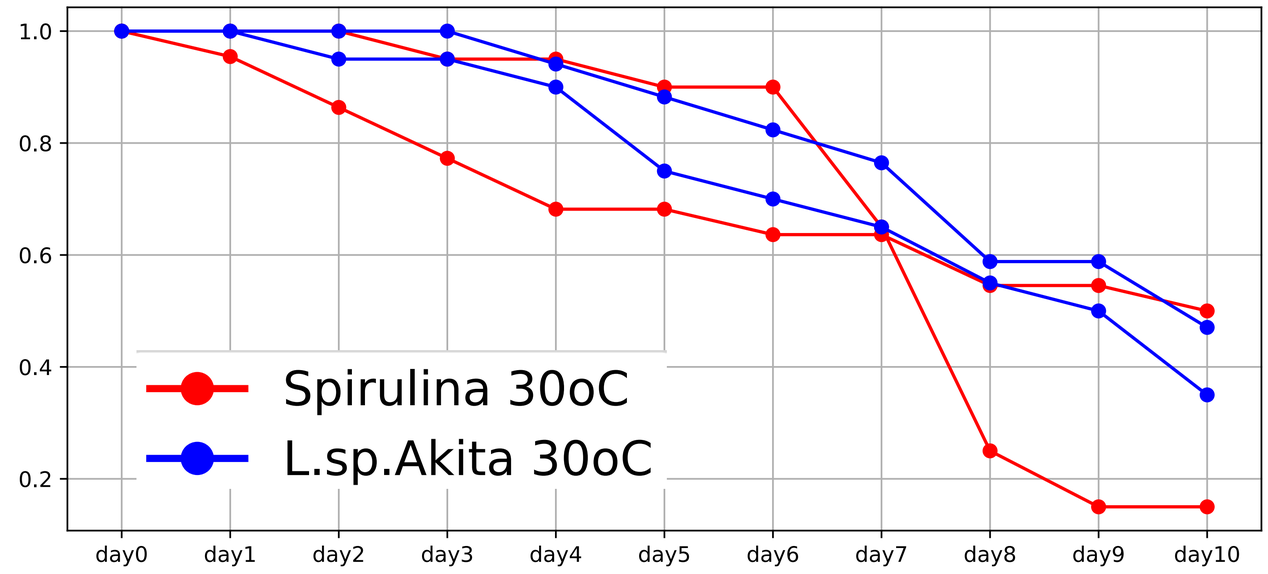

A
B
C
RBH
BH
NH
cluster
#0 #1 …
#16
Clusters


Genus rank
Pseudomonas
Unknown
Plasmodium
Leptolyngbya
Vibrio
Betacoronavirus
Streptomyces
Danio
Escherichia
Other
Relative abundance
100
80
60
40
20
0
B. buergeri (26 ℃)
B. buergeri (28℃)
B. buergeri (37 ℃)
B. japonica (40 ℃)

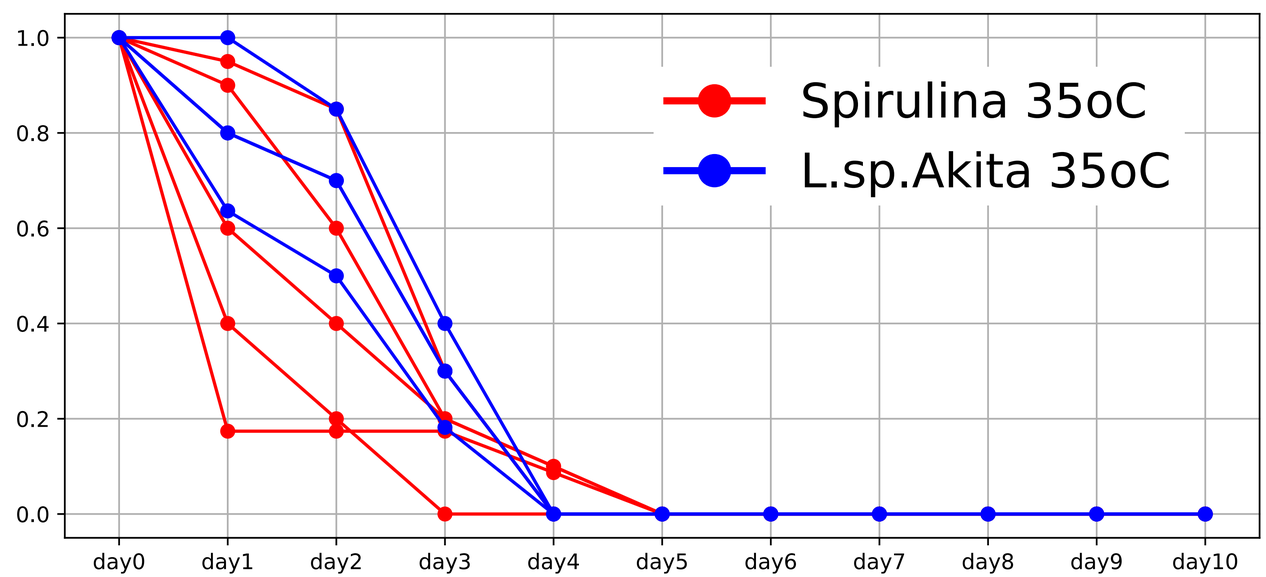
A


1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
Spirulina 25oC
L.sp.Akita 25oC
day0
day1
day2
day3
day4
day5
day6
day7
day8
day9
day10

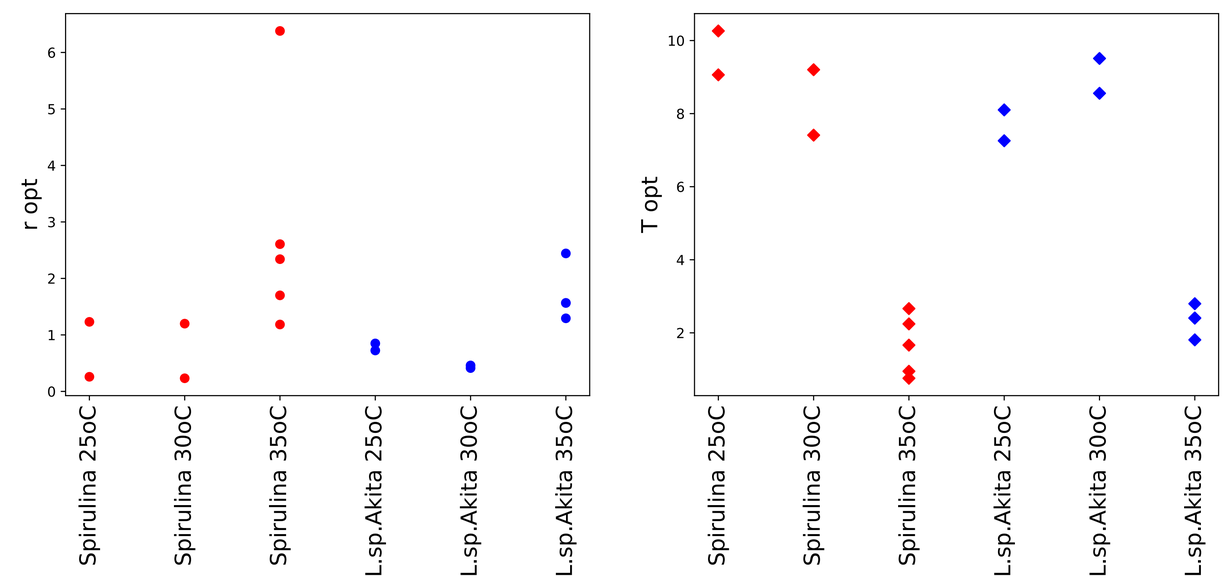
B


1.0
0.8
0.6
0.4
0.2
Spirulina 30oC
L.sp.Akita 30oC
day0
day1
day2
day3
day4
day5
day6
day7
day8
day9
day10

C


1.0
0.8
0.6
0.4
0.2
0.0
Spirulina 35oC
L.sp.Akita 35oC
day0
day1
day2
day3
day4
day5
day6
day7
day8
day9
day10

D


r opt
T opt
Spirulina 25oC
Spirulina 30oC
Spirulina 35oC
L.sp.Akita 25oC
L.sp.Akita 30oC
L.sp.Akita 35oC