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The Phylogenetic Relationship of Several Oscillatorian Cyanobacteria, Forming Blooms at Daecheong Reservoirs, Based on Partial 16S rRNA Gene Sequences

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Abstract The partial 16S rRNA gene sequences of six filamentous cyanobacterial strains, *Oscillatoria limosa* KCTC AG10168, *Oscillatoria princeps* KCTC AG10153, *Oscillatoria* sp. KCTC AG10184, *Phormidium tenue* KCTC AG10158, *Phormidium parchydematicum* KCTC AG10164, and *Lyngbya hieronymusii* KCTC AG10199, which were isolated in the late summer at Daecheong Reservoirs, were determined and assigned their phylogenetic and taxonomic position among taxa of order Oscillatoriales. Most taxa of Oscillatoriales whose partial 16S rRNA gene sequences aligned in this study, were very heterogeneously clustered with other taxa. The two strains, *Oscillatoria limosa* KCTC AG10168 and *O. princeps* KCTC AG10153, formed a cluster with *O. sancta* PCC7515, which supported 64% of the bootstrap trees with high similarity (91–96.15%). Strain *Oscillatoria* sp. KCTC AG10184, that was known to produce a nasty substance, was closely related to the toxic *Oscillatoria agardhii* group. The study on morphological variation in various environments and toxin production will confirm the taxonomic status of these species. *Phormidium tenue* KCTC AG10158 and *Phormidium parchydematicum* KCTC AG10164 made a cluster with other oscillatorian species of *Phormidium*, *Oscillatoria*, and *Leptolyngbya*, which supported 100% of the bootstrap trees with a very high sequence similarity (96.8–99.8%) in this study. The sequence analysis in this study also supported that taxa of oscillatoriales are not monophyletic. Some of the features, such as the presence or absence of sheath and cell shape, which were used to define them, would be inadequate and should be reconfirmed. We suggest that sequences of partial 16S rRNA gene fragments aligned in this study should be more useful than morphological features in the identification and reconfirmation of the taxonomic status of these oscillactorian cyanobacteria.

Key words: 16S rRNA, phylogeny, Oscillatoriales, filamentous cyanobacteria

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Although the autotrophic algal service to the global environment has been widely understood, there are many taxonomic problems in classification, especially in oscillatorian cyanobacteria. The cyanobacteria are autotrophic prokaryotes, which carry out oxygenic photosynthesis, are treated as a cluster of the plant kingdom called cyanophytes, and it is in common blue green algae [16]. Woese [23] analyzed the phylogeny of the organisms based on the small subunit of ribosomal RNA gene sequences and classified the cyanophytes as the eubacteria of prokaryotes. The classification of cyanobacteria was based on morphology, inspite of a few characteristics that was divided into five groups including Chroococcales, Pleurocapsales, Oscillatoriales, Nostocales, and Stigonematales [2].

Oscillatoriales have a filamentous habit without heterocysts [1]. Another characteristic, which has been used as a criteria for genera in the Oscillatoriales, depends on its presence or absence of variable sheathes according to the physical condition in the culture. These morphological characteristics have made classification of these taxa very obscure [8]. Some nucleotide sequence analyses showed that oscillatorian species were genetically heterogeneous and it was not easy to classify based on morphology [13, 21, 22].

The 16S rRNA gene sequences have been suggested as a useful key in determining the phylogenetic position among cyanobacteria [7, 12] and are usually used in identification of other bacteria [10, 24]. However, it is a very difficult, and not to mention, tedious process to obtain axenic strains from the natural environment for the purpose of acquiring sequence data. The best way to improve this process is to develop specific probes to distinguish the 16S rRNA genes of cyanobacteria in clonal cultures from those of their bacterial contaminants [13]. Nubel *et al.* [14] developed and tested a set of oligonucleotide primers for a specific amplification of partial 16S rRNA gene segments from cyanobacteria. This enabled gene segments from filamentous cyanobacteria to be directly sequenced without pure culturing and cloning.

Several filamentous cyanobateria are known to form blooms in late summer for several years at Daecheong Reservoir in Korea [11]. These blooms have invoked some problems such as increasing the cost of treatment for water quality and decreasing the degree of dissolved oxygen. The toxic effects due to filamentous cyanobacterial blooms, such as killing of fish and domestic animal, have also been reported throughout the world [20]. However, there is little taxonomic information on these species, except for the list of species, which is made in the flora survey without giving any description, in this reservoir.

In this study, the partial 16S rRNA gene sequences of six filamentous cyanobacterial strains, which formed blooms at Daecheong Reservoir in September 1999, were determined and separated, and their phylogenetic and taxonomic position among taxa of order Oscillatoriales were inferred based on these sequences.

MATERIALS AND METHODS

The nonaxenic filamentous cyanobacterial strains used in this study are *Oscillatoria limosa* KCTC AG10168, *Oscillatoria princeps* KCTC AG10153, *Oscillatoria* sp. KCTC AG10184, *Phormidium tenue* KCTC AG10158, *Phormidium parchydematicum* KCTC AG10164, and *Lyngbya hieronymusii* KCTC AG10199. All of the strains were cultured in the ALLEN medium by incubating in cap tubes without aeration at 23°C with fluorescent lamp illumination of 1,500 Lux.

The cells were harvested by centrifugation in a 1.5 ml micro tube and washed twice with 1 ml of distilled water and dried briefly in a vacuum before being powderized with liquid nitrogen for DNA extraction. The total genomic DNA extraction was made by using 2× CTAB (2% hexadecyltrimethylammonium bromide) buffer [18].

The 16S rRNA gene segments were amplified by Polymerase Chain Reaction (PCR) in a fragment by using the primer sets, CYA106-CYA781 [14]. A negative control without the template was included in every set of PCR. The PCR reaction mixtures were prepared by following the manufacturer's instruction (Taq DNA Polymerase™, Bioneer Co., Korea), and PCR were performed in an automated thermal cycler (MJ Research, Watertown, MA, U.S.A.). The initial cycle was carried out at 95°C for 2 min, followed by 28 cycles of 30 s at 94°C, 30 s at 60°C, and 2 min at 72°C. The final extension cycle was at 72°C for 10 min for achieving a complete primer extension. The amplified DNA was further purified using a High Pure PCR Product Purification Kit™ (Boehringer Mannheim Co., Germany).

The purified DNA was sequenced directly by the BigDye Terminator Cycle Sequencing Kit (PE Applied Biosystems, Foster City, CA, U.S.A.) following the manufacturer's recommendations. The sequence data were collected with the ABI Model PRISM 311 and 377 DNA Sequencer (PE Applied Biosystems, Foster City, CA, U.S.A.). The sequences were aligned with other known 16S rRNA gene sequences of oscillatorian cyanobacteria [13, 14] by using the PHYDIT program [3]. Ambiguously aligned sites were excluded from the phylogenetic analysis, however, similarity values were calculated for all aligned sites. The tree topology based on these aligned 16S rRNA gene sequences were reconstructed with the Kimura's 2-parameter distance model and neighbor-joining methods by using the PHYLIP 3.5 package [6, 9]. The statistical significance of tree branches were assessed by incorporating the bootstrap resampling method with 1,000 replicates [5]. The parsimony tree reconstructed by the heuristic search option of the PAUP version 4.0 beta [19] was compared with the distance tree. The resultant phylogenetic tree was visualized by using the TreeView Program [15].

RESULTS AND DISCUSSION

Among the six species, *Oscillatoria limosa* was known to be a dominant species forming blooms [11], and *Oscillatoria* sp., *O. princeps*, *Phormidium parchydematicum*, *P. tenue*, and *Lyngbya hieronymusii* were first identified in Daecheong Reservoir. A few reports [11, 4] on the filamentous cyanobacteria in Daecheong Reservoir were carried out in the ecological research without any description on their morphological characteristics, which created a great confusion on identification of the species collected in this reservoir, in addition to their morphological plasticity.

The identification of PCR products of filamentous cyanobateria contaminated with other bacteria by cloning and sequencing has been a tedious and a difficult process. The probe showing high specificity for cyanobacteria [14] enabled the amplification and sequencing to be very simple and fast. Using these primers, all of the PCR amplifications were very successful for achieving nonaxenic filamentous cyanobacterial strains from Daecheong Reservoir. The sequencing strategy generated partial sequences ranging from position 76 to 699 of *Arthrospira* sp.'s sequences (accession number X75044). The total number of nucleotides compared was 623 base pairs, after eliminating gaps or sites that were not determined. The sequences were submitted to the GenBank database under the accession numbers from AF337649 to AF337654.

A phylogenetic tree based on aligned partial 16s rRNA gene sequences was constructed by using the neighbor-joining method and the most-parsimony method. The topology of the tree was consistent between these trees (the most-parsimony trees were not shown). A phylogenetic tree constructed by the neighbor-joining method is shown in Fig. 1. Most taxa of Oscillatoriales whose partial 16S rRNA gene sequences aligned in this study are heterogeneously

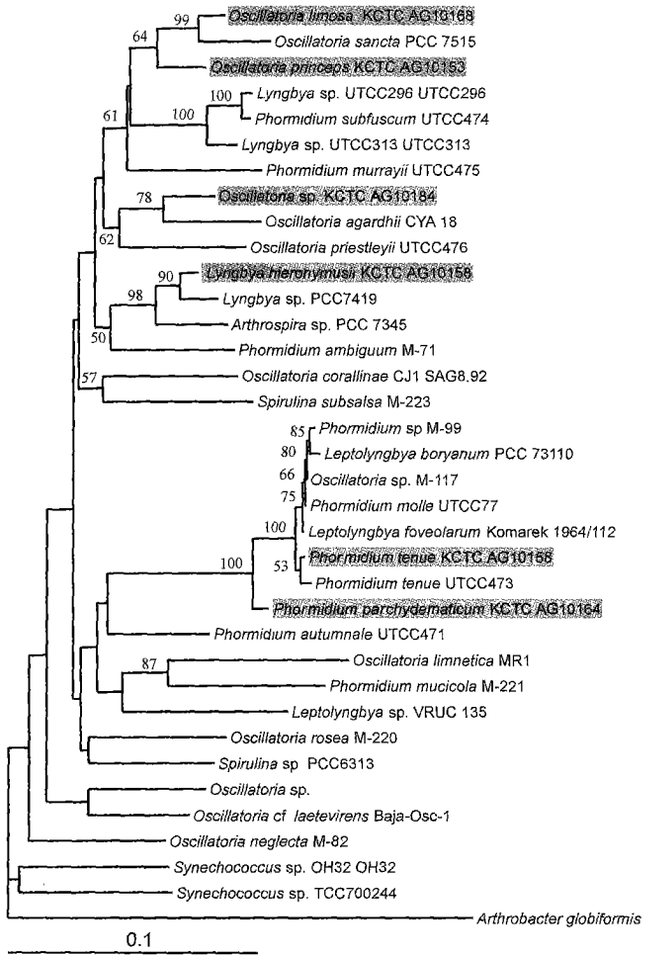

Fig. 1. Distance tree based on an alignment of 35 partial 16S rRNA gene sequences (623 positions) from filamentous cyanobateria, constructed by means of the neighbour-joining method.
Bootstrap percentages higher than 50% are placed alongside the node considered. The scale bar indicates 0.1 substitutions per nucleotide.

clustered with other different taxa. The six strains whose partial 16S rRNA gene sequences were determined in this study, involved in four separate clusters. The two strains, *Oscillatoria limosa* KCTC AG10168 and *O. princeps* KCTC AG10153 formed a cluster with *O. sancta* PCC7515 which supported 64% of the bootstrap trees with high similarity (91–96.15%). The *O. princeps* strain was characterized by a dark blue green habit, slight constriction at the cross wall, round and inflated terminal cells, and 15–55 μm in width. The *O. limosa* strain had a dark green or brown habit that was distinguished from *O. princeps* strain by a nonconstriction at the cross wall, and a thickened terminal cell wall. However, it was very difficult to confirm the taxonomic position of these strains, because of obscurity in the classification of oscillatorian cyanobacteria that was based on the topology of the phylogenetic tree.

Strain *Oscillatoria* sp. KCTC AG10184, isolated from Daecheong Reservoir, was confirmed to produce a nasty substance, and formed another cluster with *O. agardhii* CYA18 and *O. pristleyii* UTCC476, which supported 62% of the bootstrap trees. Strain *Oscillatoria* sp. had trichomes 5.0–6.5 μm wide, slight constriction at the cross wall, cell length shorter than its width, and gas vacuoles. *Oscillatoria* sp. was also similar to *O. agardhii* CYA18 except for the trichome width in morphology. Skulberg and Skulberg [17] reported that the *O. agardhii* and *O. bornetii* groups create blooms in lakes and make water inappropriate for use as a result of producing toxins and odor-substance. We could not confirm whether *Oscillatoria* sp. KCTC AG10184 produced the toxins or not. However, this partial 16S rRNA gene sequence comparison suggested that strain *Oscillatoria* sp. was closely related to these toxic *Oscillatoria* species. More studies on morphological variation in variable environments and toxin production would confirm the taxonomic status of this *Oscillatoria* sp.

Phormidium tenue KCTC AG10158 whose sequence closely related to *P. tenue* UTCC473 is characterized by dense constriction at the cross wall, a very thin filamentous habit with 1–2 μm width, and an oblique terminal cell. *Phormidium parchydematicum* KCTC AG10164 is characterized by not constricting at the cross wall and a very thick habit with 5–10 μm width. *P. parchydematicum* is known to live on rocks and soil, but it was isolated from the water surface of Daecheong Reservoir in this study. In fact, this species could be washed off by heavy rain. These strains made a cluster with other oscillatorian species of *Phormidium*, *Oscillatoria*, and *Leptolyngbya*, which supported 100% of the bootstrap trees and had a very high sequence similarity (96.8–99.8%) in this study. Although these species were identified and classified into other taxa based on their morphological features, they were found to be highly genetically related to each other, reconfirming their taxonomic status.

Lyngbya hieronymusii KCTC AG10199 also belongs to a heterogeneous lineage group of *Lyngbya*, *Phormidium*, and *Athrospira*, which supported 98% of the bootstrap trees. *L. hieronymusii* is characterized by a colorless sheath, no constriction at the cross wall, pale green habit, and 8–12 μm width. Although the sequence similarity does not necessarily represent the relationship of the species, the topology of the tree showed that this strain should be closely related to *Lyngbya* sp. PCC7419.

The six strains isolated from Daecheong Reservoir belong to four heterogeneous lineages that involve other taxa. *Oscillatoria* species are clustered in a lineage, except for the presumed toxic *Oscillatoria* species strain, and *Phormidium* species was also in a lineage. However, the oscillatorian strains aligned in this study are so heterogeneous that the taxonomic status of these strains can not be confirmed (Fig. 1). The total number of nucleotide sequences compared in this analysis may be too small to explain all the genetic information of these strains, however, it is

enough to clarify the phylogenetic relationship among them. Ishida *et al.* [8] concluded that phylogenetic tree topology based on the partial 16S rRNA gene sequences was consistent with those that were based on aligned 1,009 nucleotide sequences of several blue green algae.

The phylogenetic relationship among taxa of the order Nostocales, Stigonematales, and Pleurocapsales was supported well by the 16S rRNA gene sequence analysis, but the order Chroococcales and order Oscillatoriales were very divergent among the species, not forming a cluster [8]. The fact that a few unstable morphological characteristics have been used in construction of the genus concept may cause the oscillatorian cyanobacteria to be very heterogeneously clustered among taxa in genus and species levels which are constructed on the basis of the gene sequence. The sequences analysis in this study also supported that taxa of oscillatoriales were not monophyletic. Therefore, some of the features, such as the presence or absence of sheath and cell shape, which were used to define taxa, would not reflect their phylogenetic relationship in the classification process. Other more stable characteristics such as nucleotide sequences and chemical traits should also be adopted to elucidate this taxonomic problem and reconstruct the classification system. Additional sequences of partial 16S rRNA gene fragments aligned in this study would be more useful than morphological features for the identification and reconfirmation of the taxonomic position of oscillatorian cyanobacteria at a low cost.

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