Occurrence and phylogenetic analysis of *Pseudanabaena* sp. producing 2-methylisoborneol in drinking water source of South Korea

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Abstract

We investigated the abundance of *Pseudanabaena* species and the concentration of the monoterpene 2-methylisoborneol (2-MIB) from July to October at three sampling sites in South Korea. To identify the main cause of 2-MIB occurrence in drinking water source, we characterized and performed a phylogenetic analysis of the 2-MIB synthase gene. *Pseudanabaena* was the dominant cyanobacterium (68–100%) among the samples. At all three sampling sites, a strong positive correlation was detected between 2-MIB concentrations and *Pseudanabaena* cell numbers. A phylogenetic analysis of 222 MIB sequences isolated from the water samples showed that all of the clones were affiliated with the *Pseudanabaena* MIB synthase gene, demonstrating that the 2-MIB in Han River drinking water source was produced by *Pseudanabaena* sp. Using a clone of the 2-MIB gene, network-based analysis and unweighted pair group method with arithmetic mean (UPGMA) analysis were used to examine temporal and spatial variation in the 2-MIB concentration and *Pseudanabaena* abundance. The network analysis showed greater temporal than spatial similarity among the 2-MIB gene clones. Together, our results demonstrate that *Pseudanabaena* was the main producer of 2-MIB. These findings provide important information for odor management in drinking water source.

Keywords: 2-Methylisoborneol, Cyanobacteria, Drinking water source, Han River, Phylogeny *Pseudanabaena*

1. Introduction

Many cyanobacteria (blue-green algae) produce earthy or musty odor compounds that impact the quality of water stored in reservoirs (Westerhoff et al. 2005). These odors are caused by a secondary metabolite of cyanobacteria, 2-methylisoborneol (2-MIB), a common off-flavors monoterpene. Reports of 2-MIB in drinking water have gradually increased in recent decades, most of which were associated with cyanobacteria blooms (Devi et al. 2021). Although 2-MIB is not known to pose a serious health risk to humans or aquatic animals, many consumers judge water containing 2-MIB to be unsafe to drink due to its unpleasant odor (Huang et al. 2018, Jüttner and Watson 2007, Yu et al. 2014).

It is difficult to remove 2-MIB from drinking water source through common treatment processes (Srinivasan and Sorial 2011) because it can be detected by humans at concentrations as low as ~10 ng/L (Watson et al. 2008), and complaints have been filed at concentrations lower than 5 ng/L. Therefore, to manage drinking water source odor caused by 2-MIB, it is essential to understand and control its production in source reservoirs.

More than 40 species of cyanobacteria have been reported as 2-MIB producers, including those belonging to the genera *Pseudanabaena*, *Lyngbya*, *Oscillatoria*, *Phormidium*, and *Planktothrix* (Giglio et al. 2011, Izaguirre and Taylor 2004, Jüttner and Watson 2007, Kakimoto et al. 2014, Suurnäkki et al. 2015, Wang et al. 2016, Wang et al. 2011). *Pseudanabaena* is an important 2-MIB-producing phytoplankton, and two 2-MIB-related genes have been identified in *Pseudanabaena limnetica* (Giglio et al. 2011). Previous studies reported the presence of *Pseudanabaena* species producing 2-MIB in various water resources (Huang et al. 2018, Niiyama et al. 2016).

Cyanobacterial species such as *Pseudanabaena* species are typically counted through microscopy; this process is time consuming and separates odorous from non-odorous cyanobacteria with difficulty (Chiu et al. 2016). Odorant concentrations are generally analyzed

using gas chromatography (GC). Several studies have revealed correlations between 2-MIB concentrations and 2-MIB synthesis genes (Giglio et al. 2011, Wang et al. 2011). Such analyses can be applied to identify cyanobacteria species that produce 2-MIB, thereby influencing drinking water source palatability.

The Han River is the drinking water source for > 25 million people, representing 48% of the South Korean population (Lee et al. 2020). Previous studies have reported that cyanobacteria appear in the Han River in summer, when water temperatures exceed 20℃, occasionally leading to major outbreaks accompanied by high concentrations of the odorous compound geosmin, which reached 3,900 ng/L in 2012 (Lee et al. 2020). The episodes of unpleasant odors have been arising by geosmin or 2-MIB but the occurrence and the origin of 2-MIB odorous remains to be studied. It makes difficult to take effective measures to either prevent of control the occurrence of the 2-MIB in drinking water resources. Therefore, the objectives of this study were to investigate 2-MIB production and its main cause, and to characterize the 2-MIB synthesis gene in Han River drinking water source.

2. Materials and Methods

2.1 Study area and water sampling

The Han River system feeds the main drinking water reservoirs for Seoul and other metropolitan areas of South Korea. These reservoirs collect water that originates from the Ui Am and Cheong Pyeong Lakes. The North Han River is the longest segment of this system, draining 50% of the Han River basin, and occupies a steep slope, which is favorable for dam construction. In this study, we sampled water from the Uiam, Cheong Pyeong, and Paldang dams (Fig. 1). We analyzed the 2-MIB concentrations and cyanobacteria occurrence in samples collected from July to October, 2018. We analyzed the 2-MIB genes obtained from environmental samples collected on October 8, 2018, and October 22, 2018.

We collected 1-L water samples in plastic bottles, which were then stored at 4°C until analysis. Cyanobacteria were harvested from 100-mL water samples by membrane filtration using a polycarbonate filter (pore size, 0.2 μm; Whatman, Maidstone, UK) and subjected to DNA extraction. Water samples collected for odorous compound analysis were stored in 50-mL glass bottles under cold, dark conditions during transportation to the laboratory.

2.2 Isolation and enumeration of cyanobacteria

Water samples collected for cyanobacteria analyses were placed in 500-mL plastic bottles and fixed immediately with Lugol's iodine solution (final concentration, 2% w/v). To quantify the cyanobacteria, 1 mL of the fixed specimens was placed in a Sedgwick-Rafter counting chamber and allowed to settle for at least 15 min, followed by observation under a phase microscope (Eclipse 80i; Nikon Corp., Sendai, Japan). The number of cells per unit area was observed at 100–1000× magnification and the total concentration was calculated.

We isolated unialgal strains using the Pasteur capillary pipette method (Wang et al. 2015). Briefly, single filaments were selected using a Pasteur capillary pipette under a dissecting microscope (Nikon Corp.). The isolates were then placed in a 24-well plate containing liquid BG-11 medium and cultured at 25°C under a 12-h:12-h dark/light cycle (40 μmol/m^2/s) for GC-mass spectrometry (MS) and molecular characterization.

2.3 Analysis of 2-MIB

We analyzed 2-MIB using the headspace solid-phase microextraction (HS-SPME) method (Lin et al. 2003, Lloyd et al. 1998) in combination with GC-MS. Prior to analysis, the fibers were activated by applying helium gas at 1 mL/min for at least 1 h at 270°C. Approximately 10 mL of the specimens and 3 g of sodium chloride were placed in a 20-mL vial and adsorbed onto solid-phase microextraction fibers for 30 min at 70°C while being spun at 400 rpm. The

adsorbed specimens were desorbed for 4 min at 270°C and analyzed by GC-MS (450-GC, 320-MS; Bruker, Billerica, MA, USA).

2.4 Primer design, DNA extraction, and polymerase chain reaction (PCR)

For 2-MIB synthase gene detection, primers were designed using BioEdit v7.2 based on sequences from *Oscillatoria* (KJ658377), *Planktothrix* (KJ658378), *Planktothricoides raciborskii* (HQ830029), and *Pseudanabaena* (HQ830028). The annealing temperature for PCR was set based on the melting temperature. The GC content and self-annealing were verified using Oligo Calc (http://www.basic.northwestern.edu/biotools/ oligocalc.html).

Cyanobacteria were collected from 1.5-mL cultures by centrifugation at 16,000 × *g* at 4℃ for 10 min. The pellet was extracted using a DNA Mini Spin Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. To extract total DNA from environmental water samples, 100 mL of water was filtered on polycarbonate filters (pore size, 0.2 μm; Whatman). Total genomic DNA was extracted using a DNeasy Power Water Kit (Qiagen) according to the manufacturer's instructions.

PCR amplification of the 16S rRNA and MIB synthase genes was performed in 2X Taq PCR Smart Mix 2 Buffer (Solgent, Daejeon, Korea) with 0.2 μM each primer and 2.5 μL of genomic DNA as a template. The PCR protocol was 95℃ for 5 min, followed by 30 cycles of 95℃ for 20 s, 59℃ (16S rRNA)/56℃ (MIB synthase) for 40 s, 72℃ for 1 min, and 72℃ for 5 min. The sizes of the PCR products were determined using 2.0% agarose gel electrophoresis and a 100-bp DNA ladder (Promega, Madison, WI, USA).

2.5 Cloning and sequence analysis

The PCR products were cloned into the pGEM-T and pGEM-T Easy Vector Systems (Promega) and transformed into T-Blunt Competent Cells (Solgent) according to the

manufacturer's instructions. Cells were plated on LB agar containing 100 μg/mL of ampicillin and 50 μL of X-gal. We sequenced 50 white colonies from each clone library using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and an ABI PRISM 3730xl DNA Analyzer (Applied Biosystems).

Putative MIB sequences were obtained from GenBank using BLAST. Sequences from each colony were aligned together with sequences from GenBank using ClustalW with BioEdit (Hall 1999). The aligned sequences were used to construct a neighbor-joining (NJ) tree algorithm, and unweighted pair group method with arithmetic mean (UPGMA) analysis using 1,000 bootstrap replicates was used to construct a phylogenetic tree with MEGA 5.2 (Tamura et al. 2011). A network-based analysis was performed using Cytoscape v2.6.3 to identify sequence relationships with sampling site and date (Shannon et al. 2003).

3. Results and Discussion

3.1 Relationship between *Pseudanabaena* abundance and 2-MIB concentration

Pseudanabaena is a major producer of 2-MIB in many countries, including the USA, China, and Japan (Izaguirre and Taylor 1998b, Niiyama et al. 2016, Zhang et al. 2016). Cyanobacteria cell counts from July to October, 2018, at the three water sampling sites (Fig. 1) are presented in Table 1. The total cyanobacteria cell density varied from < 300 cells/mL in July to a maximum of 16,639 cells/mL in August. The cell density was high at all three sites in August. *Pseudanabaena* was dominant at all sampling dates, representing 68–100% of all cyanobacteria (Table 1). These results are consistent with a previous study that reported increased *Pseudanabaena* populations in August, and that *Pseudanabaena* cell counts were similar to those of other cyanobacteria from July to August (Huang et al. 2018). The cyanobacterial cells had increased in August (Lee et al. 2020), and 2-MIB production by *Pseudanabaena* sp. increases during the summer (Izaguirre and Taylor 1998a). Other study

174 also was confirmed that the *Pseudanabaena* growth rate and 2-MIB concentration increases
175 when the temperature was more than 25 degrees (Wang et al. 2015).

176 Figure 2A shows the concentration profiles of 2-MIB and *Pseudanabaena* in the North
177 Han River. The 2-MIB concentrations (3–11 ng/L) and *Pseudanabaena* cell counts (180–4,990
178 cells/mL) were lowest at the most remote sampling site (UA), compared with those at the CP
179 (41 ng/L and 390–13,450 cells/mL, respectively) and SB sites (31 ng/L and 360–15,310
180 cells/mL, respectively).

181 At all three sampling sites, a strongly positive correlation was detected between 2-MIB
182 concentration and *Pseudanabaena* cell number (R^2 = 0.7314, P < 0.0001), indicating that 2-
183 MIB was mainly produced by *Pseudanabaena* in the Han River (Fig. 2B). This result is
184 consistent with those of two previous studies of *Pseudanabaena* and 2-MIB levels in nature
185 (R^2 = 0.899, P < 0.0001; Zhang et al. 2016) and in culture (Izaguirre and Taylor 1998a).
186 Previous

187

188 **3.2 PCR-based *Pseudanabaena* 2-MIB synthase gene detection**

189 Cyanobacteria-specific 16s rRNA primers (Nübel et al. 1997) were used to identify the exact
190 species of cyanobacteria isolated from the water samples and to detect the 2-MIB synthase
191 gene (Table 1). The PCR products were of the expected size for 2-MIB producers (899 bp).
192 Previous studies reported 2-MIB production in different cyanobacterial genera, including
193 *Pseudanabaena* (Niiyama et al. 2016), *Oscillatoria* (Schrader et al. 2004), *Planktothrix* (Su et
194 al. 2015), and *Leptolyngbya* (Wang et al. 2015).

195 Among the five cyanobacteria isolated in this study, we identified two *Pseudanabaena*
196 species, one *Planktothrix* species, and two *Oscillatoria* species through 16S rRNA analysis
197 (Table 3). PCR detected the 2-MIB synthase gene in both *Pseudanabaena* strains. GC-MS
198 showed that both *Pseudanabaena* strains produced 2-MIB (Table 3); the other strains were not

associated with 2-MIB production and were not subjected to GC-MS. Previous studies reported the successful application of PCR to detect 2-MIB production via the 2-MIB synthesis gene, according to high congruence between the results of PCR and GC-MS (Suurnäkki et al. 2015). The 2-MIB gene sequences were deposited to GenBank (accession no. MT360266).

3.3 Characterization of 2-MIB synthase gene clones in drinking water source

Next, we used the PCR products to create 2-MIB gene clones. In early and late October, when the 2-MIB concentrations were high, we analyzed the 2-MIB genes in water samples; the numbers of clones ranged from 15 to 43. Nucleotide sequence information was obtained through sequence analysis (Table 3). *Planktothrix*, *Oscillatoria*, and *Pseudanabaena* typically produce 2-MIB; therefore, to explore the origin of 2-MIB in our water samples, we performed a phylogenetic analysis of the 2-MIB gene based on 222 clone sequences. Two monophyletic branches were observed, as *Pseudanabaena* and *Planktothrix/Oscillatoria* (Fig. 3); all 222 MIB clone sequences isolated from the water samples were affiliated with the *Pseudanabaena* MIB synthase gene, indicating that 2-MIB in the Han River is produced mainly by *Pseudanabaena*. Previous studies have reported that *Pseudanabaena* produces 2-MIB in other countries, including the USA (Izaguirre et al. 1999, Izaguirre and Taylor 1998b) and Japan (Niiyama et al. 2016).

We obtained the 2-MIB gene clones on two different dates and at three different locations. UPGMA and network-based analyses were performed to analyze temporal and spatial variations among the clones. The 2-MIB gene clones were clustered among samples collected on the same date, rather than among samples obtained at the same sites, although the tree branches were well separated (Fig. 4A). Figure 4B shows the results of a network-based analysis of the MIB gene clones performed using the Cytoscape program (Shannon et al. 2003), which indicated that the 2-MIB gene clones were more similar temporally than spatially. A

previous study examined the seasonal and spatial dynamics of *Pseudanabaena* at six sampling sites along a river over a period of 14 months; *Pseudanabaena* cell abundance showed similar temporal trends at all sites (Zhu et al. 2015). Cyanobacteria are strongly affected by water currents (Liu et al. 2016). In the present study, our samples were collected at three sites along the same river; therefore, our data represent the downstream movement of *Pseudanabaena* with the flow of water, and the 2-MIB gene clones can be expected to show more temporal than spatial variation.

4. Conclusion

To manage odorous compounds in drinking water source associated with cyanobacteria outbreaks, accurate source analysis is necessary. In this study, we found that *Pseudanabaena* was the main producer of 2-MIB in the Han River, South Korea. We investigated the *Pseudanabaena* abundances and 2-MIB concentrations at three sampling sites along the Han River, and we found a strongly positive correlation according to GC-MS measurements. These results were confirmed by detection of the 2-MIB synthase gene in *Pseudanabaena*. We performed a 2-MIB synthase gene analysis using field water samples; the subsequent phylogenetic analysis showed that the 2-MIB in our samples was produced by *Pseudanabaena*. In addition, these 2-MIB gene clones showed greater temporal than spatial similarity. Although this study was performed with a limited number of sampling site and isolation, this is the first field study to identify the cause of 2-MIB production in the Han River; our results could be provided important information for drinking water source odor management in this region. Further studies using a larger samples size should be performed in the future to better characterize the source of 2-MIB occurrence.

5. Acknowledgments

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Tables and Figures

Table 1. Abundances of total cyanobacteria and *Pseudanabaena* in drinking water source collected at three sampling sites in South Korea

Sampling site	Date	Abundance (cells/mL)		Proportion of *Pseudanabaena*
		Pseudanabaena	Total cyanobacteria	
UA	July 23	180	263	68%
	August 27	4,990	4,990	100%
	September 3	490	490	100%
	September 10	380	380	100%
CP	August 20	13,450	13,749	98%
	August 27	10,520	10,520	100%
	September 10	1,130	1,130	100%
	September 17	1,120	1,120	100%
	October 22	390	390	100%
SB	August 20	15,310	16,639	92%
	October 8	360	360	100%
	October 15	1,340	1,340	100%

Table 2. Primer pairs used for polymerase chain reaction amplification and sequencing of 2-MIB synthase genes

Target gene	Primers	Sequences 5'→3'	Product length (bp)	Reference
16S rRNA	CYA106F	CGG ACG GGT GAG TAA CGC GTG A	699	NüBel et al., 1997
	CYA781R (a+b)	GAC TAC T(A)GG GGT ATC TAA TCC CA(T)T T		
2-MIB synthase	63F	TAC ATC CGC CGC TCG CTT TGT GAG	899	This study
	952R	AAT CTG TAG CAC CAT GTT GAC		

Table 3. Strain identification of 2-MIB according to 16S rRNA, PCR, and gas chromatography-mass spectrometry (GC-MS) analyses

Strain		PCR (mib)	GC-MS (MIB)
Name	16S rRNA		
HNIER134	*Pseudanabaena* sp.	+	+
HNIER134	*Pseudanabaena* sp.	+	+
HNIER149	*Planktothrix* sp.	–	–
HNIER162	*Oscillatoria* sp.	–	–
HNIER165	*Oscillatoria* sp.	–	–

+ Detected.
– Not detected.

Table 4. The concentrations of 2-MIB and the numbers of clones detected in drinking water source from the three sampling sites

Date	Sampling site (label)	2-MIB (ng/L)	No. of clones
October 8	UA (UA1)	23	42
	CP (CP1)	18	43
	SB (SB1)	15	15
October 22	UA (UA2)	86	42
	CP (CP2)	17	41
	SB (SB2)	23	39

Figure 1. Water sampling sites along the Han River, South Korea. Water flows from Ui Am to Sam Bong.

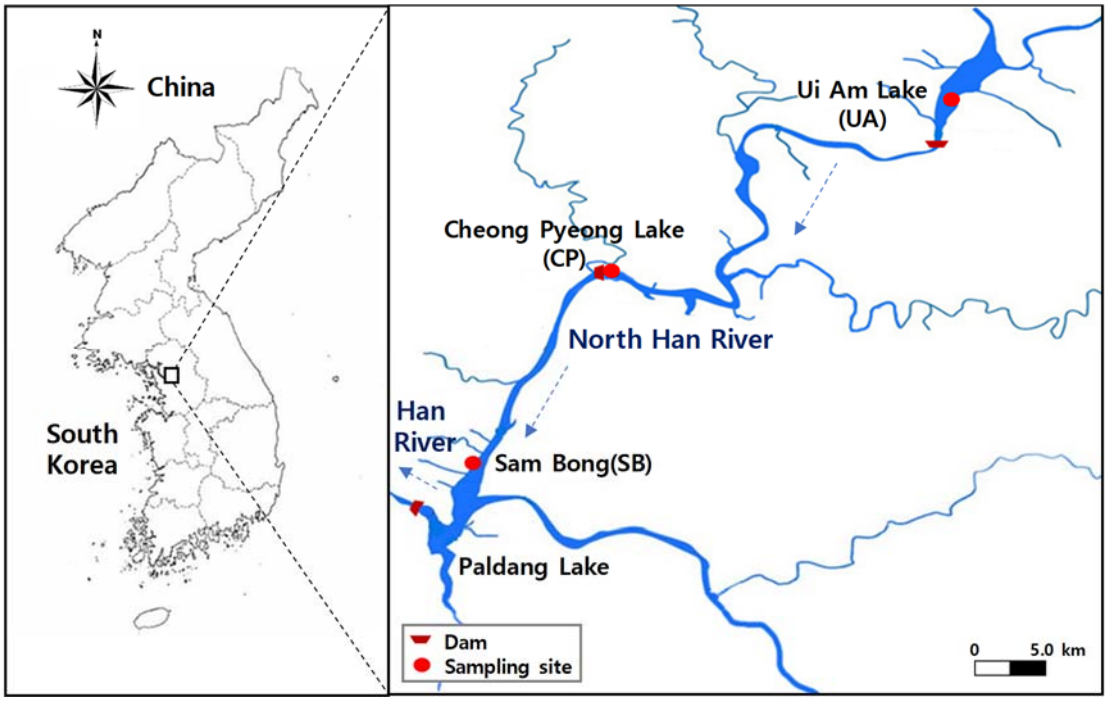
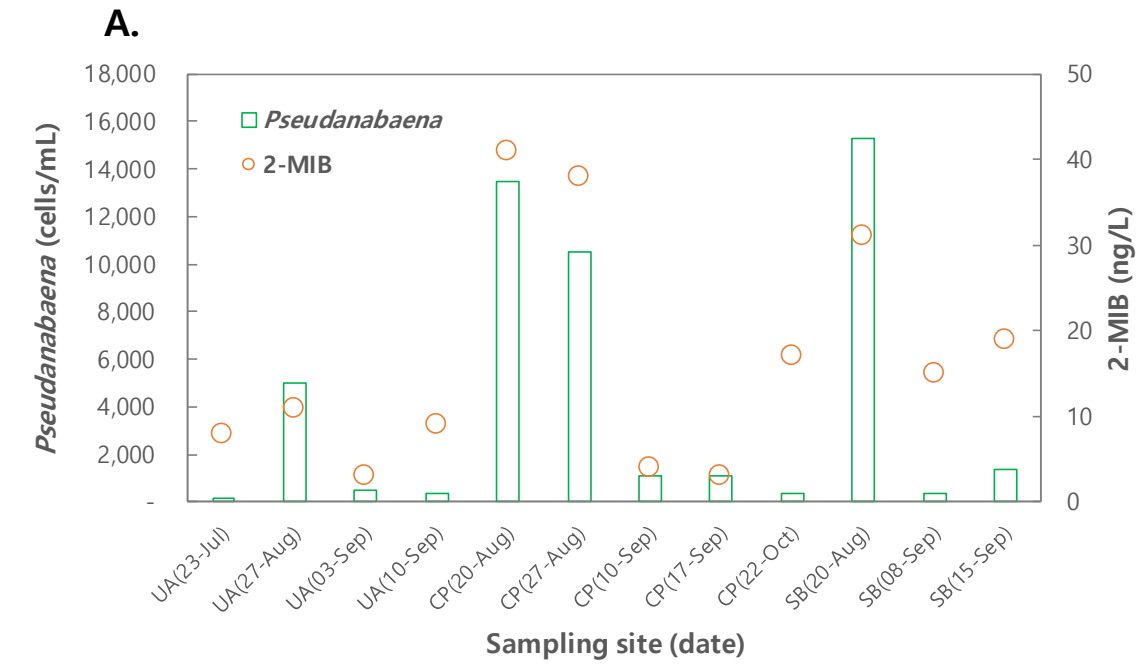


Figure 2. (A) *Pseudanabaena* abundances and 2-MIB concentrations at the three sampling sites, and (B) their correlation among all sites.



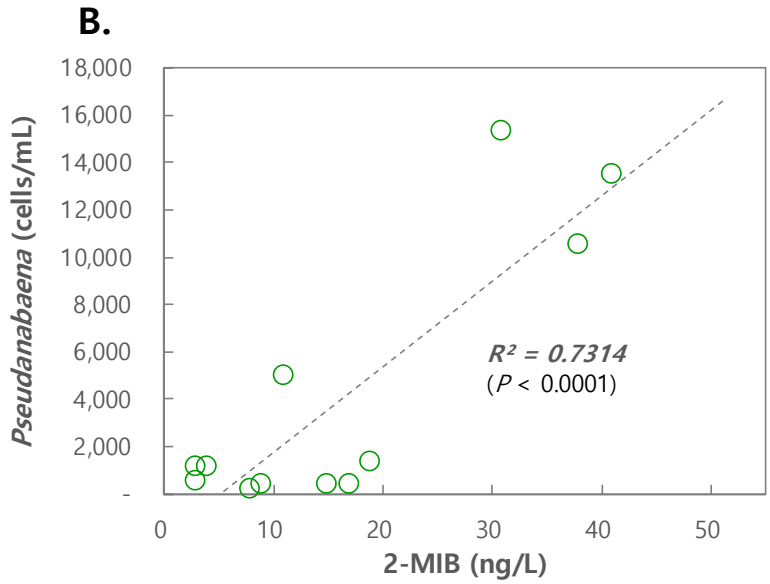


Figure 3. Neighbor-joining tree of clone sequences based on the 2-MIB synthase genes obtained using 1,000 bootstrap replicates. *Streptomyces coelicolor* A3(2) was used as an outgroup.

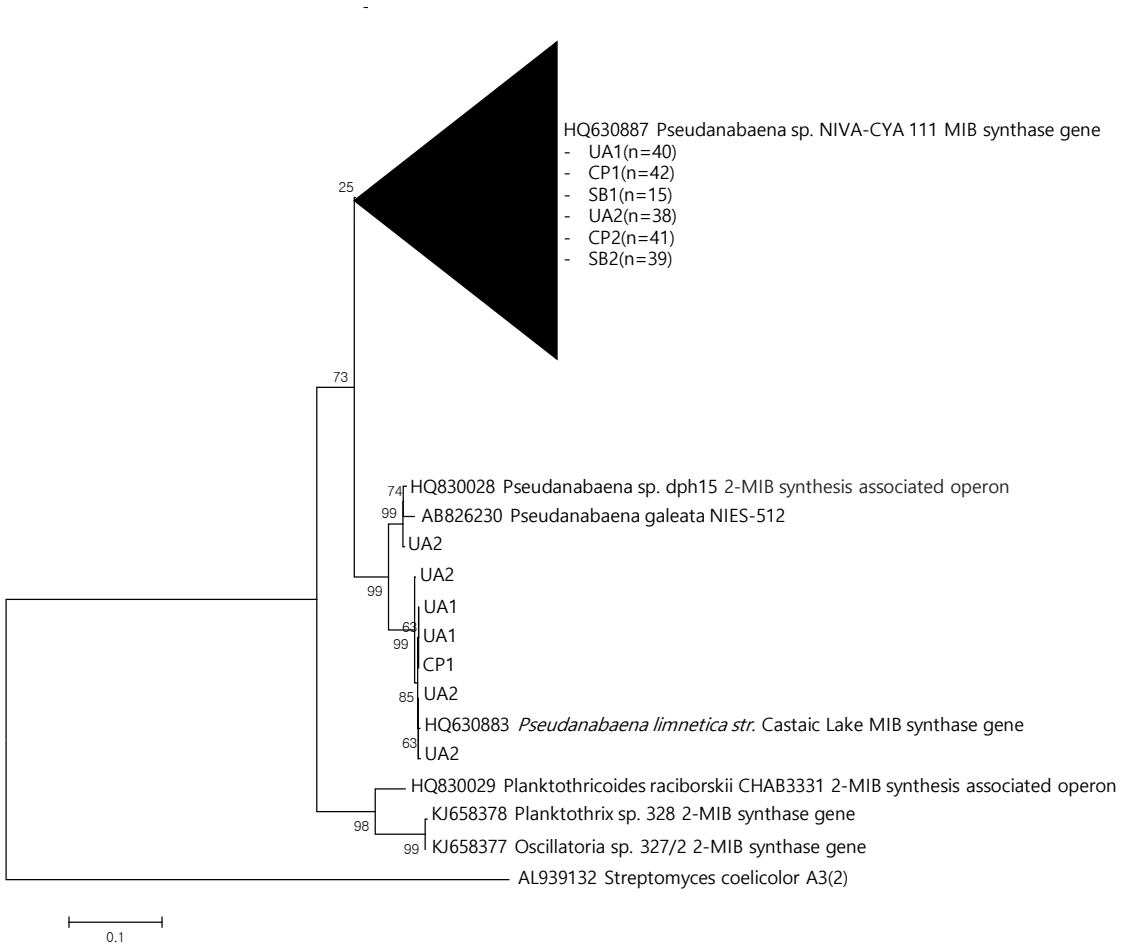
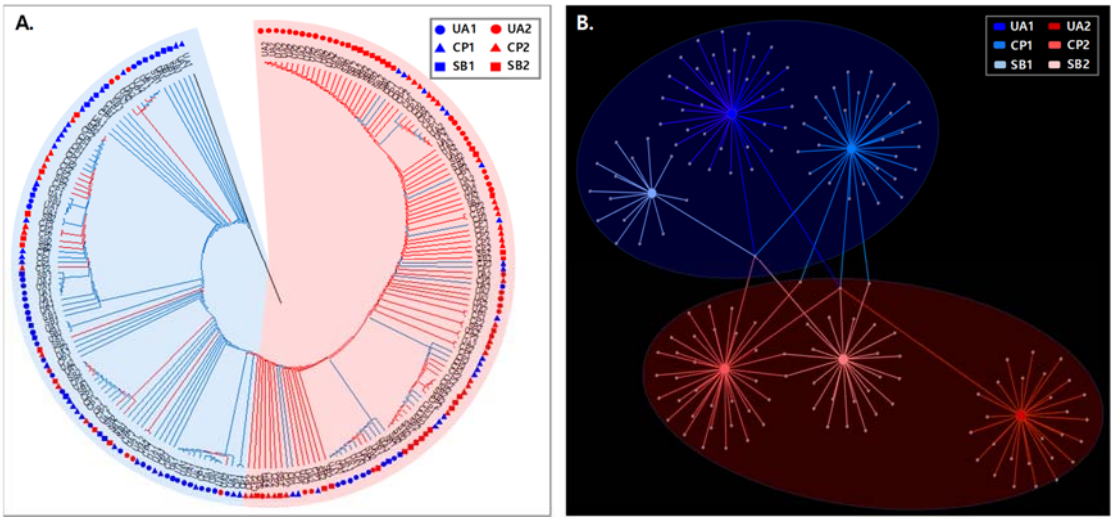


Fig. 4. (A) Unweighted pair group method with arithmetic mean (UPGMA) tree and (B) network-based analyses of the 2-MIB synthase genes at all sampling locations. Nodes represent 2-MIB genes; each line shows all of the locations where the gene was identified.



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