Presence and Function of a Circadian Clock in Marine Cyanobacteria *Prochlorococcus* and *Synechococcus*

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Abstract

This study attempts to explore cyanobacterial circadian clock-related gene expression and analysis of related environmental conditions in marine environments. A circadian clock refers to a biomechanical component that controls gene expression on a 24-hour cycle. This study aims to determine if the circadian clock is represented in the cyanobacteria Prochlorococcus and Synechococcus in natural environments in a similar manner to what has been observed in laboratory studies. Although the focus will be on the relationship between the circadian clock and light intensity, there is potential to discover new environmental connections to the cyanobacterial circadian clock by using data from cruises with greater variability in conditions. Genetic data from the Berube cycog database along with genomic data from the Gradients2 cruise was utilized to create phylogenetic trees for each component of the circadian clock. It was found that Prochlorococcus clades associated with high light adapted groups saw higher circadian clock expression values than those associated with low light adapted groups, and Synechococcus expression was generally high across all clades represented. In addition, only Synechococcus displayed a fully functioning circadian clock, and Synechococcus data illustrated increased genetic and geographical diversity when compared to Prochlorococcus.

Plain Language Summary

A circadian clock refers to a 24-hour cycle that nearly all organisms on the planet experience. In humans, we refer to it as our night-day cycle. In cyanobacteria, a critical oceanic phytoplankton, the circadian clock is an important component that assists with nearly all functions of a select few species. This study aims to explore where and under what conditions the clock is being utilized by two species of cyanobacteria, Prochlorococcus and Synechococcus. The goal is to

understand how the environment can affect how each species uses the clock, and to explore the differences in clock usage between the two cyanobacteria species. Essentially, this study sought to understand how cyanobacteria are responding to their environment and how their environment affects them through circadian clock processes. The study was not able to conclusively identify environmental conditions that could affect the circadian clock, but it was able to confirm the presence and difference in usage between Prochlorococcus and Synechococcus species. Only Synechococcus ecotypes exhibited a fully functioning circadian clock, which is congruent with previous studies on the topic.

Introduction

Marine phytoplankton are critical to aquatic and terrestrial environments and are responsible for nearly half of global primary productivity (Sánchez-Baracaldo 2015). Among marine phytoplankton, Cyanobacteria are particularly important due to their sheer numbers and controlling role in primary productivity and carbon export to deeper waters, with the latter directly impacting climate change as a sink of carbon dioxide. (Sánchez-Baracaldo 2015). Cyanobacteria are also a critical component of the global nitrogen cycle as some species are capable of nitrogen fixation (Burford, et al. 2020). Cyanobacteria have also been evaluated as a species of interest for use as a bio-agent in sustainable agriculture (Singh, et al. 2016). In 2016, Singh et al. evaluated how cyanobacteria can be used to address soil nutrient enrichment issues due to their ability to photosynthesize, fix nitrogen, and grow in a wide variety of water conditions. Due to cyanobacteria's profound impact on global atmospheric and oceanic conditions through primary production, carbon export, and nitrogen fixation, they can be viewed as a species of high interest for continued research. Understanding how the growth and function of cyanobacteria are affected by environmental factors will help determine how their key

molecular processes change as environmental parameters, such as water temperature, acidity, and salinity levels, alter with climate change and related processes.

In many eukaryotic organisms, metabolic and other molecular processes are affected by a circadian rhythm. A circadian rhythm describes a rhythmic control of cellular physiology processes set by fluctuations in temperature, light intensity, and humidity. The light dictated day and night cycle in humans is an example of a circadian rhythm (Cohen and Golden 2015). A Circadian clock can essentially determine environmental time, generally on a 24-hour cycle. (Johnson, et al. 2008). Through this synchronization with their environment, temperature, and light cycles, fitness of organisms can be improved by optimizing sunlight exposure, and by understanding when to use alternative processes to gain energy (Johnson, et al. 2008). The circadian clock was originally thought to be limited to Eukaryotes, and it was not until 1986 that a rhythm in nitrogen fixation and amino acid uptake was discovered in *Synechococcus*, a cyanobacteria (Cohen and Golden 2015). The discovery of a proper temperature compensated, resettable, and persistent circadian clock in cyanobacteria prompted a range of research into its relationship with processes such as cell division. In particular, the presence of temperature compensation is critical in defining a circadian clock, as this means that it is insensitive to immediate changes in temperature and not controlled by simple feedback mechanisms (Huang, et al. 1990). In 1997, researchers concluded that the circadian clock functioned even when cell division occurs multiple times in one circadian cycle. (Kondo, et al. 1997). This further strengthened the body of research supporting the cyanobacteria rhythm as a true circadian clock. The circadian clock in cyanobacteria allow them to adapt to environmental changes by prioritizing molecular processes that operate at higher efficiency during different light levels, such as conducting photosynthesis during high light intensity and nitrogen fixation at low light

intensity. The clock's initial state, and therefore the rhythmic expression of molecular processes, is set by environmental factors and will operate according to a 24-hour clock based on the original conditions it was exposed to (Swan, et al. 2018). The clock is not an immediate feedback loop and is reset according to input from proteins specific to the oscillator (Swan, et al. 2018). As the circadian clock's initial state is set solely by external conditions, it is important to understand how environmental changes could affect circadian rhythm of critical primary producers such as cyanobacteria.

In cyanobacteria, the molecular component responsible for the circadian clock, the core oscillator, is encoded by the KaiA, KaiB, and KaiC genes (Figure 1). The core oscillator can regulate global gene expression and cell division timings (Cohen and Golden 2015; Kitahara, et al. 2019). The oscillator is set by environmentally controlled molecules that signal cellular redox status, with input protein kinases providing information on environmental conditions to the oscillator (Cohen and Golden 2015). Essentially, the circadian clock is tuned by the environment, and then continues on light-based 24-hour cycles until conditions mandate a reset.

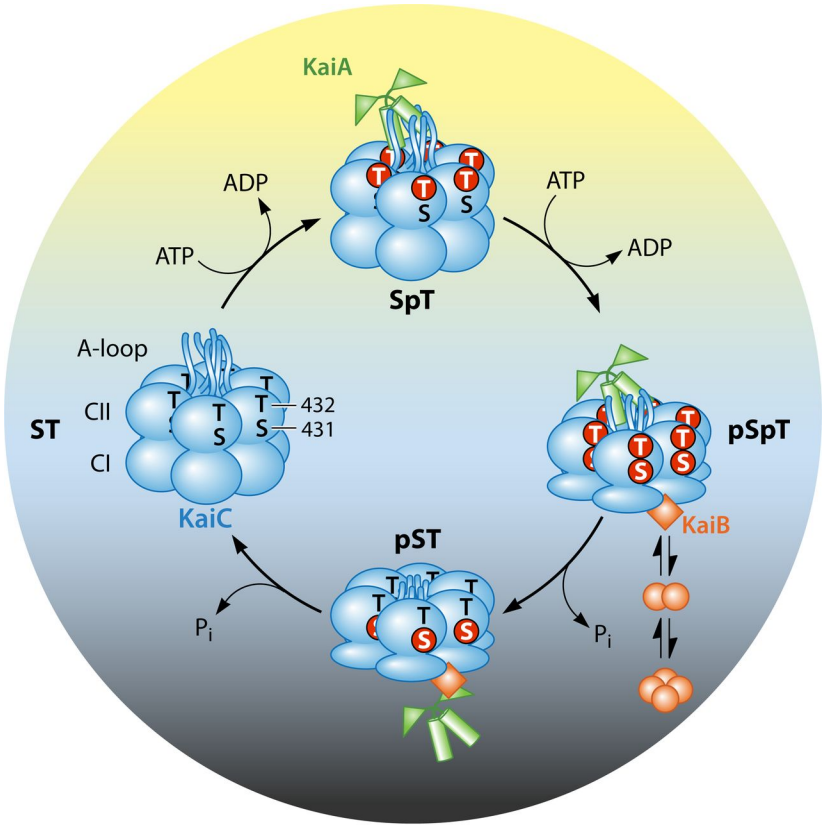

Figure 1. The mechanism behind the circadian clock, primarily comprised of KaiA, KaiB, and KaiC proteins. The complete oscillator forms a donut shape, with a cyclic relationship between the three proteins and related kinase. SpT and pST refer to phosphorylation events associated with the setting of the clock. (Cohen and Golden 2015).

Genetic analysis has shown that nearly the entire genome of *Synechococcus* is expressed rhythmically, with the majority of genes falling into two classes; class one genes show maximum expression at dusk, while class two genes show maximum expression at dawn (Cohen and Golden 2015). The separation of gene expression by sunlight exposure category further illustrates the wide-ranging control on cellular processes exhibited by the circadian oscillator. In 2008, Johnson et al. used cells with luminescence to monitor gene expression, adding a visual reference to the rhythmicity of the circadian clock's control on the genome (Figure 2).

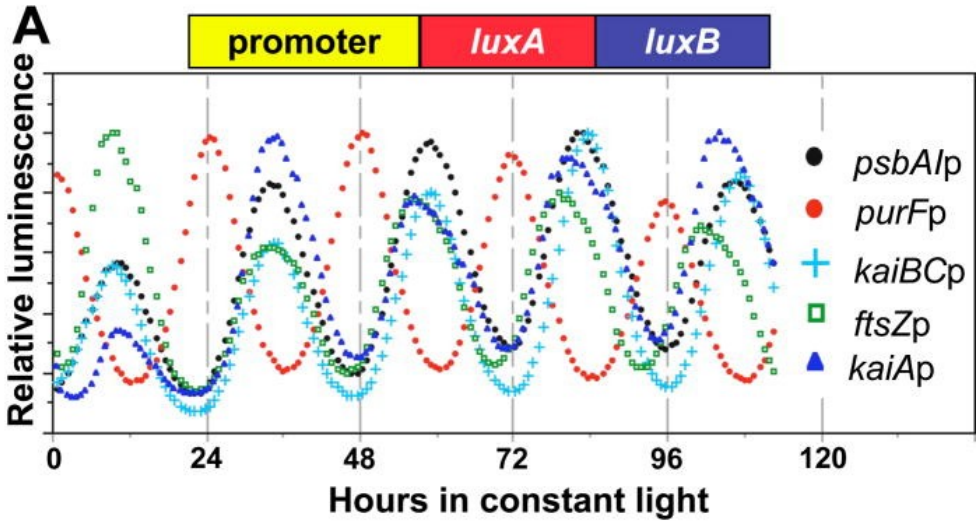

Figure 2. Circadian rhythm of gene expression monitored through different luminescent cells and proteins. Here, psbAI, kaiA, kaiBC, purF, and ftsZ refer to promotors for genes related to the circadian oscillator. (Johnson, et al. 2008).

Further research has been conducted regarding the control of metabolite partitioning by the circadian oscillator. Diamond et al. found that the loss of the core oscillator protein, KaiC, resulted in a decrease in metabolite partitioning during diurnal growth. These findings are significant as light/dark cycles should be able to influence metabolite partitioning on their own, and the immediate impact of losing KaiC demonstrates the extensive impact that the oscillator has on metabolic processes. (Diamond, et al. 2015).

While a complete cyanobacteria circadian clock is comprised of Kai A, B, and C proteins, not all cyanobacteria species express all three. The three-protein oscillator is well documented in *Synechococcus elongatus*, but less studied in other species. *Prochlorococcus marinus* is known to express only KaiB and KaiC genes, and therefore does not form the full three-protein oscillator (Axmann, et al. 2014). As a result, *Prochlorococcus* is not found to exhibit a full, robust circadian clock, instead utilizing more simple feedback loops which dampen when not provided with constant feedback (Cohen and Golden 2015). As *Prochlorococcus* requires daily

input of light, they exhibit more erratic diel gene expression patterns when observed in lab environments (Cohen and Golden 2015). The significance of a lack of a full circadian clock will be explored in this study as genetic data, gene expression data in transcripts per million, and some corresponding light intensity data is available for both *Prochlorococcus* and *Synechococcus*.

Despite a large body of research on cyanobacteria circadian rhythms, new factors that can influence circadian rhythm in cyanobacteria are still being discovered. Recent research such as how the circadian clock can slow when introduced to higher pressures (Kitahara, et al. 2019) or lower temperatures (Murayama, et al. 2017) are examples. There is much that is still unknown regarding the extent of KaiABC oscillator control on total gene expression and what factors set the circadian clock and gene expression, with lots of room for exploration through genetic analysis. The majority of current research on cyanobacterial circadian rhythm appear to be laboratory studies, so analyzing data taken from cruises could reveal new information on circadian rhythm-controlled gene expression (Hörnlein, et al. 2020). By utilizing data from natural communities of cyanobacteria, there is potential for greater variation in genetic expression and environmental variability as opposed to a more controlled lab environment. Employing cruise data allows for the research of a wide range of cyanobacteria species with equally wide-ranging environmental conditions. Through an exploration of genetic data and corresponding environmental factors, new conditions could be discovered relating to the operation or control of the circadian oscillator.

Considering the potential for significant variability in both genetic makeup and environmental conditions in cruise data, this study will initially focus on determining the presence and function of the KaiABC oscillator in field data. The focus will be on genetic analysis for *Prochlorococcus*

and *Synechococcus*, which have the most genetic data and background research available among cyanobacteria. As light intensity is often found to be the primary factor in setting the circadian clock, it will be the primary environmental parameter assessed in relation to the expression of the KaiABC oscillator alone or with large portions of the genome. However, this study is exploratory, and the KaiABC oscillator is not confirmed to be detectable in natural samples. I hypothesize that 1) KaiABC genes will be found in diverse cyanobacteria communities in natural environments due to the critical role of the clock in adapting to the light/dark cycle and regulating the genome, and 2) light intensity levels will influence the expression of the KaiABC oscillator. As this study is examining genetic data for both *Prochlorococcus* and *Synechococcus*, there is the possibility to explore the influence of light on an incomplete circadian oscillator, in the case of *Prochlorococcus*. The role of the oscillator in cyanobacteria other than *Synechococcus* is not well understood, so there is potential for exploration in this area of study.

On a broad scale, this study will aim to explore how cyanobacteria synchronize their physiology to their surrounding environments and how it affects metabolic and other physiological processes. The research explores how environmental factors, primarily light intensity, influence the three-protein KaiABC oscillator and corresponding rhythmic gene expression through proteins and input kinase, such as KaiA and CikA, which are light influenced for example (Hörnlein, et al. 2020). The first analysis will involve the creation of a phylogenetic tree of Prochlorococcus and Synechococcus KaiA, KaiB and KaiC genes, to see if the different ecotypes of the two species can be delineated based on the sequences of these genes.

Methods

Genetic sequences and Cyanobacterial clusters of orthologous groups data (CyCOG database) was obtained through data citation 1, files 6 and 12 of the Berube paper (Berube, et al. 2018)

(link), with data directly accessible here. Data from the Berube paper was collected at 13 sites across the Pacific and Atlantic oceans, primarily from GEOTRACES cruises (Figure 3).

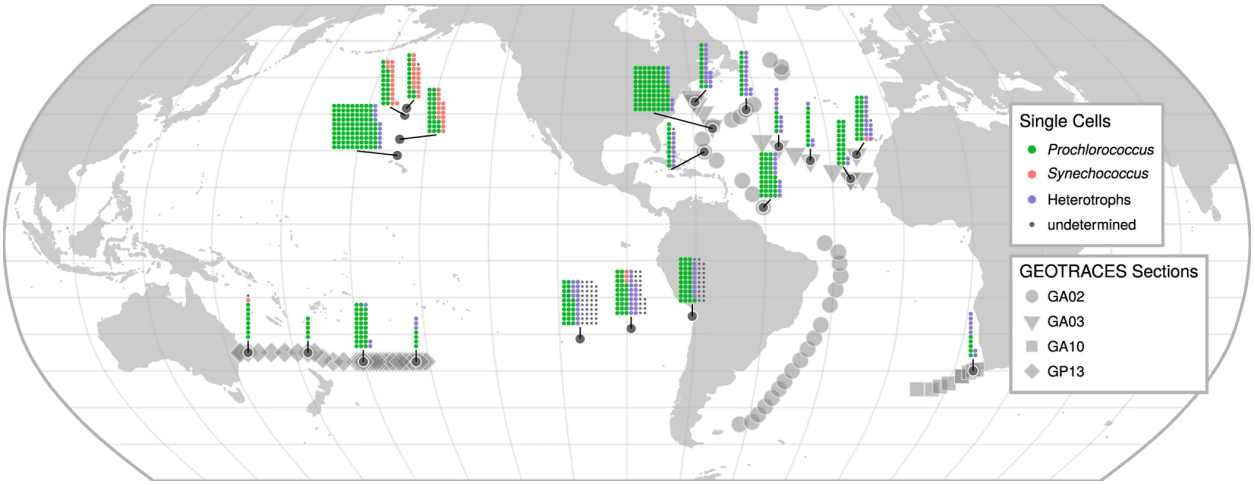

Figure 3, Collection sites for data obtained from the Berube paper and cycog database. Single cell genomes at each site are represented by miniaturized stacked dot-plots (each dot represents one single cell genome), with organism group indicated by color. Larger dots correspond to stations on GEOTRACES cruises (Berube, et al. 2018).

A metatrasncriptome dataset obtained during Gradients2 cruises was utilized for expression data and corresponding environmental data (link). The Gradients cruise data was collected between 25 and 41 degrees latitude in 2016 and 2017 (Figure 4).

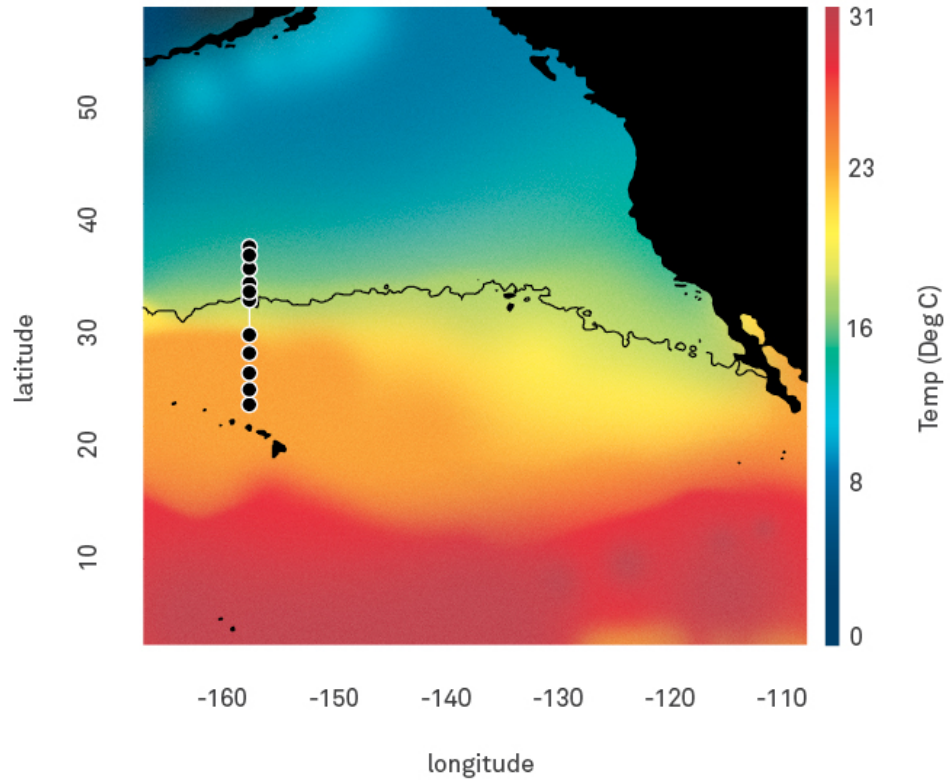

Figure 4, This image shows the 2016 Gradients cruise course, with colors corresponding to sea surface temperature. Black dots indicate sampling locations along cruise route. (Foundation 2016).

Code was written in python (link) to automatically take sequence identifiers from the cycog database on KaiA, KaiB, and KaiC, search through the larger genome sequences database (Berube data citation 1, file 12) and compile single FASTA files for each of the three genes. Genetic sequences were aligned at the DNA level in MAFFT run on auto settings in Ubuntu. Jalview was used to visualize and inspect the aligned sequences. Sequences were trimmed in Mega-X using a 90% site coverage cutoff. Initial phylogenetic tree newick files were generated in Mega-X using maximum likelihood, with the number of bootstrap replications set at 100. Newick files were uploaded to iTOL for final tree visualization. The iTOL annotation editor was used to format data within iTOL and to create heatmaps, bar charts and other phylogenetic tree annotations. Additional python scripts were written to organize and sort data and data labels in

order to finalize trees. Expression data in average TPM (average transcripts per million, a way to display gene expression) was added to iTOL trees as well. Clade data was included in the Gradients2 metatrasncriptome dataset (A clade is a group of organisms that are comprised of a common ancestor and all its descendants should be in intro) obtained from Dr. Sacha Coesel of the Armbrust lab, who is the original creator.

Results

Distribution of Synechococcus and Prochlorococcus ecotypes within KaiA data skews heavily towards Synechococcus, with all but one ecotype in the KaiA phylogenetic tree being Synechococcus. A single ecotype of Prochlorococcus was identified in the KaiA genome data (Figure 5). In the KaiA data (Figure 5), there were seven distinct Synechococcus clades identified. Total metatrasncriptome expression data (in transcripts per million or TPM) varied between and within clades, but TPM latitude was consistent within clades. Nearly all Synechococcus ecotypes expressed KaiA to some degree. Synechococcus was found throughout majority of the sampled range, with some clades such as 5.1A-IV (Figure 5) sampled consistently between 32- and 41-degrees latitude. The clades for two ecotypes were not able to be identified, which are displayed in gray on the KaiA phylogenetic tree (Figure 5).

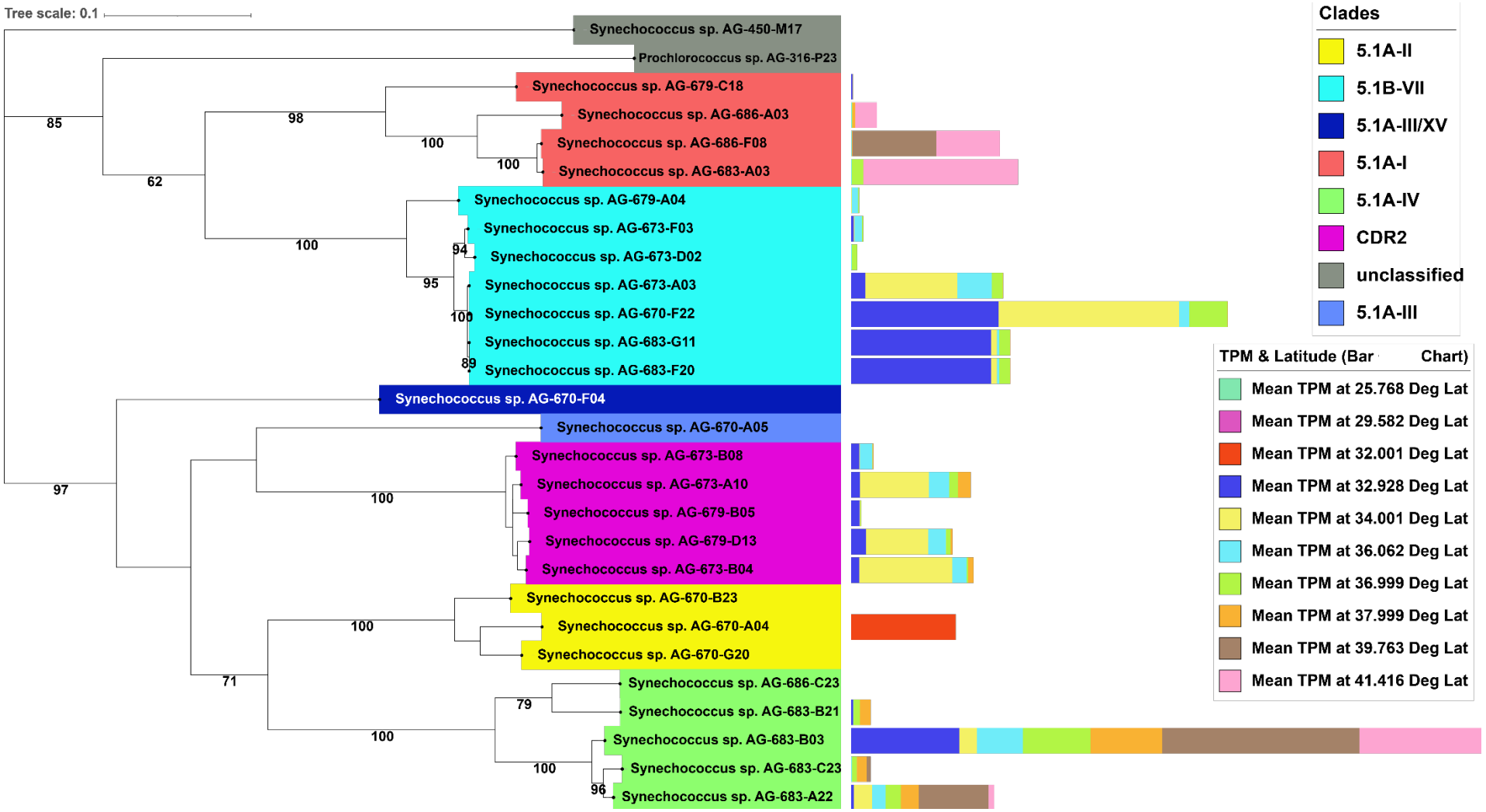

Figure 5, Phylogeny of the KaiA gene from 1 Prochlorococcus and 27 Synechococcus gDNA data. Bootstrap values are displayed on branches. Bootstrap values less than 60 are omitted. Phylogenetic clade grouping is indicated by the colored bars and labeled in the corresponding legend. Bar charts at the end of each ecotype indicate transcripts per million (TPM) for KaiA gene, with each bar broken up into different colors representing TPM at different latitudes. Bar charts are labeled in the corresponding legend.

KaiB introduces a much wider Prochlorococcus dataset, with Prochlorococcus ecotypes making up majority of the phylogenetic tree (Figure 6). The KaiB phylogenetic tree features a total of 309 ecotypes. The KaiB dataset has reduced TPM expression values for Synechococcus ecotypes (displayed in red on the innermost ring) and presents new Prochlorococcus expression data. Despite the greater number of Prochlorococcus displayed on the tree, the clade diversity is reduced when compared to Synechococcus data. While some ecotypes in Prochlorococcus clades HLI and HLII (High Light I and High Light II) display high TPM values, the other Prochlorococcus clades display little to no TPM data. Where Prochlorococcus TPM expression data is available, the expression latitude range is reduced when compared to Synechococcus expression data. Prochlorococcus clade HLII (gold/yellow colored outer ring) displayed the greatest range in latitude, ranging between 29 and 34 degrees.

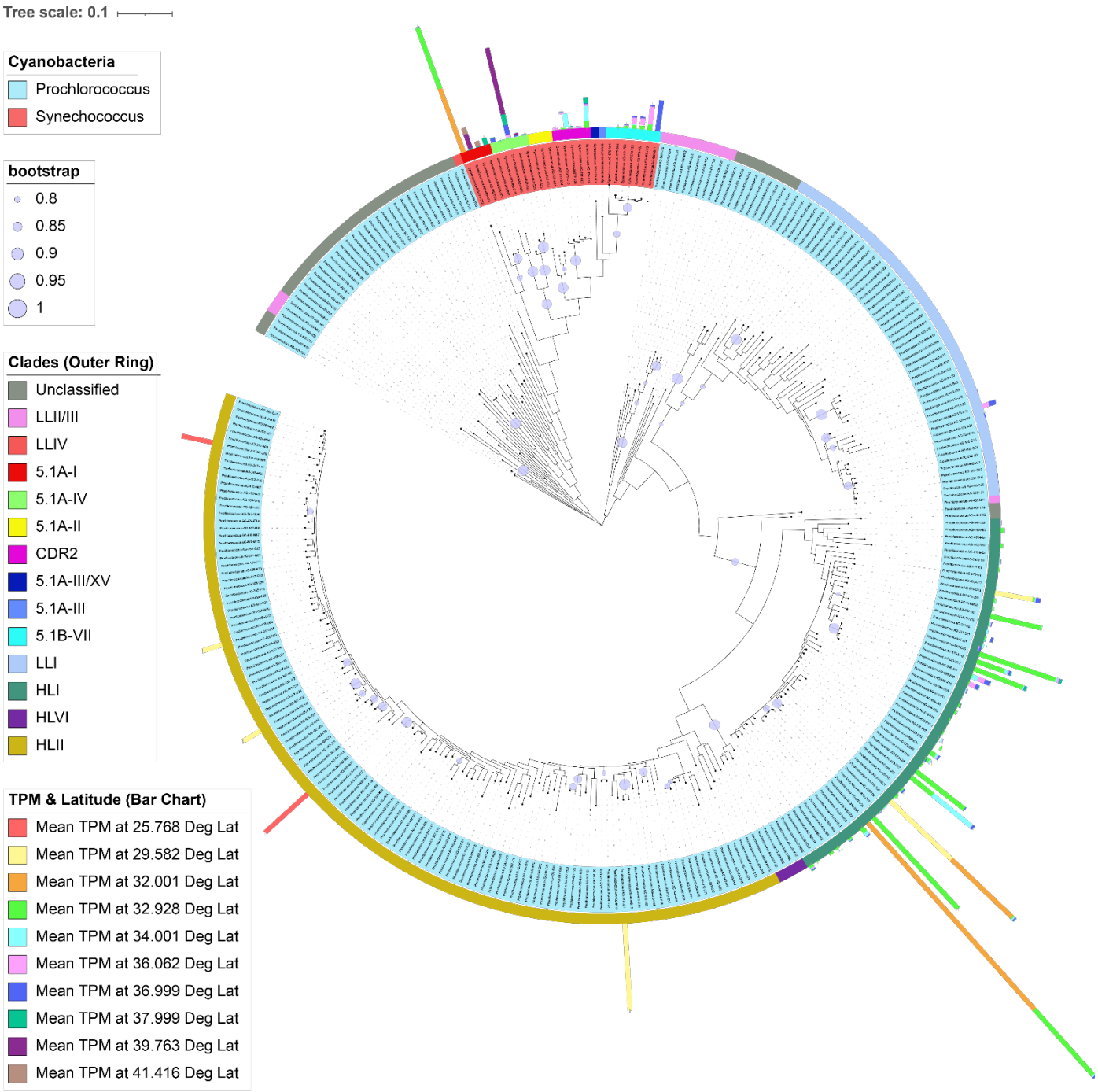

Figure 6, Phylogeny of the KaiB gene from Prochlorococcus and Synechococcus gDNA data. Cyanobacteria species is represented by the inner colored ring. Bootstrap values are represented by size-scaled dots at nodes. Bootstrap values less than 80 are omitted. Phylogenetic clade grouping is indicated by the outer colored ring and labeled in the corresponding legend. Bar charts at the end of each ecotype indicate transcripts per million (TPM) for KaiB gene, with each bar broken up into different colors representing TPM at different latitudes. Bar charts are labeled in the corresponding legend.

KaiC introduces the largest ecotype and TPM expression value dataset (Figure 7). KaiC features a total of 339 ecotypes. Expression data for Prochlorococcus and Synechococcus is higher than seen in the phylogenetic trees for KaiA and KaiB (Figures 5 and 6), with Prochlorococcus clades HLI and HLII once again displaying the highest expression values for Prochlorococcus. Except for a few ecotypes, Synechococcus expression data is more uniform across different clades, and continues the trend of having nearly all ecotypes display expression data to some extent. Latitude ranges similar to ones displayed in KaiA and KaiB trees are displayed for Prochlorococcus and Synechococcus clades on the KaiC tree. The total amount of clades in the KaiC data is the same as the KaiB dataset. Despite there being fewer total Synechococcus ecotypes, the data that was available displayed greater genetic diversity when compared to Prochlorococcus (evident by a higher number of clades compared to Prochlorococcus, visible in Figure 6 and 7). Figure 8 zooms in on the Synechococcus data within the KaiC tree, illustrating similar expression levels and latitude data when compared to KaiA (Figure 5). Overall TPM values are higher within all clades in the KaiC Synechococcus data when compared to the KaiA phylogenetic tree.

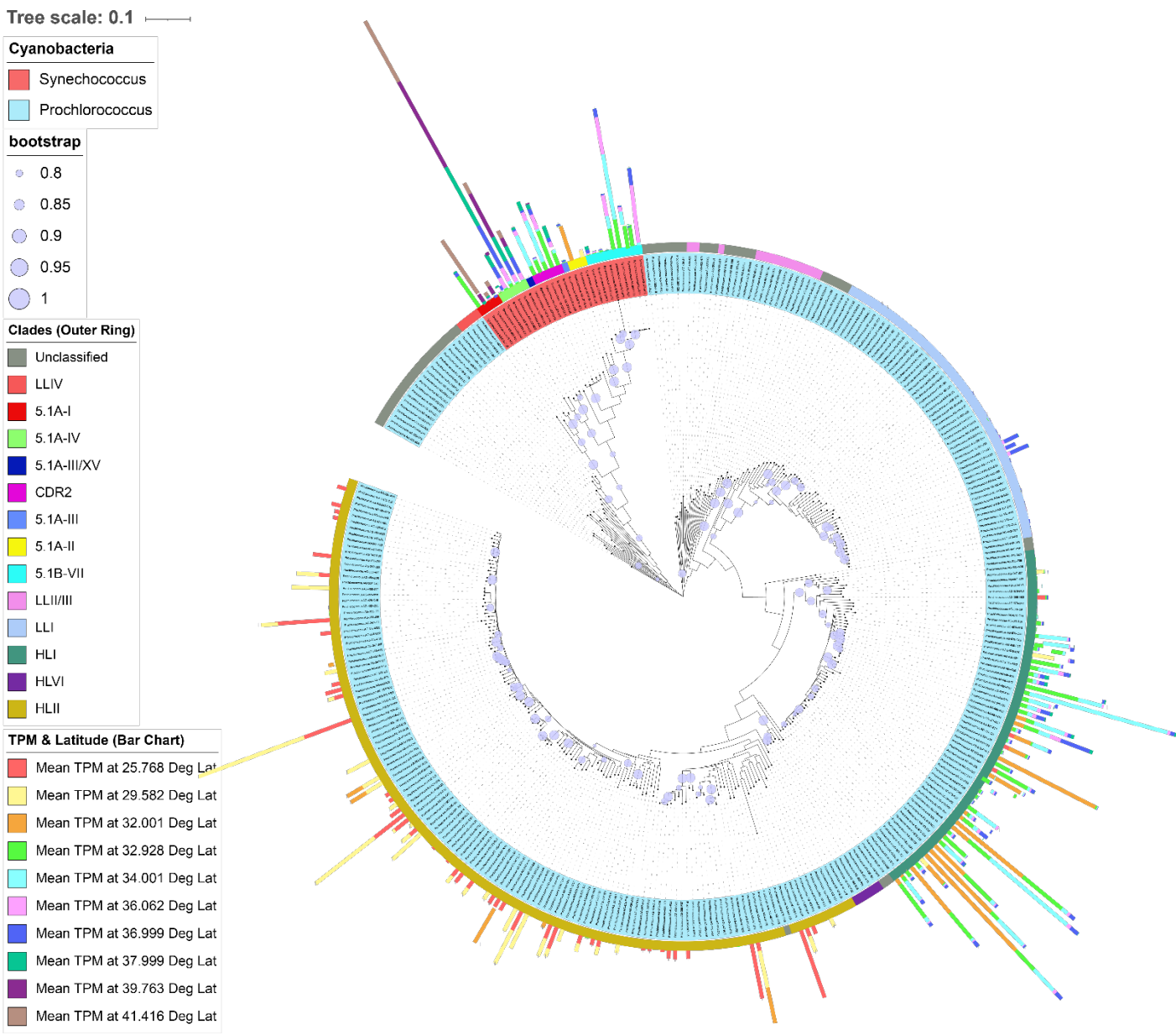

Figure 7, Phylogeny of the KaiC gene (left ring) from Prochlorococcus and Synechococcus gDNA data. Cyanobacteria species is represented by inner colored species labels. Bootstrap values are represented by size-scaled dots at nodes. Bootstrap values less than 80 are omitted. Phylogenetic clade grouping is indicated by the outer colored ring and labeled in the corresponding legend. Bar charts at the end of each ecotype indicate transcripts per million (TPM) for KaiC gene, with each bar broken up into different colors representing TPM at different latitudes. Bar charts are labeled in the corresponding legend.

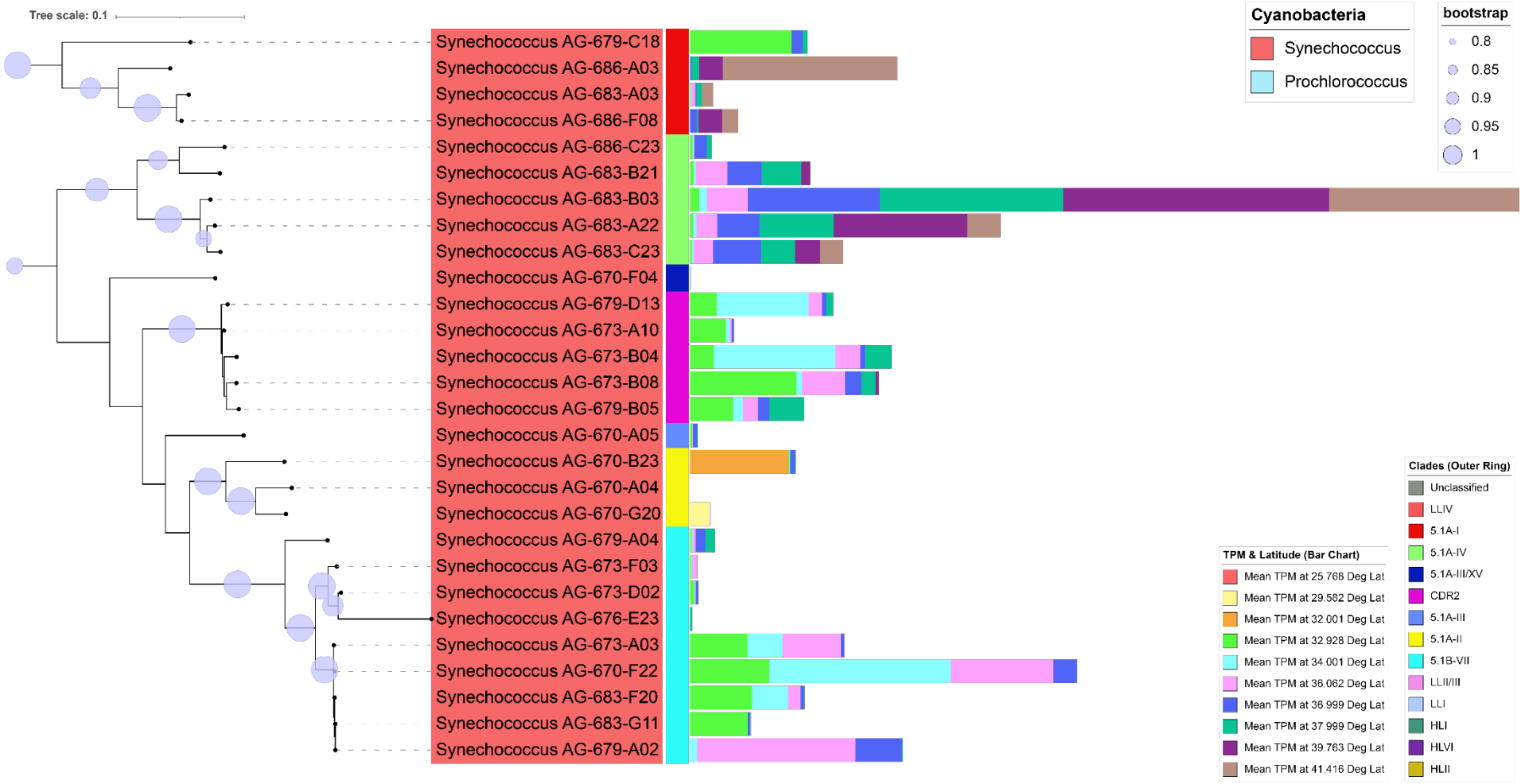

Figure 8, The Synechococcus (red part of the clade ring) data from the KaiC phylogeny (Figure 7) is displayed. Synechococcus phylogenetic clade grouping is indicated by the outer colored bars and labeled in the corresponding legend. Bar charts at the end of each Synechococcus ecotype indicate transcripts per million (TPM) for KaiC gene, with each bar broken up into different colors representing TPM at different latitudes. Bar charts are labeled in the corresponding legend.

Discussion

As this was an exploratory study, there were no clear expectations for what trends in KaiA, B, and C cyanobacterial genetic data would look like. The original hypothesis focused on identifying KaiABC presence and activity in Synechococcus and Prochlorococcus from cruise data, and this study was successful in that task. Full datasets for both Synechococcus and Prochlorococcus were created for all three genes, with corresponding genetic expression data in TPM units with collection site latitude. The second part of the hypothesis was focused on the interaction between circadian clock activity and light intensity. Unfortunately, there was no available light data in the Gradients cruise data where the genetic expression data originated from. The lack of complete light intensity data means it is difficult to draw conclusions regarding the link between KaiABC expression and light intensity. However, information on light conditions can be accessed through other means. In KaiB and KaiC phylogenetic trees where substantial Prochlorococcus data is available, there are distinct high light and low light clades (HL and LL on Figures 6 and 7). Although there is no exact light data, expression data coupled with clade identifiers can give insight into what conditions may have been present for different expression values. When examining the trees for KaiB and KaiC (Figures 6 and 7), there are much higher expression values present in HL clades when compared to LL clades. This could indicate that even though Prochlorococcus does not express KaiA (evident in Figure 5), the incomplete clock comprised of KaiB and KaiC functions to a greater extent in higher light conditions. Although there was one Prochlorococcus ecotype present in the KaiA tree (Figure 5), there was no additional information available to confirm whether it was a legitimate datapoint or an error.

The presence of only KaiB and KaiC in Prochlorococcus is in line with previous studies, which have determined that without KaiA, cycles associated with the circadian oscillator in Prochlorococcus dampen without continuous light input (Cohen and Golden 2015). In comparison, previous studies have established that Synechococcus displays robust a circadian rhythm that can be set by environmental factors (mainly light intensity) and then function without further input (Cohen and Golden 2015). Although the dataset in this study cannot conclude whether each Synechococcus ecotype is exhibiting a full robust circadian rhythm, the data does show that nearly every Synechococcus ecotype expresses all three genes to some extent, despite a wide range of genetic and geographical diversity. Such diversity is not present in Prochlorococcus, which generally inhabits open ocean habitats between 45N and 40S, whereas Synechococcus is known to inhabit a wider range of habitats with varying conditions (Berube, et al. 2018). Additional research could be conducted to examine whether the fully functioning circadian oscillator in Synechococcus contributes to a greater degree of flexibility.

Expression levels for KaiB and KaiC, thought to be tuning components of the clock, varied but the overall trend points to higher KaiC expression for most cyanobacteria ecotypes displayed in Figures 6 and 7. The reason for this difference in expression values between the two genes is unknown, and could be another subject for further research. KaiA is documented as being the critical component in a fully functioning circadian clock, but differences in the usage of KaiB and C in both in Synechococcus and Prochlorococcus has not been explored (Cohen and Golden 2015). Additional research could be conducted to determine if other environmental factors associated with each cyanobacteria clade influence expression of each clock component.

Conclusion

This study aimed to identify the presence and function of the Cyanobacterial circadian clock and was successful in doing so. Although the second part of the hypothesis that focused on the interaction between light and the function of the clock was not able to be fully explored, the study was able to identify preliminary trends in high light versus low light Prochlorococcus clades that does indicate that light can influence components within the circadian oscillator. The study was also able to confirm that only Synechococcus displayed all three clock components, with relatively high expression values for all three among most of the ecotypes studied. Synechococcus also displayed higher genetic and geographical diversity, and a potential connection to the fully functioning circadian oscillator could be explored in a later study. These findings suggest that further research into both Prochlorococcus and Synechococcus oscillator activity in cruise data samples could yield additional results linking light and other environmental factors to the function of the circadian clock.

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