Cyanobiont-bearing Dinoflagellate Ornithocercus in Temperate Coastal Waters: Cyanobiont Genetic Diversity and Host Specificity

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Abstract

Cyanobacteria are ubiquitous in marine environments and play an important role as primary producers. Some cyanobacteria, the so called cyanobionts (cyanobacterial symbionts), have a symbiotic relationship with unicellular organisms. Among these relationships, in particular, the nature (e.g., genetic diversity, host or cyanobiont specificity, and cyanobionts seasonality) of the cyanobionts-dinoflagellate host consortia remain poorly understood. In this study, 16S rDNA of cyanobionts in a total of 138 single host cells isolated over four seasons in temperate waters were sequenced using the MiSeq platform. Genetic analysis of cyanobionts from the dinoflagellate host Ornithocercus revealed that three genetic types of Synechococcales cyanobionts occurred at a wide range of water temperatures (11–24°C) and their distribution seems to be closely associated with the variation in salinity. Furthermore, this study showed the presence of some degree of host (or cyanobiont) specificity in cyanobionts (or host) among Ornithocercus species as well as among other dinophysoid species (i.e. Amphisolenia, Citharistes, and Histioneis). In addition to Synechococcales cyanobionts, this study identified some OTU sequences affiliated with the Vampirovibrionales and Chroococcidiopsidales in some Ornithocercus cells, suggesting that Ornithocercus species seem to be an additional new habitat for those bacterial groups.

Introduction

Symbiosis can be defined as the relationship between two different organisms living together, and is widespread across all taxa and in diverse habitats in marine environments. Of the symbioses at the unicellular level, the relations between cyanobacterial symbionts (cyanobionts) and protistan hosts are particularly noteworthy, as some nitrogen-fixing cyanobacteria (diazotroph) play an important role in primary production, especially in nitrogen-limited oligotrophic oceans[1-3]. Cyanobacteria, mostly pico-sized *Synechococcus* and *Prochlorococcus*, are ubiquitously distributed and are the most abundant photosynthetic organisms on Earth, accounting for a quarter of global carbon fixation in marine ecosystems [4-6]. Cyanobionts have been found in numerous protists groups belonging to dinoflagellates, tintinnids, radiolarians, amoebae, diatoms, and haptophytes[7,8]. Among these cyanobionts, little is known regarding the nature (e.g., genetic diversity, host or cyanobiont specificity, and cyanobionts seasonality) of symbiosis related to dinoflagellate hosts, in particular.

Dinophysoid dinoflagellates (class Dinophyceae, order Dinophysiales) contain genera *Amphisolenia*, *Triposolenia*, *Citharistes*, *Histioneis*, *Parahistioneis*, and *Ornithocercus* and are known to have cyanobionts, of which the first two have intracellular cyanobionts and the others have extracellular cyanobionts[9-13]. Early studies on the dinophysoid dinoflagellates have exclusively focused on the morphological features of the cyanobionts, such as shape, size, and thylakoid arrangement based on ultrastructural observations[13-16]. Later, molecular techniques were applied to the dinophysoid cyanobionts revealing their genetic diversity[17]. Regionally, those studies have been conducted exclusively in the tropical and subtropical Pacific, Atlantic, and Indian Oceans, because cyanobiont-bearing dinoflagellates are commonly found in the low-nutrient conditions of the open ocean[10,13-15,17-19]. In

contrast, free-living picocyanobacteria such as *Synechococcus* and *Prochlorococcus* are widely distributed even in temperate waters undergoing seasonal changes in water temperature and can be subdivided into cold/warm types associated with the variation in water temperature[20].

The Jeju Strait and Pohang coastal area off Korea are geographically located in temperate waters and undergo large seasonal variations in water temperature and salinity[21-23], owing to the input of the branch of the Tsushima Warm Current and the Yangtze River[24-26]. Therefore, the occurrence of dinophysoid dinoflagellates in such temperate coastal waters, if any, could raise several questions regarding their cyanobionts. For example, do the dinophysoid dinoflagellates newly acquire their cyanobionts seasonally from the surrounding waters? Does the dinoflagellate host maintain a constant type of cyanobionts irrespective of the variation in water temperature and salinity? To address these questions, a total of 138 individuals *Ornithocercus* cells were isolated over four seasons (March; winter, June; spring, September; summer, November; autumn, and December; autumn) from 13 surveys over three years. To identify the genetic type of *Ornithocercus* cyanobionts, the cyanobacterial 16S rDNA (V3-V4 regions) sequences of whole cyanobionts of individual host cells were verified using the Illumina MiSeq sequencing platform.

Results

Microscopic observations of the cyanobionts

Most *Ornithocercus* specimens collected in field samples contained the cyanobionts that inhabit between the upper and lower girdle lists of the cingulum called a symbiotic chamber (Fig. 1). The cyanobionts were rod-shaped, measuring on average 8.38 μm in length and 4.06 μm in width (n = 30), and were reddish brown in color under the light microscope, with bright orange fluorescence emitted under green-light excitation. Spherical shaped cyanobionts smaller than the rod-shaped ones were rarely observed. Among the cyanobionts, cells undergoing transverse binary fission were often observed (Fig. 1b, c). The thylakoids of cyanobionts consisted of concentric layers (Fig. 1e).

Screening for high-throughput sequencing runs of cyanobionts

A total of 4,422,006 high quality sequences of the cyanobacterial 16S rDNA (V3-V4 regions) were obtained from 138 individual *Ornithocercus* cells, with on average 32,044 reads per individual cell assigned. For each sample, the operational taxonomic units (OTUs) with sequences ≥1% of the total reads were selected. These sequences were 98%–100% similar to those of three cyanobacteria order Synchococcales, Chroococcidiopsidales, and Vampirovibrionales and clustered into 229 OTUs (Fig. 2). The most common order in the dataset was Synchococcales, accounting for 86% (197 OTUs) of the total OTUs, followed by Vampirovibrionales (Melainabacteria) with 9.6% (22 OTUs) and Chroococcidiopsidales with 4.4% (10 OTUs). Based on the MiSeq data, *Ornithocercus* cyanobionts belonging to the Synchococcales could be divided into three major genetic types as Type 1, Type 2, and Type 3,

accounting for 111 (48.5%), 74 (32.3%), and 8 (3.5%) OTUs, respectively. The maximum proportional abundance of each OTU relative to the host species and season is represented as outer and inner heatmaps, respectively, using a color gradient from black (100%) to white (0%) (Fig. 2). All types of the cyanobionts showed no significant pattern of occurrence for the different host species and seasonal changes.

Phylogenetic analyses of *Ornithocercus* cyanobionts

Ornithocercus cyanobionts formed a monophyletic clade and were a sister group to *Synechococcus* subcluster 5.1 within the picocyanobacterial groups. The phylogenetic analyses based on the cyanobacterial partial 16S rDNA-internal transcribed spacer (ITS) gene revealed that *Ornithocercus* cyanobionts comprised three distinct clades (A, B, and C): clade A containing six clone sequences (V3–V4 region of 16S rDNA) of *Ornithocercus* cyanobionts isolated from the Atlantic Ocean and Pacific Oceans and Type 1 sequences obtained in this study, clade B containing Type 2 sequences, which were 100% identical to sequence (OmCyn01) isolated from Japan, and Type 3 sequences newly discovered in this study, and clade C containing one sequence of *Ornithocercus* cyanobionts isolated from the Atlantic Ocean (red circles in Fig. 3). The Type 1 was further subdivided into Type 1a and 1b. The two subtypes displayed only one sequence difference in 16S rDNA, but distinct difference of 25 nucleotides with one gap in the ITS sequences. Overall, while partial 16S rDNA genes (1140 bp) of *Ornithocercus* cyanobionts were quite conservative owing to low sequence variations with each other, ITS genes were characteristic of being highly variable, resulting in considerable variations in both length and sequence (Table 1).

Table 1
Genetic p-distance matrix of the partial 16S rDNA and entire ITS gene among three genetic types Ornithocercus cyanobionts obtained from this study.

Ornithocercus cyanobionts	partial 16S rDNA					entire ITS				
	Size (bp)	Type 1A	Type 1B	Type 2	Type 3	Size (bp)	Type 1A	Type 1B	Type 2	Type 3
Type 1a	1140	-	-	-	-	754	-	-	-	-
Type 1b	1140	0.1	-	-	-	753	3.3	-	-	-
Type 2	1140	1.2	1.1	-	-	697	18.1	17.6	-	-
Type 3	1140	1.3	1.2	0.3	-	708	17.9	17.6	4.0	-

Hydrographical characteristics of the study area

Seawater temperature showed a typical seasonal change in temperate waters. Seasonal mean temperature ranged from 11.1 to 25.2°C, and minimum and maximum temperatures were 10.5°C and 27.6°C, respectively (Fig. 4). Vertical temperature differences (surface to 30 m in depth) in June and

September were 4 °C and 2.7 °C, respectively, which were relatively large compared to those in November and March (Supplementary Table S3). Surface seawater temperature tended to increase from station W1 toward station W9 along the transect throughout the year. Salinity showed a seasonal mean of 31.3 to 34.5 (Supplementary Table S3). Considering only March, June and November, the range in salinity variation was slightly narrow, with a minimum of 32.2 and a maximum of 34.2 (Fig. 4). In September, however, the salinity variation was very large, from a minimum of 29.6 to a maximum of 33.9 because of a large amount of runoff input from the Yangtze River in this season. The seawater and salinity of the Pohang station in December was 11.7 °C and 34.3, respectively (Fig. 4).

Cyanobionts diversity and abundance relative to seasons and host species

Individually isolated host cells were classified as 74 *O. magnificus*, 16 *O. orbiculatus*, 10 *O. quadratus*, 5 *O. steinii*, 24 *O. thumii*, and 8 unidentified *Ornithocercus* species. *O. magnificus* was the most dominant host species in the study area and was the most abundant in November (autumn), but rarely found in June (spring) (Fig. 5a). The cyanobionts Type 1 and 2 occurred together in most samples except in March. and June 2018 (Fig. 5b), with a temperature range of 11.7–24.3°C and salinity range of 31.0–34.6 (Fig. 4a, b). In contrast, the cyanobiont Type 3 occurred mostly in the autumn season (November and December), when the temperature and salinity were 11.7–22.6°C and 33.5–34.3, respectively, except for one cell found in low salinity of 31.4 (Figs. 4c, 5b). The Synechococcales cyanobionts Type 1 and 2 were observed in all host species examined (Fig. 6). In particular, a large portion of the Type 2 (66%) was observed in *O. magnificus*, and was even predominant in the individual host (Fig. 6). While the cyanobiont Type 1 was occasionally observed in low abundance specifically in the *O. magnificus* cells, it was found predominantly in the *O. orbiculatus*, *O. quadratus, O. steinii*, *O. thumii*, and unidentified *Ornithocercus* cells (except for one *O. orbiculatus* and one *O. thumii* cell in which Type 2 dominated) (Fig. 6). The cyanobiont Type 3 was observed exclusively in a few *O. magnificus* cells except for one unidentified *Ornithocercus* cell (outer heatmap in Fig. 2 and Fig. 6). Over half (59.4%) of the host species had different types of the Synechococcales cyanobionts simultaneously, most of which were a combination of Type 1 and 2 (89%), rarely of types 1 and 3 (1.2 %), and of types of 2 and 3 (1.2%). In seven specimens (8.5%), all three types were detected at the same time (Fig. 6). The cyanobiont Type 1 was found in 81.2% of *Ornithocercus* populations followed by the Type 2 at 75.4%, and the Type 3 at 7.2% (Fig. 6). In addition to the Synechococcales cyanobionts Type 1, 2, and 3, cyanobacterial symbionts Chroococcidiopsidales (9.4%) and Vampirovibrionales (18.8%) (Melainabacteria) were detected with low abundance in some cells that were sampled in September (summer), November, and December (Figs. 2, 6).

Phylogenetic clustering of cyanobionts from dinophysoid hosts of different origin

Based on the 16S rDNA sequences (V3–V4 regions) of cyanobionts of dinophysoid dinoflagellates, *Amphisolenia*, *Citharistes*, *Histioneis*, and *Ornithocercus* that are registered in GenBank, including sequences obtained from the present study, the phylogenic relationships among them were divided into four clades and revealed moderately host-cyanobiont relationship to some extent at the genus level (Fig. 7). All but one *Ornithocercus* cyanobionts (AY444957) clustered together with one *Amphisolenia* cyanobiont (AY444918) and two *Histioneis* cyanobionts (AY44954 and AY44955) (clade 1; Fig. 7). The cyanobionts of *Histioneis* and *Amphisolenia* formed clades 2 and 3, respectively, although each clade included one to three cyanobionts of different host genera. Except for two *Citharistes* cyanobionts (AY444931 and AY444928) placed in different positions, all the other *Citharistes* cyanobionts clustered into clade 4, which comprised only one host species among the dinophysoid cyanobionts. In addition, neither of two *Histioneis* cyanobionts (AY444950 and AY444940) nor a *Citharistes* cyanobiont (AY444928) were included in any clade, and the latter two were closer to *Prochlorococcus* with 97.4 to 98.7% similarity rather than taxa of dinophysoid cyanobionts.

Discussion

Dinophysoid dinoflagellates are among the rare species in planktonic communities, and have not been established in laboratory cultures available in the long term. Nonetheless, they have been receiving much attentions because they harbor unique cyanobacterial symbionts (i.e., cyanobionts) inside or outside the cell, thereby providing good materials for studying several aspects of the cyanobionts-dinoflagellates host consortia. In the present study, genetic analysis of cyanobionts from the dinoflagellate host *Ornithocercus* isolated from samples covering four seasons in temperate waters revealed that the dinoflagellate host harbors moderately host-specific and consistent types of cyanobionts throughout the year.

In general, the ITS gene has proved to be successful in distinguishing closely related cyanobacterial species strains[27-29]. Indeed, the result from the present study also showed that Synechococcales cyanobiont Type 1 could be further subdivided into Type 1a and 1b when based on ITS sequences, revealing the presence of four genetic types of *Ornithocercus* cyanobionts (Supplemental Fig. S2a, b). In addition, the phylogenetic position of the *Ornithocercus* cyanobiont (OmCyn) was better represented when based on the ITS sequence than on the 16S rDNA sequence[30]. Despite the indication of the presence of at least four distinguishable genetic types of *Ornithocercus* cyanobionts based on ITS sequences, they were treated as three types in this study because all samples were processed based on the V3-V4 regions of cyanobacterial 16S rDNA gene.

The Synechococcales cyanobionts Types 1 and 2 were very similar or identical to the *Ornithocercus* cyanobionts found in the Atlantic and Pacific Oceans, and coastal waters of Japan, respectively [17,30]. Most of the cyanobionts of *Ornithocercus* cells were a rod-shape with orange fluorescence as previously reported [18,30], and had a mean of 8 µm and 4 µm in length and width, respectively (Supplemental Fig. S2a, b). Unfortunately, the cell shape of the Type 3 could not be identified in this study; however, considering the observation that most cyanobionts were rod-shaped, it is likely rod-shaped. Alternatively,

the Type 3 may have a different shape in morphology and ultrastructure from that of the Types 1 and 2. Previous ultrastructural studies have reported the several different morphotypes of *Ornithocercus* cyanobionts such as rod/ellipsoid or spherical shapes, and concentric, central/peripheral, or transverse thylakoids, and were especially much smaller (1–3.5 μm) than those currently observed [10,13,14,18]. The small-sized cyanobionts may have been underestimated by the direction of the TEM section in the previous studies or overlooked in this study.

Unlike the free-living picocyanobacterial community, of which the distribution changes dynamically with water temperature and nutrient levels[20], it is noteworthy that the composition and distribution of *Ornithocercus* cyanobionts belonging to Synechococcales were not significantly affected by seasonal changes in water temperature. In this study, all three types of Synechococcales cyanobionts occurred at a wide range of water temperatures from 11 to 24 °C. Rather, their distribution seems to be closely associated with the variation in salinity. Types 1 and 2 occurred in the salinity range of 31–34.6, while the distribution of Type 3 was mainly confined to a relatively narrow range of 33.5–34.6 except for one cell detected in low abundance of a host specimen with Type 3 (Fig. 4c and 2019-09-W7-01 in Fig. 6), suggesting that the former is a euryhaline species and the latter is a stenohaline species. The picocyanobacterium *Prochlorococcus* has been known to be advected from warm, oligotrophic open oceans to temperate waters by currents[20]. Similarly, it seems likely that already established cyanobionts-*Ornithocercus* host consortia from the tropical region were moved to temperate regions such as to the current study area by the Tsushima Warm Current rather than newly forming the consortia by either acquiring cyanobionts or choosing a host in the study area. If this is true, then, why do the three types of Synechococcales cyanobionts display different patterns of distribution relative to salinity? Perhaps, this is because while Types 1 and 2 are able to tolerate variation in salinity and readily adapt to temperate waters, Type 3 may be not tolerant to such variations in salinity and thus fails to adapt to the new environment. Further study is needed to address ecophysiological adaptation of the Synechococcales cyanobionts relative to variations in salinity.

In the cyanobionts-dinophysoid host consortia, whether the dinophysoid host actively searches for and selects specific cyanobiont(s) as the symbiotic partner or cyanobiont(s) choose each preferred host remains unknown. Nonetheless, the results from this study revealed the presence of some degree of host (or cyanobiont) specificity in cyanobionts (or host) among *Ornithocercus* species as well as among the dinophysoid species. Given that Types 1 and 2 were detected in 81.2% and 75.4%, respectively, of the 138 *Ornithocercus* specimens isolated in the present study, it seems evident that both two types are dominant Synechococcales cyanobionts. Therefore, it is likely that Foster et al.[17] have also commonly detected Synechococcales cyanobionts genetically similar to the Type 1 in most *Ornithocercus* hosts isolated from subtropical and tropical regions. More importantly, the results from the present study demonstrate that more than half of the *Ornithocercus* specimens (59.4 %) have multiple types of Synechococcales cyanobionts simultaneously, most of which were a combination of Types 1 and 2 (89%). The relative dominance of these types within each specimen, however, tended to be dependent on the *Ornithocercus* species. While Type 2 tended to be predominant in *O. magnificus*, Type 1 was predominant in other

Ornithocercus species (i.e., *O. orbiculatus*, *O. quadratus*, *O. steinii*, and *O. thumii*) except in *O. magnificus*. Very recently, the new finding of a cyanobiont (OmCyn), which is the same as the Type 2 in the present study, by a Japanese group[30] must have resulted from the use of a single *O. magnificus* specimen, thereby making it easy to detect this type. It should also be noted that Type 3 was observed exclusively in *O. magnificus*, except for one unidentified *Ornithocercus* cell. Furthermore, phylogenetic analysis of the Synechococcales cyanobionts from dinophysoid species (i.e., *Amphisolenia*, *Citharistes*, *Histioneis*, and *Ornithocercus*) displayed four distinct clades, with each clade consisting of specific Synechococcales cyanobionts, except for a few exceptional cases (Fig. 6). We frequently observed that the cyanobionts are in the process of cell division in the host cingulum and are transmitted vertically along with the division of the host cell (Supplemental Fig. S2c), as reported in previous studies[7,14]. In terms of the symbiont transmission mode, the host dependence on vertically transmitted symbionts tended to be higher than that of horizontally transmitted symbionts[31]. In addition, according to the size fractioned global cyanobacterial sequences based on Tara ocean metagenomics data, *Ornithocercus* cyanobiont sequences have been predominantly retrieved in the host-size category (20–180 μm) rather than in the cyanobionts-size (0.8-20 μm)[30], which suggests that the life history of the cyanobionts is obligately dependent on their host than the free-living life stage. Such a highly dependent host-cyanobiont relationship may have allowed them to have either host specificity or cyanobiont specificity. Taken together, the results from this study suggest that host (and/or Synechococcales cyanobionts) niche separation is present among *Ornithocercus* species as well as dinophysoid species.

In addition to Synechococcales cyanobionts, this study identified some OTU sequences affiliated with the Vampirovibrionales (Melainabacteria) and Chroococcidiopsidales in some *Ornithocercus* cells. Both cyanobacterial groups have frequently been found in extreme and dynamic environments such as marine sediment, rocks, grassland soil, and guts[32-34]. *Ornithocercus* species seems to be an additional new habitat for those bacterial groups, which has not been known before. Melainabacteria group has been known to lack the photosynthetic function[32-34], and the physiological characteristics of the two bacterial groups remains still unknown. Further study is needed to better understand the role and function between these cyanobionts and the host.

Methods

Study area and sample collection

This study was conducted along the coast of the East sea (1 station, Pohang) and the coast of the South Sea of Korea (9 stations between Wando and Jeju) seasonally affected by the flow in from Jeju Warm Current, a branch of the Tsushima Warm Current (Supplementary Fig. S1). The Pohang sample was collected from the coastal water using a 0.3 m diameter plankton net with 20 μm mesh in December 2017. Except for the Pohang sample, all samples were vertically hauled through the water column from a depth of 30m to the surface using a 0.6 m diameter bong net with 20 μm mesh on a cruise ship every March, June, September, and November for 3 years from 2017 to 2019. Fifty mL of the highly

concentrated plankton samples were immediately fixed with 2% neutral Lugol's solution in a polyethylene bottle and wrapped in foil for a microscopic observation later. The remaining sample was poured into a 10 L bucket containing seawater for a live-cell observation in the laboratory. The oceanographic parameters were measured from discrete depths using a CTD profiler (SBE 19plus V2, Sea-Bird Electronics, USA) mounted on a rosette sampler at each station.

Single-cell isolation for DNA extraction and light microscopy

From the fixed and live plankton samples, 138 single *Ornithocercus* host cells with cyanobionts were isolated under an inverted microscope (Axio Vert.A1, ZEISS, Germany). The host cells were first photographed for host species identification and observation of their symbionts. Light micrographs of the fixed host cells were obtained at magnifications of 200× and 400× using the inverted microscope equipped with a full HD mini box camera (MediCAM-Z, Comart System, Korea). After obtaining the micrographs, individual host cells with cyanobionts (at least five cells per station) were drawn using Pasteur glass pipettes, gently washed five times in sterile seawater, and put into separate 0.2 mL PCR tubes containing 50 µL of 10% Chelex (Bio-Rad, USA). In addition, only one cyanobiont cell from a live-host cell was isolated and processed in the same manner as described above in order to obtain a single symbiont DNA. In contrast, the live host cells were isolated using the glass pipettes, placed on a microscope slide, and photographed at magnifications of 630× and 1000× using an AxioCam HRc (Carl Zeiss Inc., Germany) coupled to a Zeiss Axio Imager A2 microscope equipped with differential interference contrast optics. Subsequently, the individual live cells were carefully taken back from the slide and then treated in the same way as above. The PCR tubes were boiled at 95 °C for 1h and then centrifuged at 13,000 rpm for 5 min. Supernatant (30 µL) was harvested as genomic DNAs of single host cells and its extracellular symbionts.

Amplification of cyanobacterial 16S rDNA for sequencing using an MiSeq platform

Extracted DNAs were amplified using the V3-V4 hypervariable regions of the 16S rDNA of the cyanobionts. The libraries of the 16S rDNA were prepared for Illumina MiSeq sequencing using two modified primer pairs with ligated Illumina overhang adapter sequences on both forward and reverse primers (Supplementary Table S1). PCR was performed in 25 µL reaction mixtures including 3 µL genomic DNA, 0.7 µLeach of 10 µM primers, 0.5 µL of 10 mM dNTPs, and 0.6 units Diastar^{TM} Taq polymerase (Solgent, Korea). The first-round PCR reaction consisted of a 10 min denaturation step at 94 °C, followed by 40 cycles of 30 s at 94 °C, 30 s at 53 °C, and 50 s at 72 °C. Each amplicon was then amplified using an Illumina universal index primer i5 and i7 for multiple samples to be pooled and sequenced in a single run. In nested PCR reactions, 3 µL of the PCR product from the first- round PCR was used as a template and PCR conditions were run as described above, except for 35 instead of 40 cycles

and an annealing temperature 60 °C instead of 53 °C. Resultant products were purified using a LaboPass^{TM} PCR purification kit (Cosmogenetech, Korea), normalized by a Qubit 3 fluorometer (ThermoFisher, USA) and pooled into a 1 mL tube. Amplicons sequencing was performed by Chunlab, Inc. (Korea) using an Illumina MiSeq platform.

Data analyses

The sequence data obtained through the MiSeq platform were processed using the Mothur software program. The paired-end reads were assembled and aligned to the SILVA database v. 132 and chimeric sequences removed using UCHIME. The clustered sequences were utilized to construct OTU tables with 96% identity level. Output data was analyzed using Microsoft Excel 2016. The relative abundance of OTUs for individual cells was ranked as the observed total OTUs number, and those less than 1% of the total number were not considered for further analysis.

Amplification of partial 16S rDNA and the ITS region of the cyanobiont

To verify additional genetic information of the cyanobionts, the DNA samples of a single host cells, that predominantly contained one type of symbiont, were selected based on the results of the MiSeq sequencing data. Type 1A: 2018-09-W3-03 (single-host DNA), Type 1B: 2018-11-W5-05 (single-host DNA) and 2019-11-W9-01 (single-cyanobiont DNA), Type 2: 2018-11-W9-04 (single-host DNA) and 2019-11-W4-02 (single-cyanobiont DNA), and Type 3: 2018-11-W9-05 (single-host DNA). Additionally, individual cyanobionts were isolated from the single host cells (sampled in November 2019) and single-cell DNA was extracted following the method described by Kim and Park (2019)[35]. DNA samples of single hosts and single cyanobionts were subjected to PCR amplification of cyanobacterial 16S rDNA (partial)-entire ITS region. The amplification of each gene was necessarily accompanied by nested PCR assay because the amount of the single-cell DNA was not sufficient to be detected in one PCR assay. All amplifications were amplified using 3 μL of genomic DNA and primer sets (Supplementary Table S1) under the PCR condition of 10 min at 94 °C, followed by 40 cycles of 30 s at 94 °C, 30 s at 55 °C, and 2 min at 72 °C. The nested PCR condition was the same as that used in the first-round amplification, except for 35 instead of 40 cycles and an annealing temperature of 57 °C instead of 55 °C. Amplified DNA fragments were purified using a LaboPass^{TM} PCR purification kit (Cosmogenetech, Korea) and sequence by Applied Biosystems (Cosmogenetech, Korea).

Phylogeny

For the phylogenetic analyses, two model-based methods, Maximum Likelihood and Bayesian inference were applied based on the data sets of cyanobacterial 16SrDNA and ITS gene sequences. The alignment

data were generated by combining the cyanobacterial 16S rDNA and ITS gene sequences which were obtained from GenBank (Supplementary Table S2). Alignment was constructed by eye using Genetic Data Environment (GDE 2.4) and positions which could not be aligned unambiguously were omitted from analysis. Maximum likelihood tree analysis was performed using the edge-linked partition model in IQ-TREE v.1.6.12 [36] with 1000 replicates of fast standard nonparametric bootstrap. The Bayesian inference analysis was carried out with the Markov chain Monte Carlo process for 20,000,000 generations, retaining one tree in every 1,000 generations and the first 10% of each tree was discarded using the MrBayes 3.2.7a program. Trees were visualized in Figtree v1.4.2.

Measurement of cyanobionts cell size

Thirty individual cyanobionts were measured for their cell size. On the micrographs of the *Ornithocercus* host with cyanobionts, one to two cyanobionts per host cell were randomly selected and size measured using a software AxioVision SE64 Rel. 4.8.

Data Availability

The partial 16S rDNA- ITS sequences in four types of *Ornithocercus* cyanobionts are deposited in GenBank under the accession numbers xxxxxx-xxxxx. The raw sequence data obtained through the MiSeq platform are deposited in the NCBI Sequence Read Archive (SRA) database with the BioSample accession ID xxxxx under the BioProject xxxxx.

Declarations

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Author Contributions

M.K. and M.G.P. conceived and designed the study. M.K. collected plankton samples, observed microscopy for the enumeration and identification of the cells, and performed molecular work. M.K and D.H.C analyzed and interpreted the sequencing data obtained through the MiSeq platform. M.K. drafted the manuscript and M.G.P. and D.H.C contributed to the final paper. All authors read and approved the final manuscript.

Additional information

Supplementary information.

Competing interests

The authors declare no competing interests.

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Figures

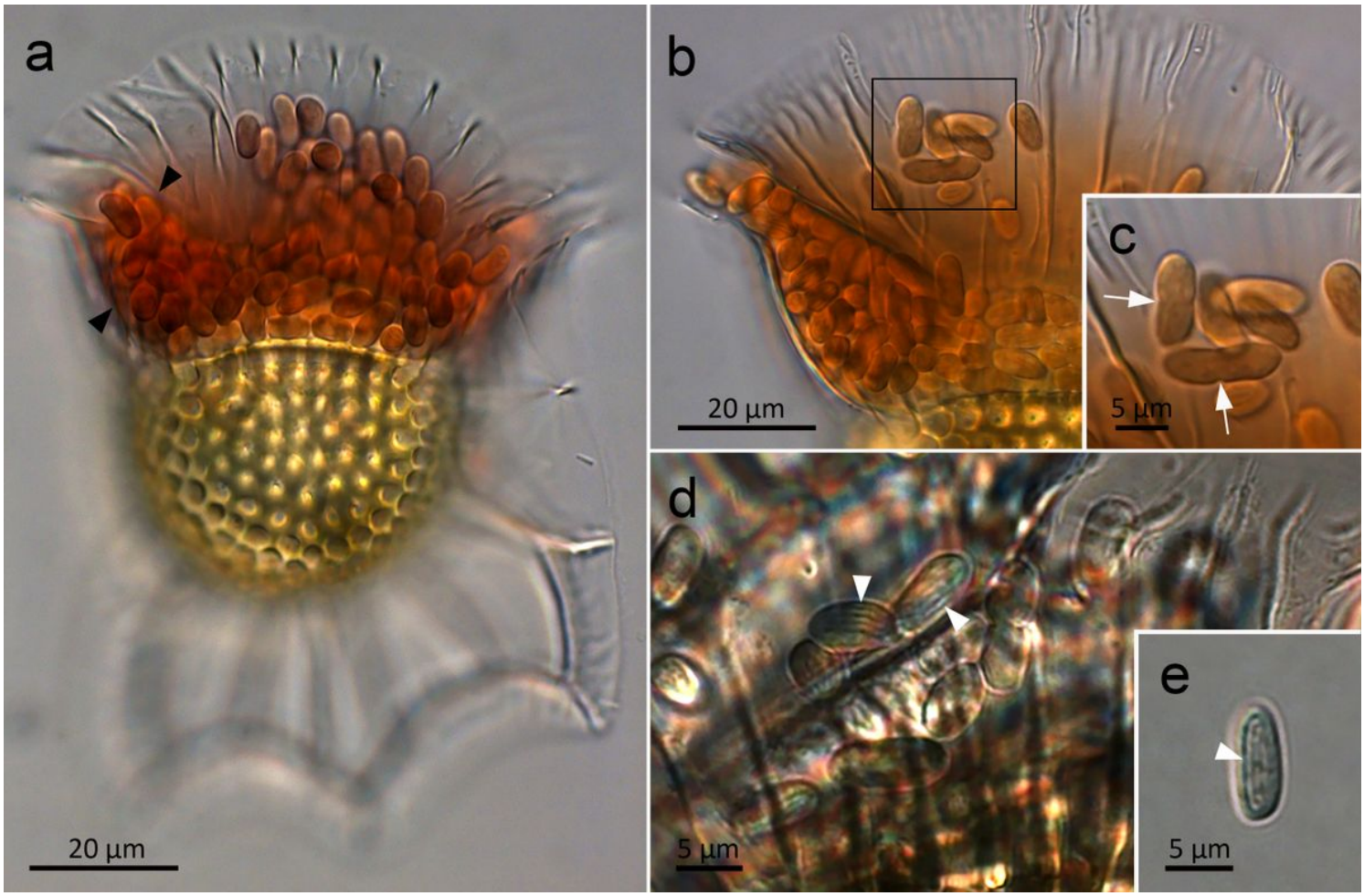
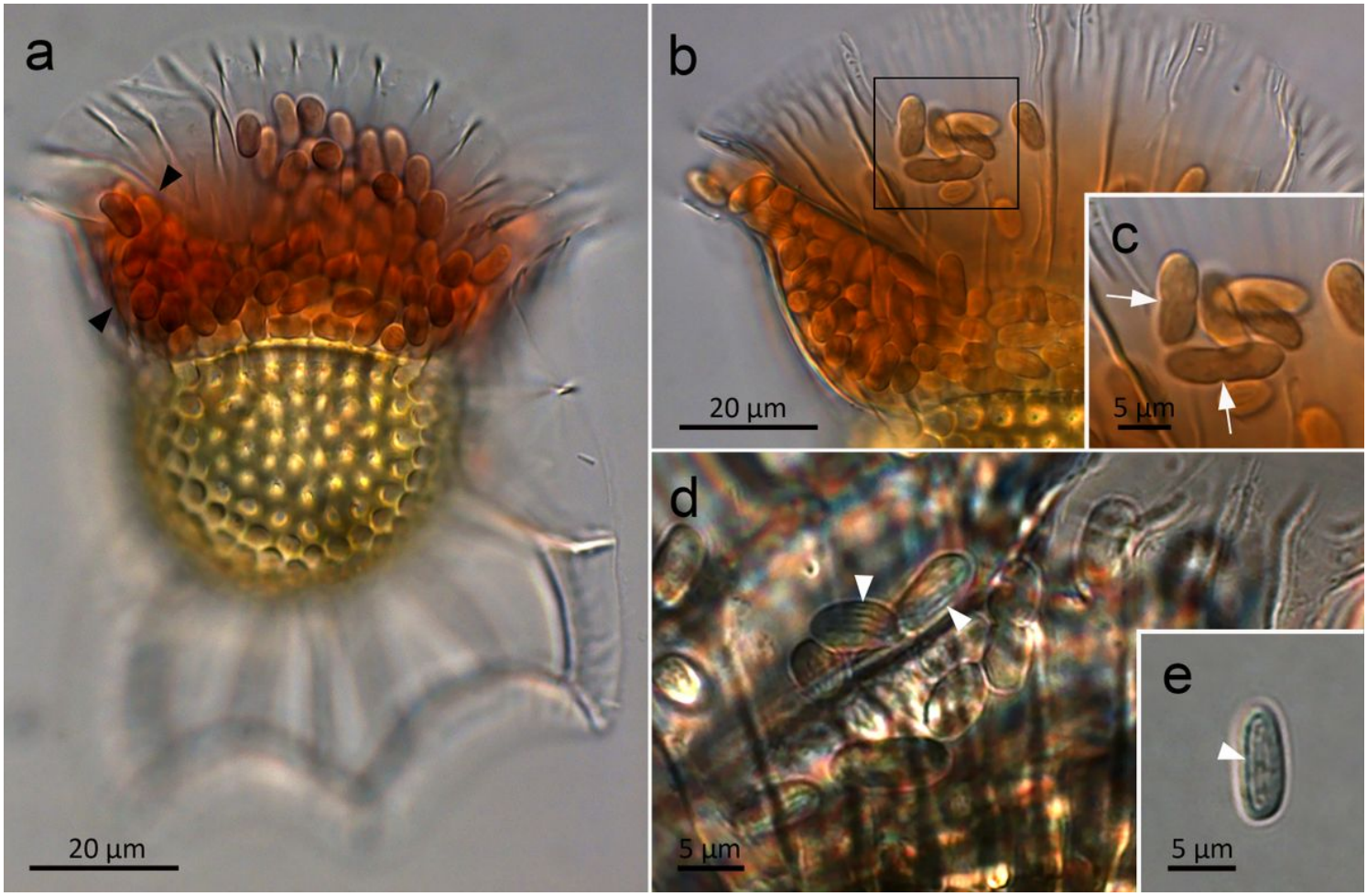


Figure 1

Micrographs of live cyanobacterial symbionts (cyanobionts)-dinoflagellate Ornithocercus host consortium. (a) O. magnificus with numerous cyanobionts present in the upper and lower girdle lists (black arrowheads) of the cingulum called a symbiotic chamber. (b) O. steinii with numerous cyanobionts inhabiting the symbiotic chamber. (c) Enlargement of the area indicated by the box in 2B showing two cyanobionts that are being divided by binary transverse fission (white arrows). (d) Thylakoid membranes (white arrowheads) of O. steinii cyanobionts. (e) O. steinii cyanobiont escaped from the same O. steinii host as shown in 2D showing thylakoids in concentric layers (white arrowhead).



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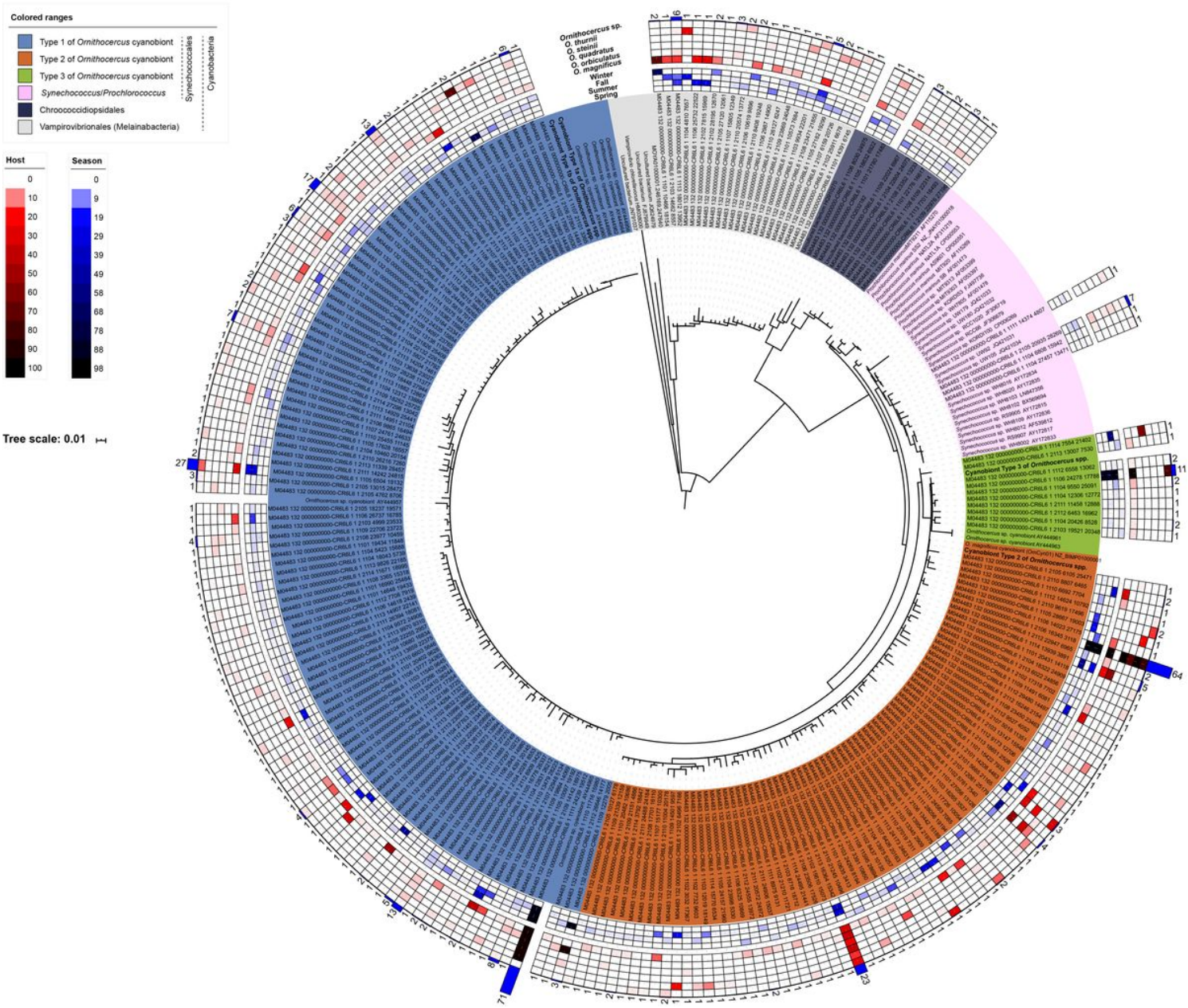
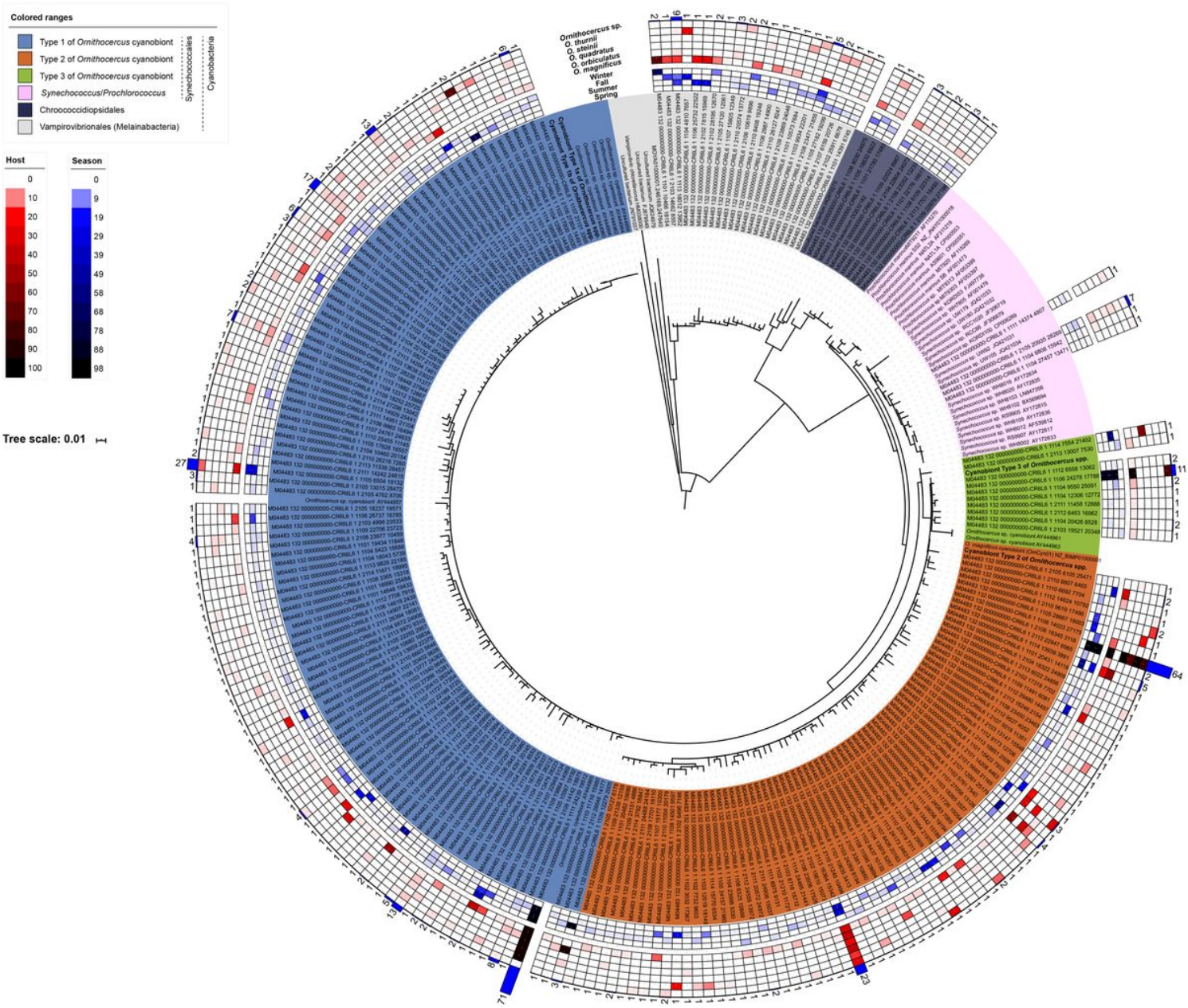


Figure 2

Circular phylogenic tree with heatmaps of the cyanobacterial operational taxonomic units (OTUs) obtained from the Ornithocercus symbionts. The tree was constructed based on the V3–V4 regions of cyanobacterial 16S rDNA gene. The two heatmaps represent the maximum proportional abundance of each OTU to the host species (outer) and season (inner). The color ranges of the heatmaps represent the relative percentage for each OTU. The outermost bars indicate the number of Ornithocercus cells with each OTU.



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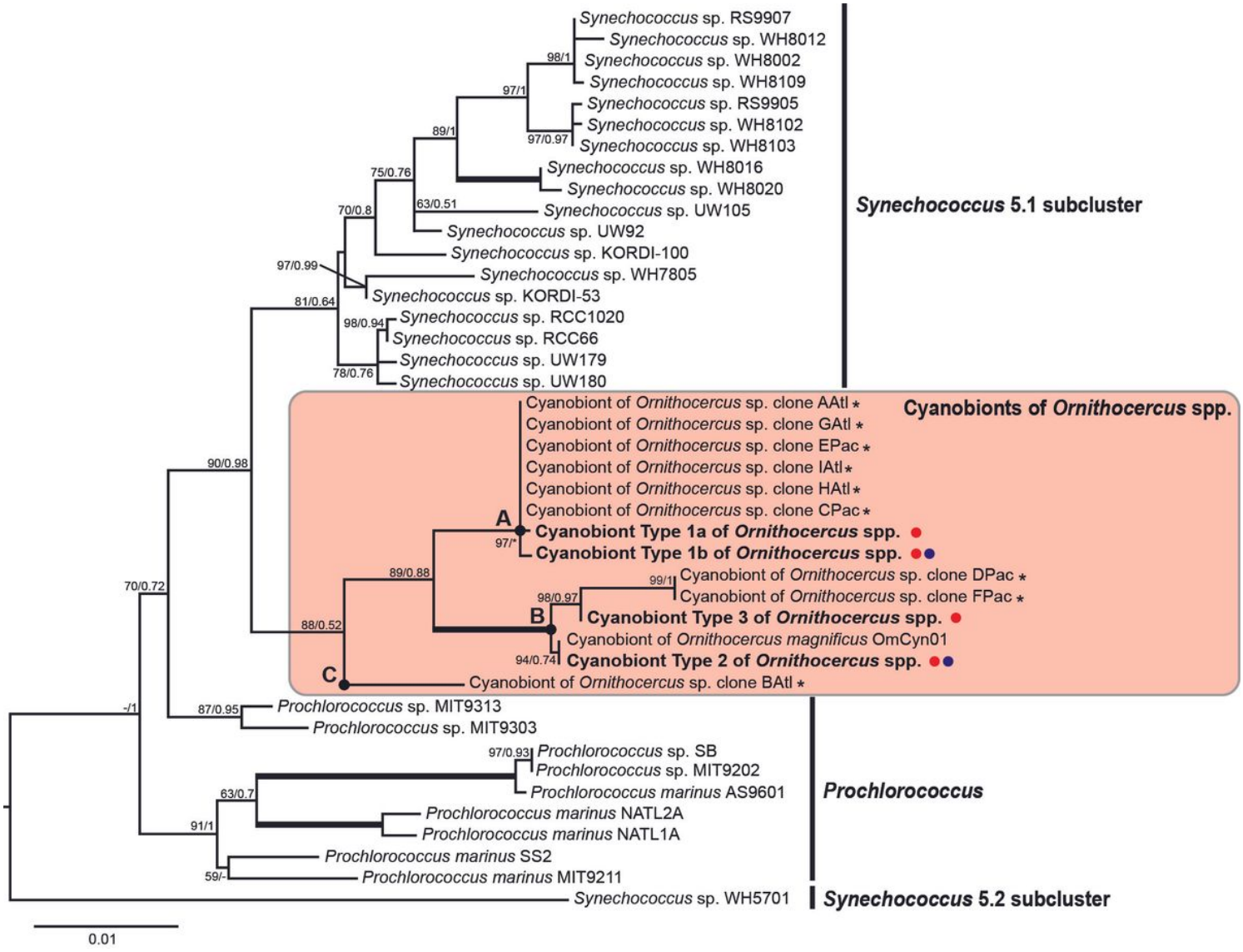

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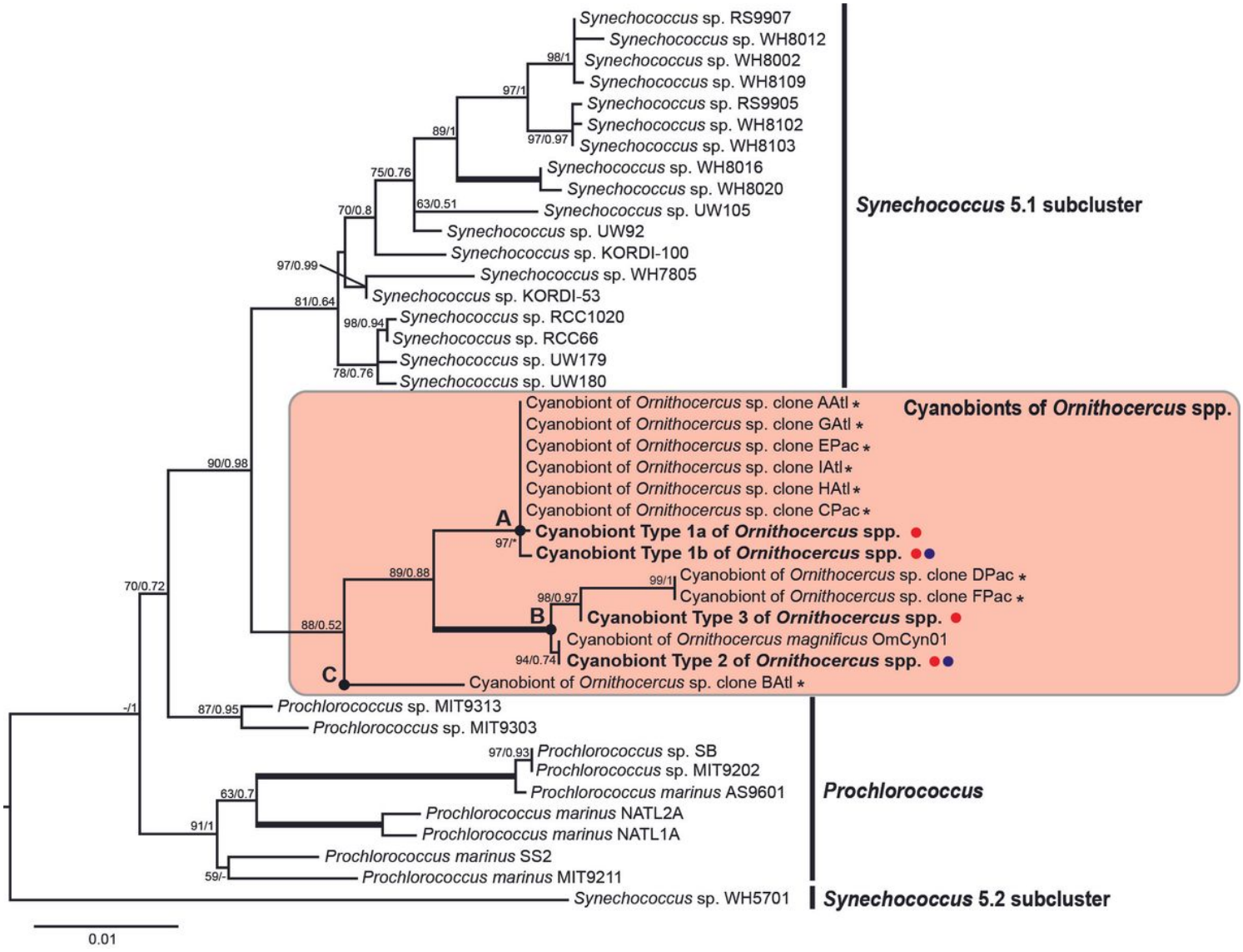
Maximum likelihood (ML) phylogeny of the cyanobacterial symbionts (cyanobionts) of dinoflagellate Ornithocercus host and marine picocyanobacteria based on combined s data of cyanobacterial partial 16S rDNA-entire ITS gene. Bold text indicates sequence of Ornithocercus cyanobiont obtained in this study. Red and blue circles represent amplified sequences from single-host DNA and single-cyanobiont DNA, respectively. The ML tree was inferred using IQ-TREE. Branch support was obtained from bootstrap values and Bayesian posterior probability. Thick branch denotes a strongly supported bootstrap value of 100% and the highest posterior probability (1). Hyphen represents the unmatched tree topology with the Bayesian tree. Letters on branches (A-C) refer to clades of Ornithocercus cyanobionts. Asterisks represent the taxa of which only short 16S rDNA sequences (V3–V4 regions) are analyzed.



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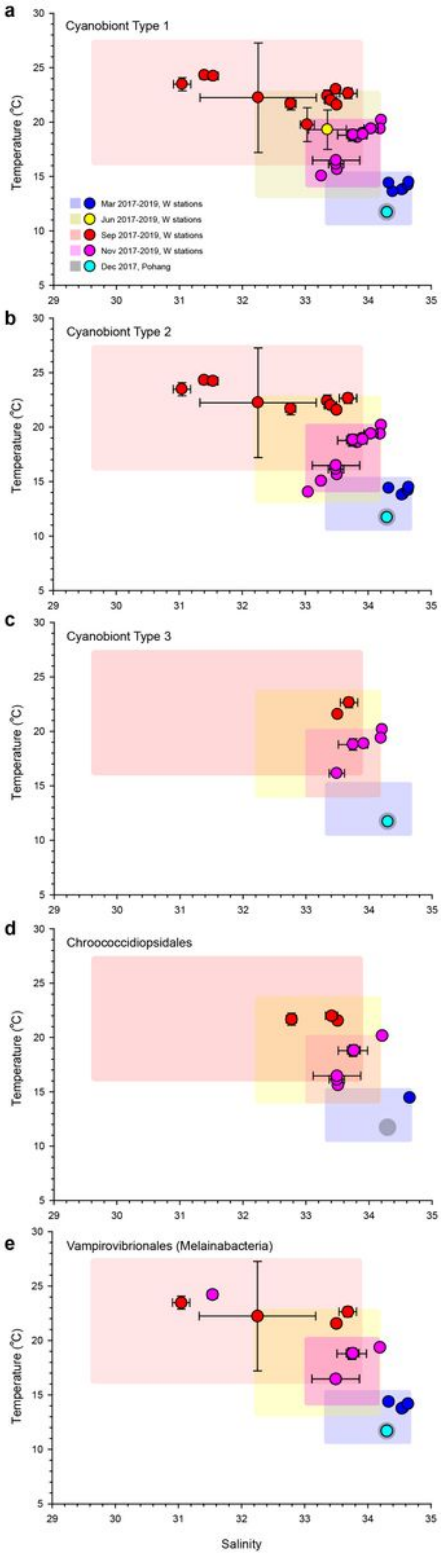
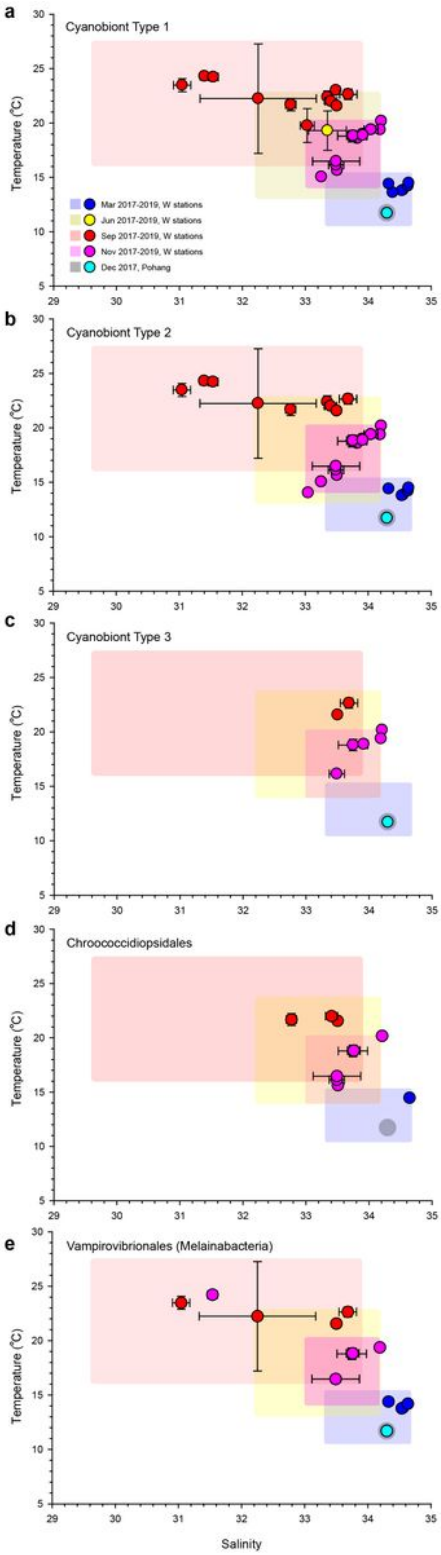


Figure 4

Temperature and salinity in March, June, September, and November 2017-2019 for the three genetic types (a-c) of Ornithocercus cyanobionts in the study areas. Circle indicates individual cyanobiont and square means the temperature-salinity range of the study area where the cyanobionts appeared. Error bar is a standard deviation (SD) for temperature and salinity from the surface to a depth of 30 m.



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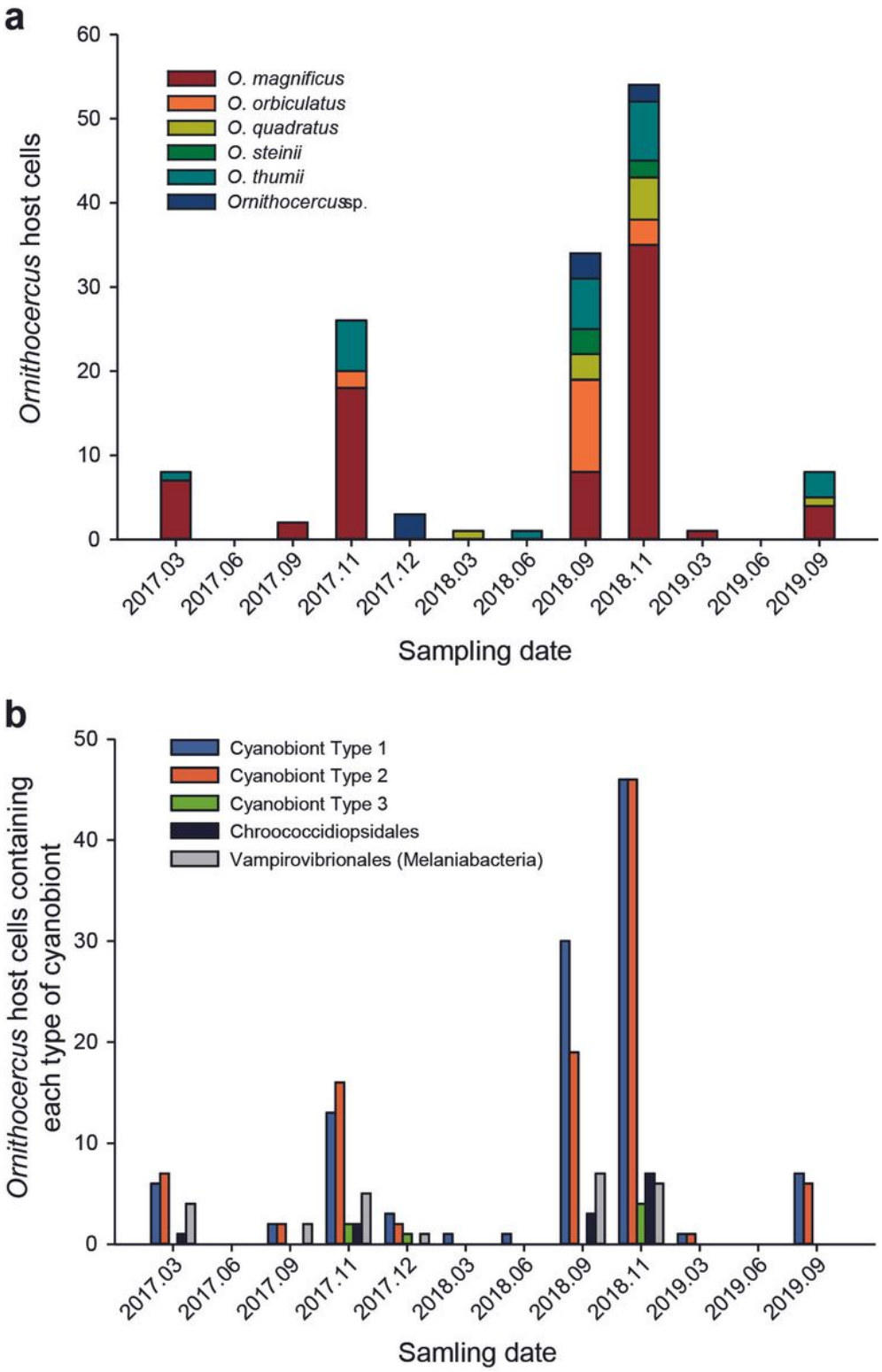

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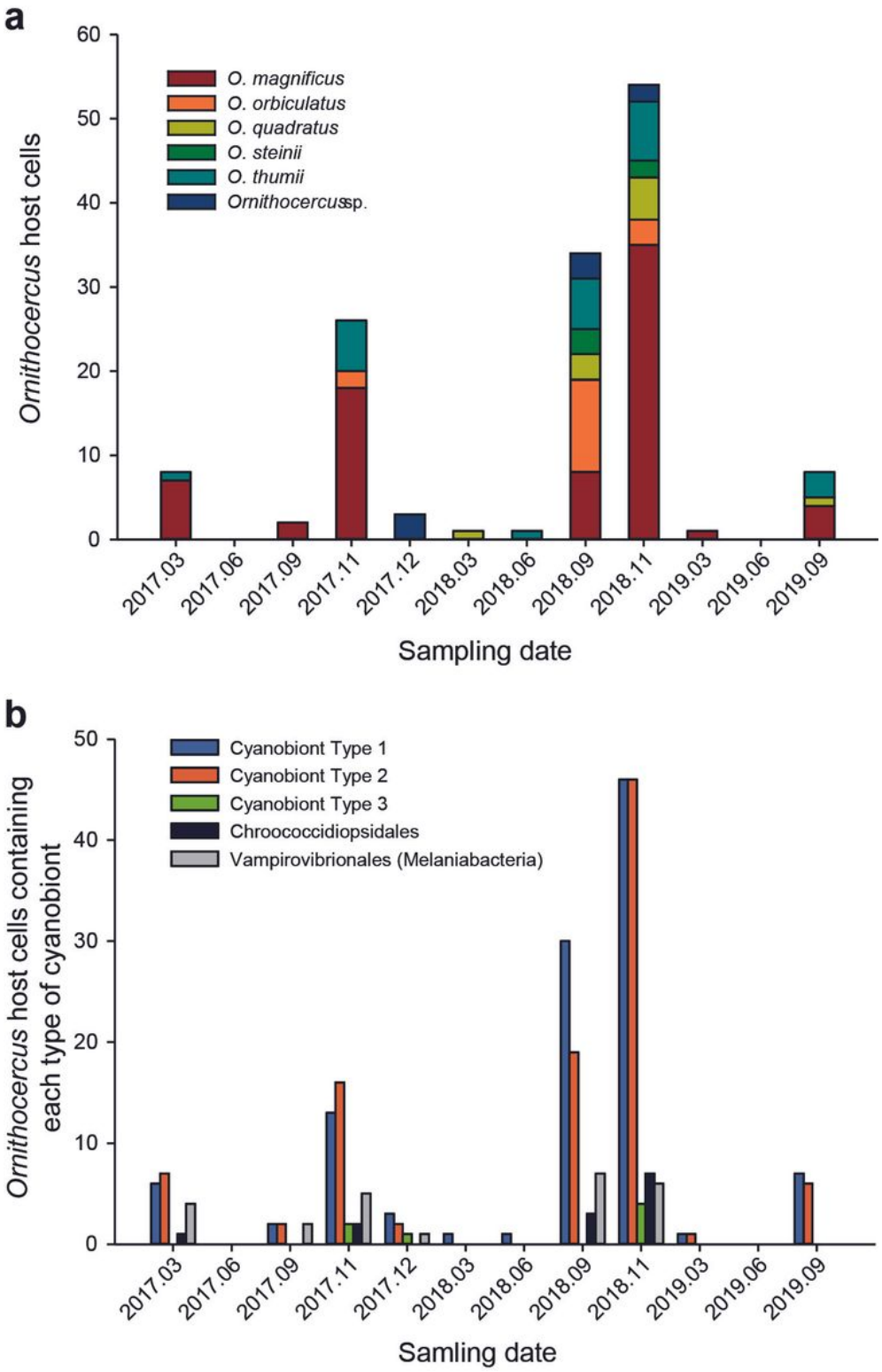
Number of cyanobiont-bearing Ornithocercus species in temperate waters. (a) Seasonal changes in Ornithocercus populations showing the five host species and unidentified species encountered in the study areas (b) Number of Ornithocercus hosts showing seasonal variations in the types of symbionts that the host has. (c) Number of Ornithocercus hosts showing the symbiont types according to host species.



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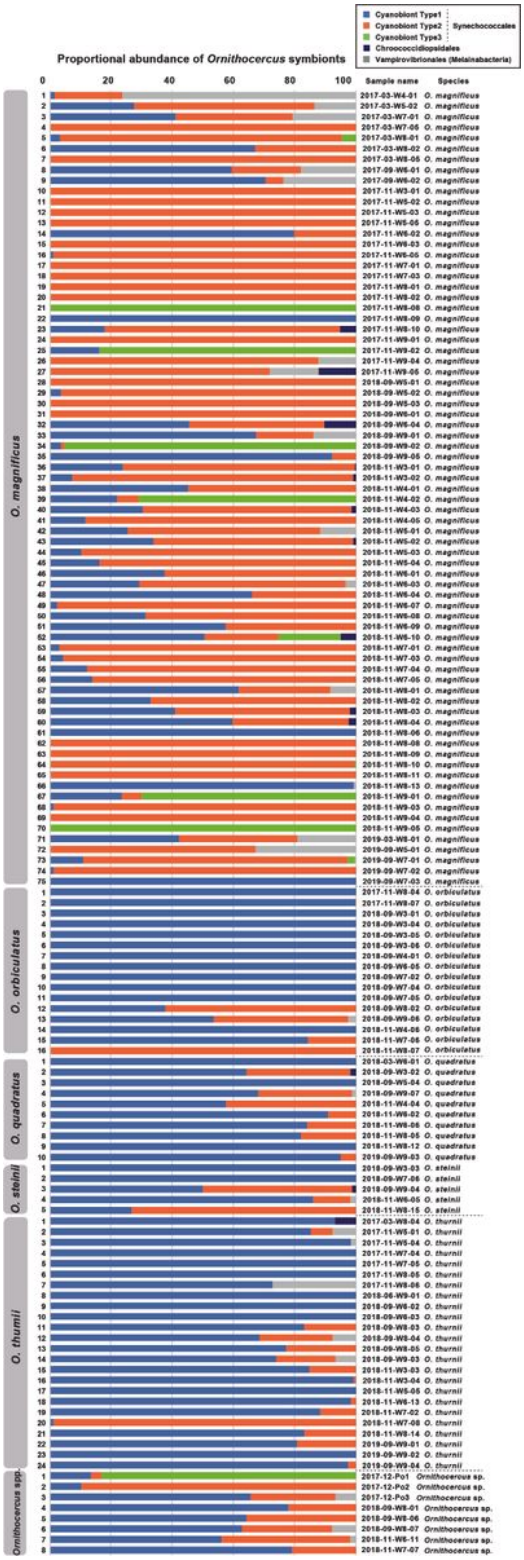

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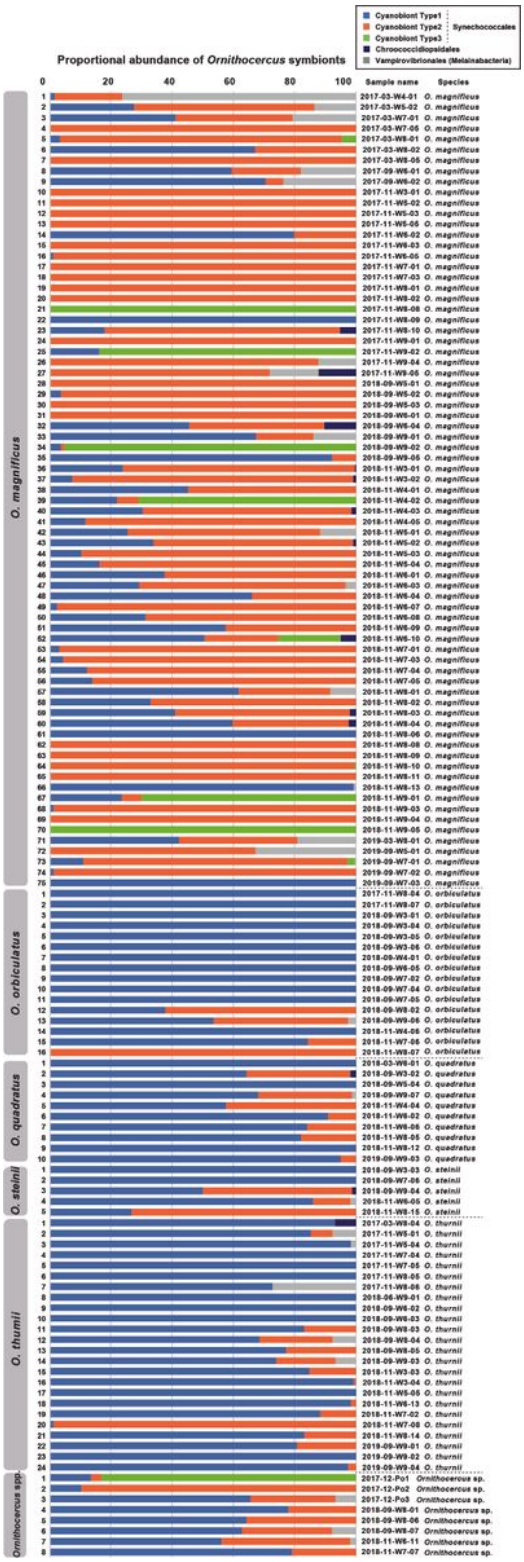
Proportional abundance of three cyanobionts types to the single Ornithocercus cells. Note that it is listed by host species.



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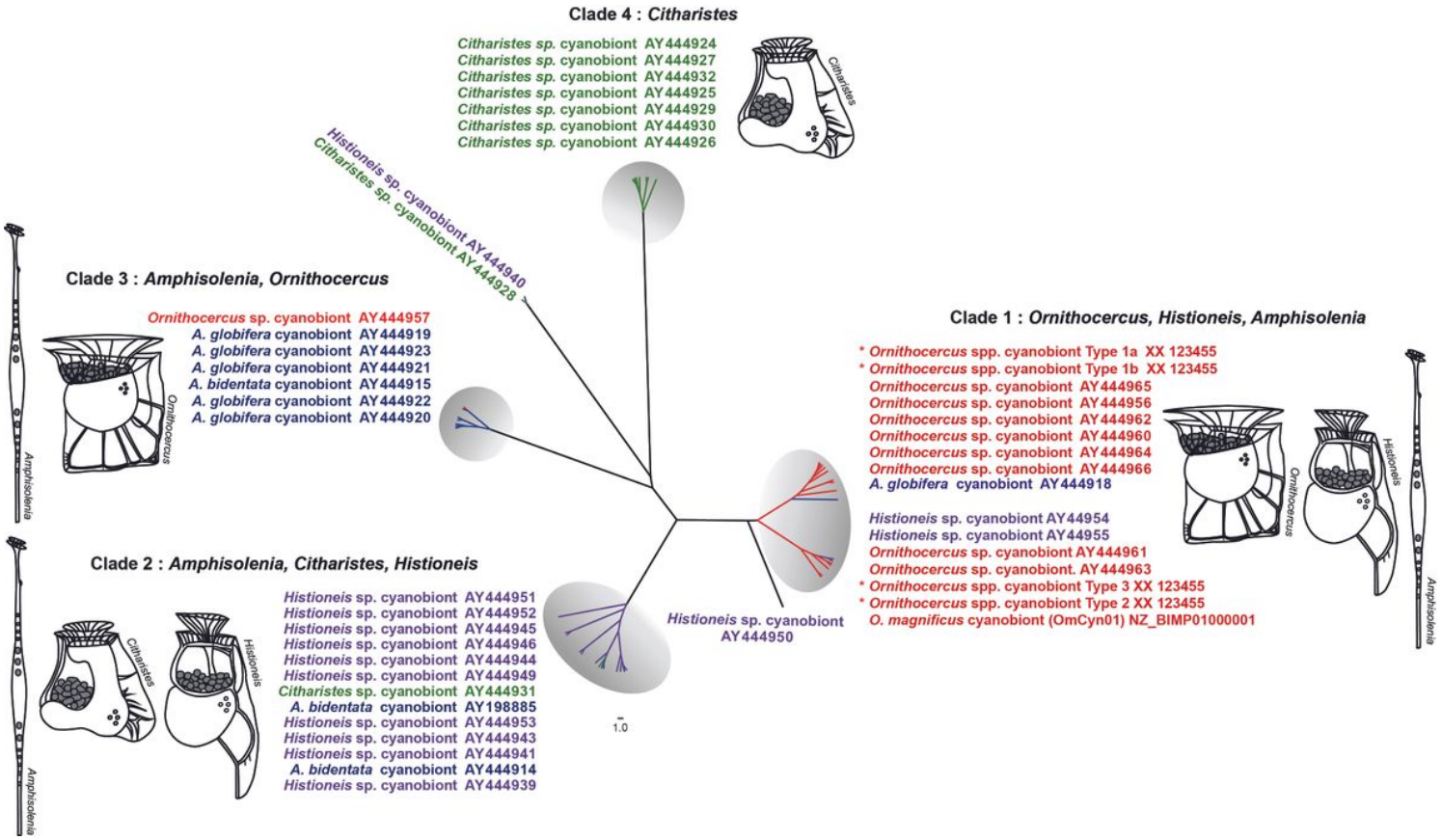
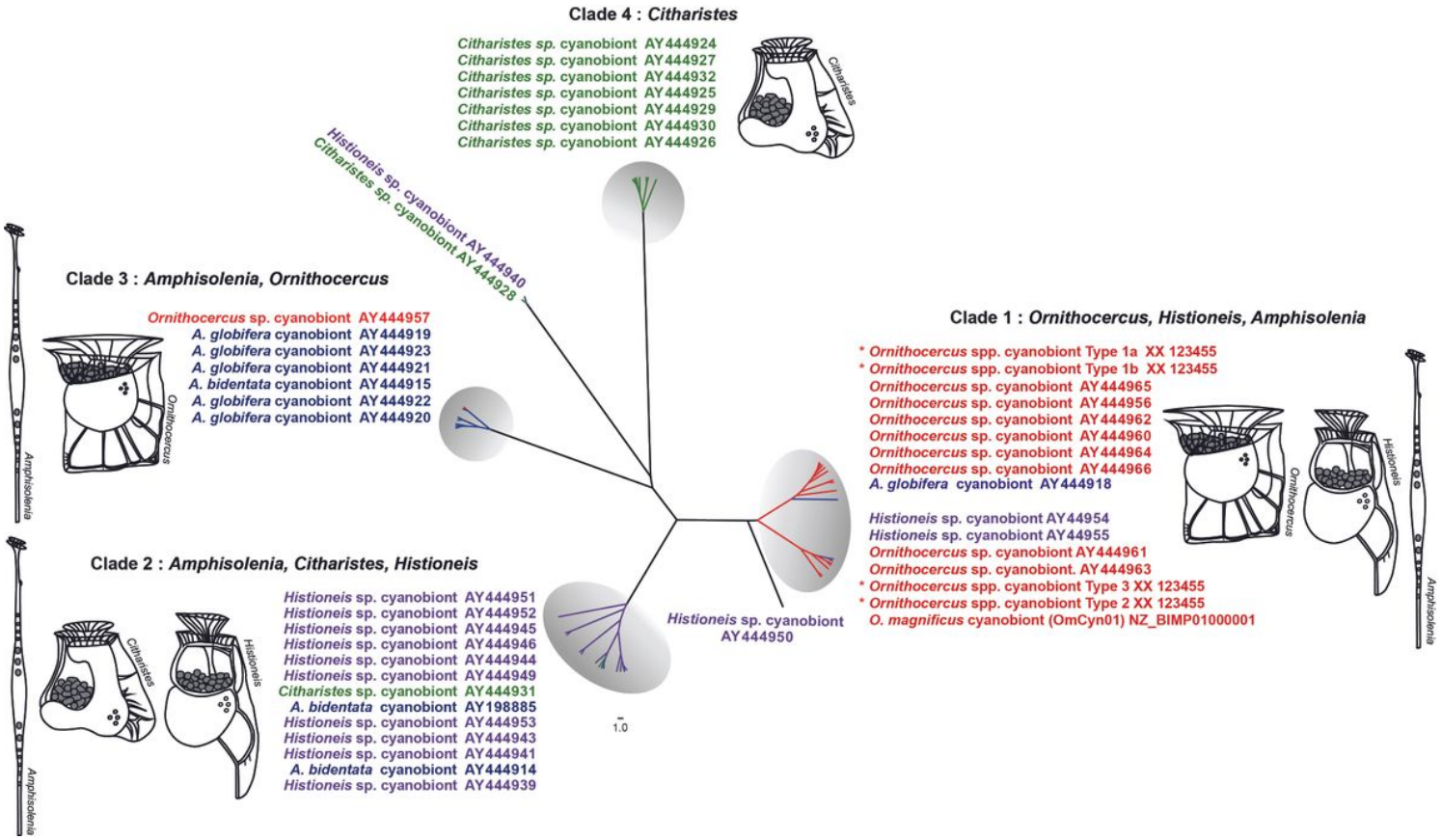


Figure 7

Phylogenetic clustering of dinoflagellate hosting-cyanobionts. Maximum Likelihood tree based on the V3–V4 regions of cyanobacterial 16S rDNA gene. Each host genus with cyanobionts (grey) is illustrated. Different colors of text and lines represent each host genus.



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