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Detection of bioluminescent dinoflagellates based on luciferase genes during the 'blue tears' season around the Matsu archipelago

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ABSTRACT: *Noctiluca scintillans* is considered to be a bioluminescent bloom-forming species in the coastal water around the Matsu archipelago. To identify the bioluminescent dinoflagellates and their distributions around the Matsu archipelago, metatranscriptome and luciferase (*lcf*) gene sequencing were conducted from June 2016 to July 2017. Metatranscriptomes retrieved *lcf* genes mainly from *Noctiluca* and other bioluminescent dinoflagellates. This result demonstrates that *lcf* genes were actually expressed in multiple dinoflagellate species. An analysis of the *lcf* composition of dinoflagellates indicated that *N. scintillans* was the dominant bioluminescent species during May and July. In late summer, this dominance was replaced by other bioluminescent dinoflagellate species, such as *Alexandrium affine* and *Ceratium fusus*. No *lcf* gene from the known toxic bioluminescent dinoflagellates was obtained during the period of investigation. Our results suggest that *N. scintillans* is not the only dinoflagellate species producing bioluminescence around the Matsu archipelago.

KEY WORDS: Bioluminescence · Luciferase gene · Matsu · Dinoflagellates · Metatranscriptome

1. INTRODUCTION

Nocturnal bioluminescence in tides has been reported in many coastal waters of the world (Haddock et al. 2010, Widder 2010). With elevated nutrient loads from anthropogenic activities, the occurrence of algal bloom events is increasing in coastal waters (Anderson et al. 2008, Heisler et al. 2008, Glibert et al. 2016). In recent years, the phenomenon of bioluminescence has also been observed from April to August around the Matsu archipelago (see Fig. 1), and it has been locally named 'blue tears'. This phenomenon not only stimulates people's curiosity but also arouses awareness of environmental protection. Therefore, it is necessary to provide the correct scientific information to understand the phenomenon of bioluminescence along the coast.

Blooms of *Noctiluca scintillans* have been reported to occur in bioluminescent seas from field observations along the coast of the East China Sea (Tseng et al. 2011, Tsai et al. 2018, Qi et al. 2019). *Noctiluca scintillans* appeared when the seawater temperature was between 16 and 27°C from April to June (Tsai et al. 2018), which is consistent with the wide temperature range of 10 to 25°C in coastal zones worldwide (Harrison et al. 2011). However, according to image

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inspection and spectral analysis through satellite remote sensing, blooms of *N. scintillans* have also been reported in August and September when the water temperature rose above 28°C in the East China Sea (Qi et al. 2019). Blue bioluminescence at night has often been reported after July, although it has not been determined whether bioluminescence is from *N. scintillans* or from other dinoflagellate species. However, bioluminescence has been considered a warning signal if it occurs in relation to toxic dinoflagellates (Le Tortorec et al. 2014). For example, *Protoceratium reticulatum*, *Gonyaulax spinifera* and *Lingulodinium polyedrum* have been confirmed to produce yessotoxins, which are responsible for diarrhetic shellfish poisoning (Paz et al. 2008, Howard et al. 2009, Cusick & Widder 2014). *Alexandrium ostenfeldii*, *A. tamarense* and *Pyrodinium bahamense* are known as saxitoxin producers that cause paralytic shellfish poisoning (Gribble et al. 2005, Landsberg et al. 2006, Hakanen et al. 2012, Le Tortorec et al. 2014, Zou et al. 2014).The presence of toxic bioluminescent dinoflagellates during the 'blue tears' season has yet to be confirmed. Therefore, regular investigations of the distribution of bioluminescent dinoflagellates would provide fundamental information to explain the source of bioluminescence along the coast.

In coastal waters, dinoflagellates are the most ubiquitous and abundant protists that exhibit bioluminescence (Haddock et al. 2010, Widder 2010). The generation of bioluminescent light in dinoflagellates, including key genes coding for the enzyme luciferase (*lcf*) and luciferase binding protein (*lbp*), is well characterized (Li et al. 1997, Li & Hastings 1998, Liu et al. 2004, Fajardo et al. 2020). The most primitive *lcf* gene has been reported in *N. scintillans*, which possesses only one catalytic domain of *lcf* and *lbp* in a single gene (Liu & Hastings 2007). In other dinoflagellate species, 3 repeated catalytic domains of the *lcf* gene and a separate *lbp* gene have been found (Li et al. 1997, Li & Hastings 1998, Liu et al. 2004, Fajardo et al. 2020). As dinoflagellate strains of normally bioluminescent species always contain the *lcf* gene, this gene has been proposed as a powerful tool for identifying bioluminescent species (Valiadi et al. 2012). Detection of the *lcf* gene has been applied as a promising tool for investigating the distribution of bioluminescent dinoflagellates (Valiadi et al. 2014, Cusick et al. 2016, Le Tortorec et al. 2016).

Therefore, the aims of this study were to evaluate whether the 'blue tears' phenomenon is caused by *N. scintillans* alone or with other dinoflagellates, and whether there are toxic bioluminescent dinoflagellates in the natural assemblages around the Matsu archipelago. Metatranscriptomes were performed to broadly explore the expression of *lcf* genes from natural assemblages. Sequencing of *lcf* genes was conducted to detect the temporal and spatial distributions of bioluminescent dinoflagellate species around the Matsu archipelago.

2. MATERIALS AND METHODS

2.1. Sample collection

Six sampling cruises in the coastal waters around Nangan Island were conducted in 2016–2017. The sampling stations were in the vicinity of Nangan Island (Fig. 1). Seawater and plankton sampling were performed from a boat during the daytime, and not all stations were visited depending on sea conditions. The seawater temperature and salinity were measured with a thermometer and a salinometer (HI98194, Hanna). The surface seawater samples were collected using a clean plastic bucket and transported to the laboratory for chlorophyll *a* (chl *a*) analysis. Chl *a* concentrations were measured using a Turner Designs Fluorimeter (10AU-005) using nonacidification extraction (Gong et al. 2000). Plankton samples for the metatranscriptome were collected using a 20 µm mesh plankton net with a 0.5 m mouth diameter. The plankton net was towed in surface water for 10 min. The collected samples were stored in a lightproof icebox and transported to the laboratory immediately. In the laboratory, plankton samples were sequentially filtered through 200 µm and 20 µm mesh screens, and then plankton collected from both screens were preserved separately with RLT buffer (Qiagen). Plankton samples for mixed community DNA isolation were collected using a 20 µm mesh plankton net with a 0.2 m mouth diameter. At each station, the net was towed in surface water for 10 min. The collected samples were fixed in 95% ethanol.

2.2. Total RNA isolation and metatranscriptome sequencing

High-throughput sequencing was performed to obtain metatranscriptomes from Stns M4 and M7 in June 2015 and Stns M7 and M8 in July 2015. The plankton samples preserved with RLT buffer were disrupted by supersonic disruption (VCX600, Sonics & Materials). Total RNA was isolated using an RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. To remove the residual genomic

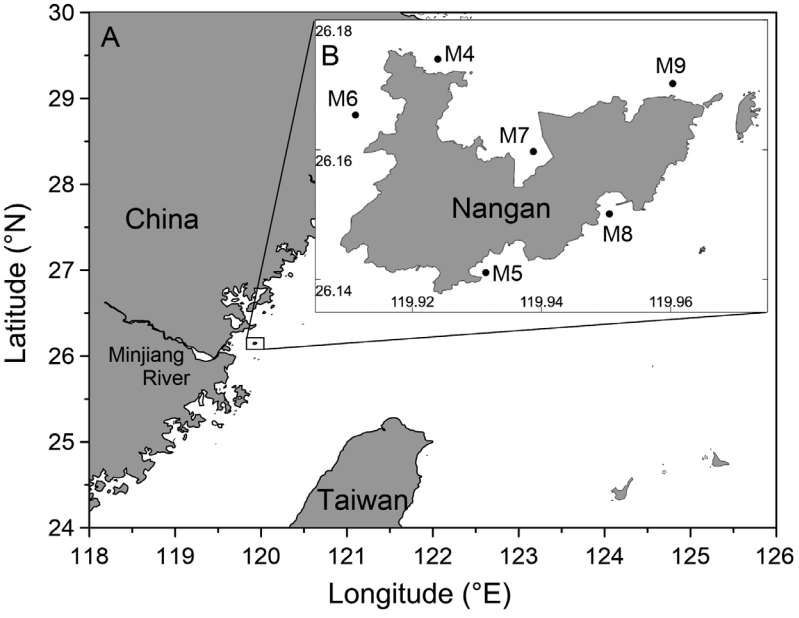

Fig. 1. (A) The location of Nangan island in the southern East China Sea. (B) Details of the sampling stations around Nangan Island

DNA, on-column digestion for 30 min with RNase-free DNaseI (Qiagen) was performed. The concentration of isolated RNA was determined by using a spectrophotometer (ND-1000, NanoDrop Technologies). An aliquot of 5 µg total RNA was taken from each sample, and mRNA was purified using oligo (dT) polyA selection. The eluted mRNA was fragmented and reverse-transcribed into cDNA. After second-strand cDNA synthesis, specific adapters were ligated to both ends, and these cDNA fragments were ready for bridge PCR amplification (Illumina). Transcriptome sequencing was performed by the NGS Core Lab (Academia Sinica, Taipei, Taiwan) using an Illumina MiSeq system. Raw sequence data have been deposited in the NCBI Sequence Read Archive under accession number PRJNA750338.

2.3. Genomic DNA extraction and *lcf* gene sequencing

Genomic DNA was extracted according to a modified phenol-chloroform method with cetyltrimethylammonium bromide (Clark 1992). Ethanol-fixed samples were transferred to Tris-EDTA (TE) buffer before DNA extraction. The concentration of extracted DNA was determined using a NanoDrop spectrophotometer (ND-1000). In addition, partial ethanol-fixed samples were used for single-cell PCR following a previously published method (Kang et al. 2011). Dinoflagellate cells were isolated using an inverted microscope (IX51, Olympus) equipped with a microinjector (IM-9B, Narishige). Single-cell DNA was extracted following the enzymatic DNA extraction method (Ki et al. 2004). To amplify the catalytic domains of the *lcf* gene, nested PCR was developed. In the first round of amplification, a newly designed primer pair DNlcfF3 (5′-CCN TGY GGC CCN CTN CCN TGG CC-3′)/DNlcfR4 (5′-CCN GAC TCC ATC TCC CAR AAR AAN CC-3′) based on the alignment of known dinoflagellate *lcf* catalytic domain sequences from GenBank was used to generate a predicted 395 bp amplicon. In the second round of amplification, universal primers DinolcfF4 (5′-CGG CTA CGT GCC CAA RAC NAA YCC-3′) and DinolcfR2 (5′-CAC CAG GGG CTC GTA RAA RTA RTG-3′) were used according to the protocol described by Valiadi et al. (2012), and a predicted 270 bp amplicon was amplified. The PCR products were purified from agarose gels and then cloned into pGEMT vectors (Promega). The plasmids were transformed into competent cells of *Escherichia coli* (DH5α) by the heat shock method. To screen the positive clones, more than 25 randomly chosen white colonies were checked by colony PCR with primers DinolcfF4 and DinolcfR2. Finally, the positive plasmidial DNA was purified, and Sanger sequencing of the cloned DNA fragments was performed by Genomics (Taipei, Taiwan).

2.4. Sequence analysis and taxonomic assignation

For each sample, approximately 20 clones were sequenced. The obtained sequences were trimmed to remove sequences of vector and primers and checked using the BLASTn tool of the NCBI (http://blast.ncbi.nlm.gov/Blast.cgi). For a more reliable taxonomic assignment of these *lcf* fragments, a reference database including the long *lcf* sequences that can be split into the 3 catalytic domains was collected from GenBank and the MMETSP database. Alignments were performed using ClustalW. The phylogenetic tree was constructed using the maximum likelihood method bootstrapped at 100 replications using MEGA 7.0 software (www.megasoftware.net/). According to the phylogenetic analysis, sequences grouped under the same supported clade and sharing over 95% identity were combined as an opera-

tional taxonomic unit (OTU), and the most conserved sequence within the same clade was chosen as the representative sequence. The representative sequences were deposited in GenBank under accession numbers MZ645793–MZ645833.

2.5. Statistical analyses

A spatio-temporal clustering analysis of *lcf* compositions around Matsu Island was performed using the PRIMER 7 program (Clarke & Gorley 2015). The Bray-Curtis similarity matrices were computed using the OTU proportional abundances from each station. A square-root transformation was applied to the OTU proportional abundances before construction of the similarity matrices. A hierarchical cluster analysis was performed using the group average linkage method, and the significance of the cluster groups was determined using the similarity profile test (SIMPROF, $p < 0.05$) (Clarke et al. 2008).

3. RESULTS

3.1. Hydrographic conditions around Nangan Island

In this study, field samples were collected during 6 cruises from June 2016 to July 2017 (Table 1). Higher surface water temperatures of 29.5 to 29.9°C were observed in August 2016. Lower salinities ranging from 25.4 to 29.3 psu in May were a result of high discharge from the Minjiang River (Fig. 1) during the monsoon season (Yu & Chen 2012). The surface water chl *a* concentrations varied between 0.87 and 5.81 mg m^{-3} during the investigation period.

3.2. Metatranscriptomes for the expression of *lcf* genes

To investigate the bioluminescent dinoflagellates through the expression of *lcf* genes in metatranscriptomes, MiSeq sequencing was performed using field samples collected in June and July 2016. Eight transcriptomes were constructed, and the sequencing depth of each metatranscriptome ranged from 3.0 to 3.6 million raw reads (Table 2). Using BLAST with known dinoflagellate *lcf* genes from GenBank, a total of 8636 reads were assigned to dinoflagellates. *Alexandrium*, *Protoceratium*, *Pyrocystis* and *Noctiluca* were the common genera found in each metatranscriptome. *Noctiluca* was the genus with the most abundant reads. Assembling all of the *Noctiluca* reads, nearly the full *lcf* gene, including the complete *lbp* sequence, was obtained (MZ645834). These results indicated that not only *Noctiluca* but also other bioluminescent dinoflagellates existed around the Matsu archipelago. As most of these genera could be detected by universal *lcf* primers (Valiadi et al. 2012), these primers were used for routine investigation of the potential bioluminescent dinoflagellates around the Matsu archipelago.

3.3. Dinoflagellate *lcf* genes

Universal *lcf* primers targeting the conserved region of the luciferase catalytic domains were used to amplify an approximately 270-bp fragment. Approximately 20 *lcf* clones were sequenced from each of the 25 samples, resulting in a total of 400 clones with confirmed sequences. To distinguish the 3 catalytic domains, the bioluminescent dinoflagellate species with long *lcf* sequences, which can be divided into 3 separate domains (D1, D2 and D3), were aligned in our reference database (Fig. 2). In addition, 2 assembled *lcf* contigs from *Ceratium fusus*, a well-known bioluminescent species (Sullivan & Swift 1994, Swift et al. 1995), were also included from the MMETSP transcriptomes (Keeling et al. 2014). As the *N. scintillans lcf* gene contains only one catalytic domain and has been considered the most primitive luciferase among the sequenced dinoflagellate *lcf* genes (Widder 2010), its sequence was used as an outgroup in the phylogenetic analysis. All 3 catalytic do-

Table 1. Surface water characteristics among the sampling stations in Matsu during 2016–2017. –: water samples for temperature and salinity measurements were not taken

	Jun 2016	Jul 2016	Aug 2016	Sep 2016	May 2017	Jul 2017
Temperature (°C)	25.6–26.1	26.6–27.9	29.5–29.9	27.0–27.2	–	27.0–28.2
Salinity (psu)	25.4–29.3	31.8–32.4	31.7–32.5	28.8–30.0	–	31.3–32.8
Chl *a* (mg m^{-3})	1.55–3.57	1.03–3.11	1.09–3.03	0.87–1.37	1.35–2.47	0.90–5.81

Table 2. Read counts of the luciferase gene from 8 metatranscriptomes

	June 2016				July 2016			
Station:	M7	M7	M4	M4	M7	M7	M8	M8
Size fraction:	>200 µm	20–200 µm	>200 µm	20–200 µm	>200 µm	20–200 µm	>200 µm	20–200 µm
Total reads:	3 285 932	3 058 911	3 451 414	3 197 510	3 340 330	3 595 148	3 232 481	3 665 125
Order Noctilucales								
Noctiluca	88	9	179	29	2284	1339	1305	2966
Order Gonyaulacales								
Pyrocystis	10	10	12	66	2	5	5	1
Ceratocorys	0	0	1	0	0	0	0	0
Pyrodinium	2	2	1	20	0	1	3	0
Alexandrium	3	17	11	62	3	5	22	10
Protoceratium	1	8	8	23	2	9	23	14
Lingulodinium	1	1	0	1	0	0	0	0
Gonyaulax	1	2	0	2	0	1	2	1
Ceratium	0	1	1	2	26	25	0	0
Fragilidium	0	0	0	1	0	1	0	0
Order Peridiniales								
Protoperidinium	0	0	0	2	0	1	3	0

mains of *L. polyedra* were grouped under the same clade (Fig. 2). However, sequences of each catalytic domain from *Pyrocystis* species tended to group in individual clusters of D1, D2 and D3 with bootstrap support. Similarly, *Alexandrium*/*Protoceratium* tended to form 3 individual catalytic domain clusters with bootstrap support. Interestingly, the *lcf* catalytic domains of D1 and D2 in *C. fusus* were also clustered with *Alexandrium*/*Protoceratium* D1 and D2, respectively, but D3 of *C. fusus* formed an independent clade (Fig. 2). *Lcf* genes from field samples were aligned with the reference dataset, and sequences sharing more than 95% identity were combined as an OTU with the most conserved sequence as a representative. A total of 41 unique OTUs was obtained. The OTU numbers were assigned according to the order of total read abundance, where MZlcfOTU01 had the most reads. Overall, the most abundant OTU was affiliated with *N. scintillans*.

3.4. Temporal and spatial distribution of dinoflagellate *lcf* genes

The hierarchical cluster analysis of dinoflagellate *lcf* compositions within the stations during 5 cruises generated 4 significant groups (SIMPROF, $p < 0.05$; Fig. 3A). Most samples from the same cruise were grouped together, indicating that there was no obvious spatial distribution around Nangan Island. Comparing the composition of total *lcf* clones from each cruise, higher proportions of *Noctiluca* (MZlcfOTU01) and *Protoceratium* (MZlcfOTU02) clones were found in July 2016 (Fig. 3, Group B). Then, the proportion of *Noctiluca* clones decreased dramatically and was replaced by the other dinoflagellate species in August and September 2016 (Fig. 3, Groups C and D). In May and July of the next year, *Noctiluca* dominated as the major component, consistent with the observation of a higher cell abundance of *Noctiluca* from April to July around the Matsu archipelago (Tsai et al. 2018).

4. DISCUSSION

Metatranscriptomic approaches are promising techniques for characterizing the functions of ocean microbial communities (Gilbert et al. 2008, Gifford et al. 2011, Helbling et al. 2012, Aguiar-Pulido et al. 2016, Salazar et al. 2019). In the present study, our results demonstrated the expression of *lcf* genes from various bioluminescent dinoflagellates around the Matsu archipelago. With sufficient sequencing depth, identifying all of the bioluminescent dinoflagellates in the collected samples is possible. However, during the analysis, due to the limited number of known species, we might underestimate the species that have not been established in the database. On the other hand, although a large number of short gene transcripts were generated by next-generation sequencing, chimeras could be generated during the assembly of the sequence from the metatranscriptome, especially the highly conserved regions of *lcf*

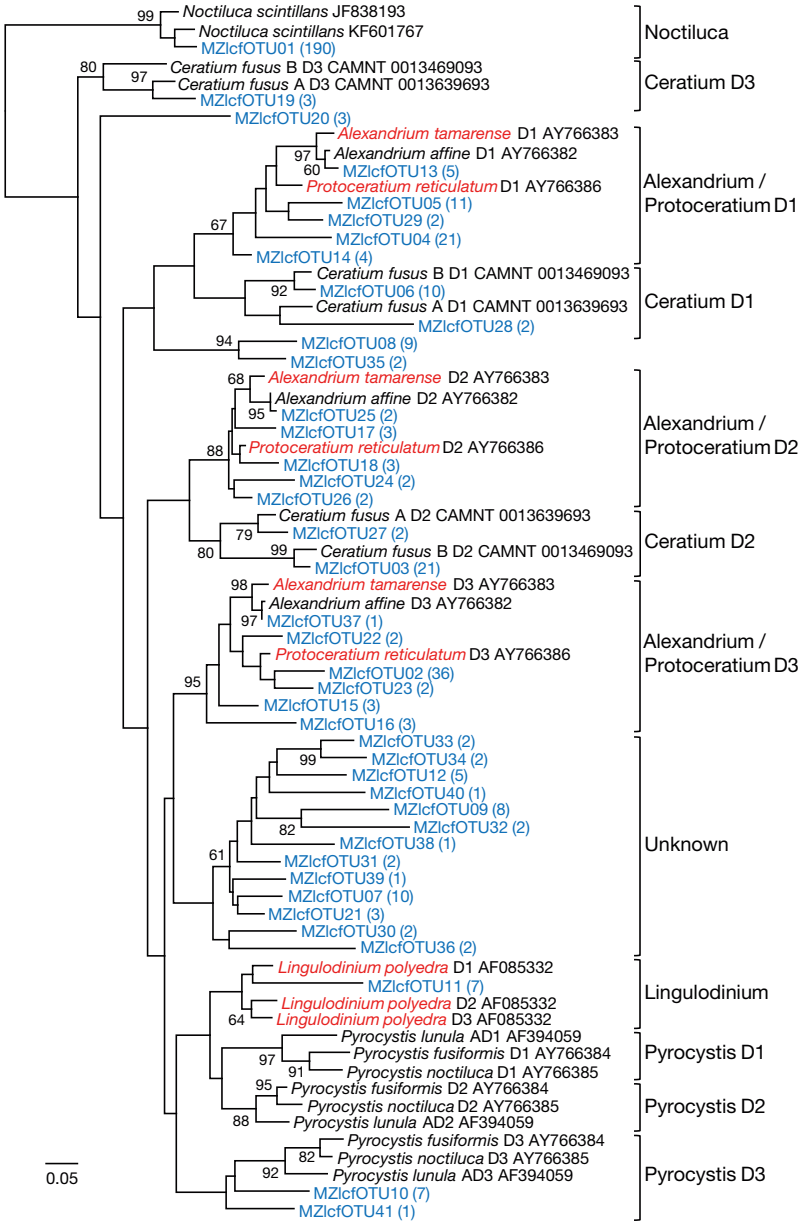

Fig. 2. Phylogenetic tree based on the *lcf* catalytic domains from dinoflagellates. A total of 270 bp aligned positions were analyzed by the maximum likelihood method with 100 bootstrap resamplings. Only significant values (>60%) are shown at internal nodes. The known sequences obtained from GenBank or MMETSP are shown in black type, and the sequences from toxic species are shown in red type. The GenBank accession numbers are shown after the species name, and the sequences from MMETSP are followed by the CAMNT number. Sequences obtained in this study are shown in blue type. The number of sequencing clones grouped to each OTU is shown in parentheses. Scale bar: mean number of nucleotide substitutions per site

catalytic domains among closely related species. These results show that the major problem for the application of metatranscriptomes to ocean eukaryotic communities is still limited by the database of known species.

Comparing the sequences of a functional gene among related species provides clues for the evolution of their functional roles and can also be useful for distinguishing the species. The complete sequences of the *lcf* gene in various dinoflagellates not only would provide information for functional and evolutionary approaches but also could be utilized for taxonomic identification (Baker et al. 2008, Valiadi et al. 2014, Fajardo et al. 2020). In this study, the use of universal primers amplifying all 3 *lcf* catalytic domains increased the credibility for taxonomic identification. For example, all 3 *lcf* catalytic domains of *A. affine* were found to have significant bootstrap support (OTU51, OTU10 and OTU04). With the MMETSP transcriptome data, all catalytic domains of the *lcf* gene in *C. fusus* were also found to have significant bootstrap support (OTU20, OTU15 and OTU16). These results strongly supported that *A. affine* and *C. fusus* should be the bioluminescent dinoflagellate species in Matsu. However, an unknown clade containing 15 OTUs was found to not match any *lcf* gene of known species. This clade was grouped with *Alexandrium*/*Protoceratium* clades without bootstrap support in our phylogeny. The placement of this clade in dinoflagellates remains to be further confirmed. We utilized single-cell PCR to amplify multiple genes (Kang et al. 2011) to identify the unknown clade; however, this strategy is useful only for species with clear morphological characteristics, such as *C. fusus* (data not shown). As there was no clear clue for these unknown clades, single-cell PCR might be combined with other sequence labeling methods, such as fluorescence *in situ* hybridization, to target potential cells (Hosoi-Tanabe & Sako 2005, Piwosz et al. 2021).

In this study, our results demonstrated a temporal transition of bioluminescent dinoflagellates around Nangan Island. According to the *lcf* data, *N. scintillans* was the dominant bioluminescent species between May and July, and it was replaced by other

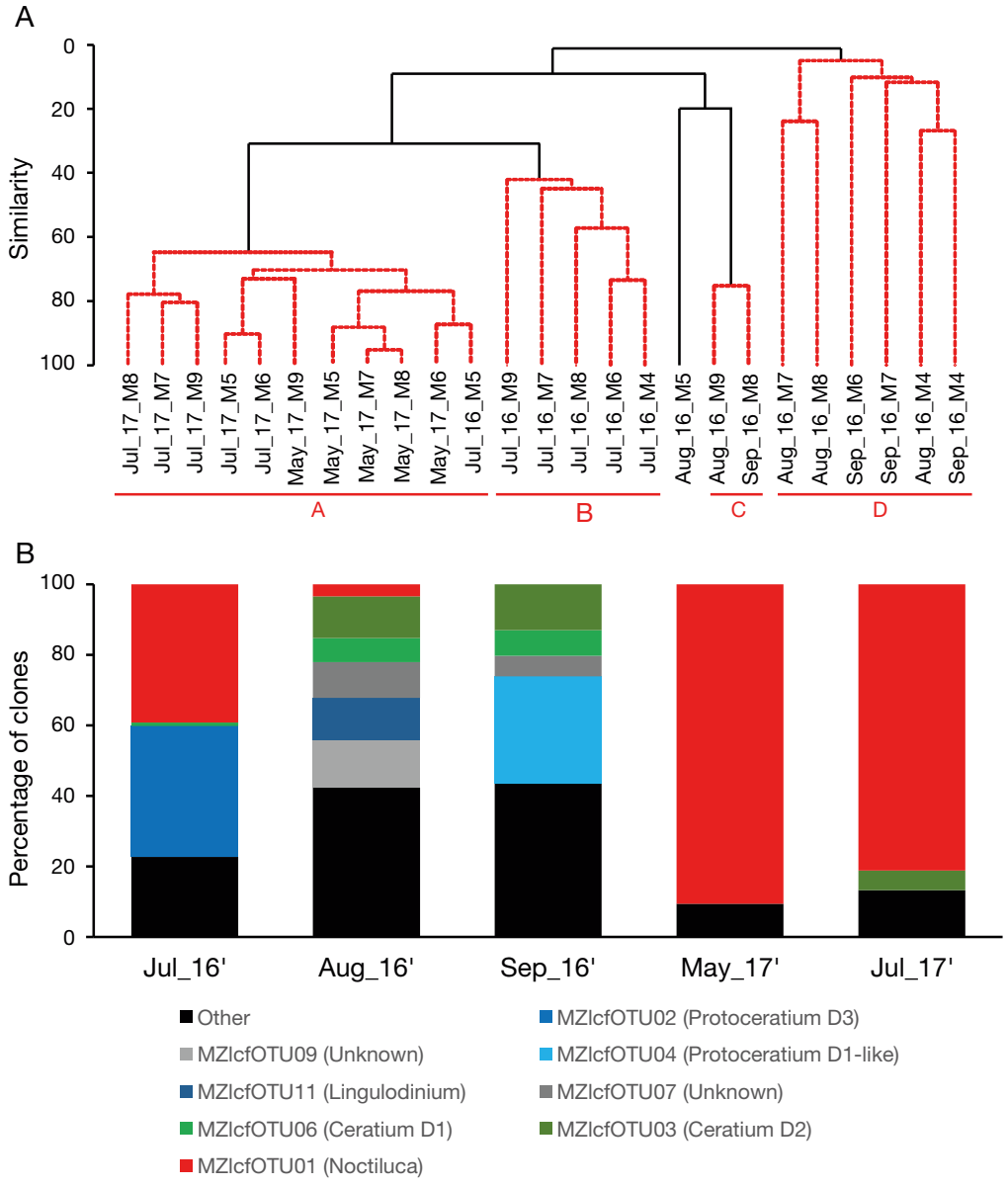

Fig. 3. Comparison of *lcf* clone composition among sampling locations and cruises. (A) Hierarchical cluster analysis of the correlation among stations and cruises based on sequencing clones of *lcf* genes. Red dashed branches indicate no significant differences in grouping by SIMPROF ($p < 0.05$). (B) Percentage abundance of the major *lcf* OTUs among cruises

bioluminescent dinoflagellates in late summer (August to September). The dominance of *Noctiluca* mainly occurred during the flood season from April to June (Tsai et al. 2018). After the flood season in late summer, *Noctiluca* disappeared. Our results indicated that other bioluminescent dinoflagellates, such as *C. fusus*, existed around Matsu. Among these dinoflagellates, 4 OTUs (MZIcfOTU02, 11, 18 and 23) were grouped close to the toxic species, including *L. polyedrum*, *P. reticulatum* and *Alexandrium tamarense*, with weak bootstrap support (<60%). As these OTUs shared 93% fewer identities with the toxic clades, they could not be regarded as toxic species. Interestingly, MZIcfOTU11 was affiliated with *Lingulodinium* with weak bootstrap support, and these sequences shared an average of 98% identity to an environmental sequence group (Lp-like Group G3), which dominated the *lcf* sequencing retrieved from the Patagonian Shelf (Valiadi et al. 2014). Although our results indicate the existence of other bioluminescent dinoflagellates around the Matsu archipelago, the current methods used in this study

are not suitable for quantitative work. Quantitative analysis is still needed to determine the precise abundance of these species to explain the relationship with the phenomenon of 'blue tears' in the summer season.

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