Research Article

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Bioluminescence capability and intensity in the dinoflagellate *Alexandrium* species

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Some species in the dinoflagellate genus *Alexandrium* are bioluminescent. Of the 33 formally described *Alexandrium* species, the bioluminescence capability of only nine species have been tested, and eight have been reported to be bioluminescent. The present study investigated the bioluminescence capability of seven *Alexandrium* species that had not been tested. *Alexandrium mediterraneum*, *A. pohangense*, and *A. tamutum* were bioluminescent, but *A. andersonii*, *A. hiranoi*, *A. insuetum*, and *A. pseudogonyaulax* were not. We also measured the bioluminescent intensity of *A. affine*, *A. fraterculus*, *A. mediterraneum*, *A. ostenfeldii*, *A. pacificum*, *A. pohangense*, *A. tamarense*, and *A. tamutum*. The mean 200-second-integrated bioluminescence intensity per cell ranged from 0.02 to 32.2 × 10^4 relative luminescence unit per cell (RLU $cell^{-1}$), and the mean maximum bioluminescence intensity per cell per second (BL_{Max}) ranged from 0.01 to 10.3 × 10^4 RLU $cell^{-1}$ s^{-1}. BL_{Max} was significantly correlated with the maximum growth rates of *Alexandrium* species, except for *A. tamarense*. A phylogenetic tree based on large subunit ribosomal DNA (LSU rDNA) showed that the bioluminescent species *A. affine*, *A. catenella*, *A. fraterculus*, *A. mediterraneum*, *A. pacificum*, and *A. tamarense* formed a large clade. However, the toxicity or mixotrophic capability of these species was split. Thus, their bioluminescence capability in this clade was more consistent than their toxicity or mixotrophic capability. Phylogenetic trees based on LSU rDNA and the luciferase gene of *Alexandrium* were consistent except for *A. pohangense*. The results of the present study can provide a basis for understanding the interspecific diversity in bioluminescence of *Alexandrium*.

Key Words: harmful algal bloom; luciferase gene; luminescence; protist; red tide

INTRODUCTION

Bioluminescence, the production of light by living organisms, is a widespread phenomenon in nature (Haddock et al. 2010, Valiadi and Iglesias-Rodriguez 2013). Light is produced when luciferase (enzyme) binding with luciferin causes an oxidation reaction of luciferin (substrate) (McCapra 1976, Wilson and Hastings 2013). A majority of the bioluminescent organisms reside in the ocean (Shimomura 2006, Widder 2010).

Dinoflagellates are ubiquitous protists and one of the most common bioluminescent producers (Haddock et al. 2010, Le Tortorec et al. 2016, Kang et al. 2019, Cusick and Widder 2020, Jang and Jeong 2020, Lee et al. 2020).

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In dinoflagellates, bioluminescence is a defense mechanism against predation and helps attract secondary predators (a function sometimes called a burglar alarm) (Esaias and Curl 1972, Haddock et al. 2010, Valiadi and Iglesias-Rodriguez 2013, Lindström et al. 2017). Several species of bioluminescent dinoflagellates form red tides, dense algal blooms (Valiadi et al. 2012, Cusick and Widder 2014, Jeong et al. 2015); due to their brightness, fish, mammals, and ships passing red-tide patches can be detected at night (Cram and Schülein 1974, Rohr et al. 1998, Miller et al. 2005). Thus, dinoflagellate bioluminescence has been studied in terms of ecophysiology and military uses. However, the bioluminescent capability of dinoflagellates has been tested in only a small portion of 3,450 formally described dinoflagellate species (e.g., Sweeney 1963, Kelly 1968, Swift et al. 1995, Latz and Jeong 1996, Jeong et al. 2021). Among the tested dinoflagellate species, only 70 (<3% of 3,450 formally described dinoflagellates) have been reported to be bioluminescent (Marcinko et al. 2013, Cusick and Widder 2014, 2020). Furthermore, the bioluminescence intensity of only 14 species (<1% of 3,450 formally described dinoflagellates) has been reported, including that of *Alexandrium tamarense*, *A. catenella*, *Ceratocorys horrida*, *Lingulodinium polyedra*, *Noctiluca scintillans*, *Pyrodinium bahamense*, *Protoperidinium* spp., *Pyrocystis* spp., and *Tripos* spp. (Seliger et al. 1969, Esaias and Curl 1972, Esaias et al. 1973, Swift et al. 1973, White 1979, Widder and Case 1981, Lapota et al. 1989, Latz and Lee 1995, Sullivan and Swift 1995, Latz et al. 2004, Cussatlegras and Le Gal 2007, Eckert 2015). Thus, the bioluminescent capability and intensity of more dinoflagellate species should be explored.

The genus *Alexandrium* is widely distributed across coastal waters and is a major organism causing red tides or harmful algal blooms (Anderson et al. 2012, Jeong et al. 2017). However, among the 33 known *Alexandrium* species (Guiry and Guiry 2021), only nine species have been tested for the bioluminescence capability (Esaias and Curl 1972, Esaias et al. 1973, Liu et al. 2004, Baker et al. 2008, Latz et al. 2008, Kremp et al. 2009, Martínez et al. 2016). Furthermore, bioluminescence intensity has been reported in only three *Alexandrium* species (Esaias and Curl 1972, White 1979), and it widely ranged from 2×10^4 to 7×10^7 photons per cell. As the bioluminescence intensity of *Alexandrium* spp. may be species-dependent, it should be measured in more *Alexandrium* species. In addition, bioluminescence intensity should be assessed using the same technique across species, in order to reveal the potential factors that affect the bioluminescence intensity.

In the present study, the bioluminescence capability of seven *Alexandrium* species (*A. andersonii*, *A. hiranoi*, *A. insuetum*, *A. mediterraneum*, *A. pohangense*, *A. pseudogonyaulax*, and *A. tamutum*) was investigated. The bioluminescence intensity of eight *Alexandrium* species (*A. affine*, *A. fraterculus*, *A. mediterraneum*, *A. ostenfeldii*, *A. pacificum*, *A. pohangense*, *A. tamarense*, and *A. tamutum*) was also explored. Moreover, the effects of cell size, mixotrophic ability, toxicity, maximum swimming speed, and maximum growth rate on bioluminescence intensity were investigated in eight *Alexandrium* species. In addition, the sequence of the luciferase gene (*lcf*) was analyzed in *A. fraterculus*, *A. mediterraneum*, *A. pohangense*, and *A. tamutum*, and the phylogenetic trees based on the *lcf* and large subunit ribosomal DNA (LSU rDNA) of *Alexandrium* spp. were compared. The results of the present study can provide a basis for understanding the interspecific diversity in the bioluminescence capability and intensity of *Alexandrium*.

MATERIALS AND METHODS

Cultures of experimental organisms

Cells of *A. affine*, *A. fraterculus*, *A. mediterraneum*, *A. pacificum*, *A. pohangense*, and *A. tamutum* were isolated from Korean coastal waters and then established using two serial single-cell isolations (Table 1). Cultures of the other six *Alexandrium* species (*A. andersonii*, *A. minutum*, *A. insuetum*, *A. ostenfeldii*, *A. tamarense*, and *A. hiranoi*) used in the present study were obtained from the National Center for Marine Algae and Microbiota (NCMA; USA), Roscoff Culture Collection (RCC; France), and the Microbial Culture Collection at the National Institute for Environmental Studies (NIES Collection, Japan). All experimental cultures were maintained at 20°C in a chamber under cool white fluorescent lights (20 µmol photons m^{-2} s^{-1}) on a 14 : 10 h light / dark cycle. The culture of *A. pohangense* was grown mixotrophically by feeding on *Margalefidinium polykrikoides*. Other cultures were grown autotrophically in F/2 (Guillard and Ryther 1962) or L1 (Guillard and Hargraves 1993) seawater medium without silicate (Table 1).

Bioluminescence capability

The bioluminescence capability of each *Alexandrium* species was tested when the cultures reached their late exponential to early stationary phases. Cultures of

the seven *Alexandrium* species were mechanically and chemically stimulated (Table 1). For mechanical stimulation, dense *Alexandrium* cultures were placed in the dark for 3 h and then shaken by hand in a dark room. During mechanical stimulation, bioluminescence images were captured using a Canon EOS R with a Canon RF 50 mm F1.2L lens (Canon, Tokyo, Japan) at aperture F1.2, shutter speed 30 s, ISO4000.

If mechanically stimulated bioluminescence was not visible using the digital camera, chemical stimulation was conducted. For the chemical stimulation, four 200 µL aliquots of dense cultures of each *Alexandrium* species were placed in four wells of Corning 96-well white plates (Corning Life Sciences, Amsterdam, Netherlands). Four culture media without *Alexandrium* cells were also placed in four wells as controls, and analysis of these wells confirmed no bioluminescence. The bioluminescence analysis of each *Alexandrium* species was performed 3 h after the beginning of the dark phase, when the cells are known to be fully dark-adapted and produce highly stimulated bioluminescence (Biggley et al. 1969, Krasnow et al. 1980). Chemically stimulated bioluminescence was measured after the addition of 50 µL of 1 M acetic acid to the well (Hastings and Sweeney 1957, Fogel and Hastings 1972, Sweeney 1986). Acidification by the addition of acetic acid, which reduces the intracellular pH to <6–7, is known to bind the luciferase and luciferin of bioluminescent species (Fogel and Hastings 1972, Sweeney 1986, Von Dassow and Latz 2002). The bioluminescence response was assayed for 200 s using a GloMax Navigator microplate luminometer (Promega, Madison, WI, USA).

Measurement of bioluminescence intensity

To measure the bioluminescence intensity of eight *Alexandrium* species, triplicate 1 mL aliquots were removed from a culture of each *Alexandrium* species and then fixed with 5% acid Lugol's solution, and *Alexandrium* cells were enumerated under a light microscope to determine the density of each culture. Cells were added to a 50 mL culture flask to reach the target cell density of 1,000 cells mL^{-1}, using media in which the cells were grown. A 200 µL aliquot of diluted culture was placed in a well in a Corning 96-well white plate (Corning Life Sciences), with four replicates. The analysis of bioluminescence was conducted using the method described above.

Bioluminescence data analysis

To obtain the bioluminescence intensity per cell per second of each *Alexandrium* species, the mean value obtained from the four control wells (containing seawater without bioluminescent *Alexandrium* cells) was subtracted from the bioluminescence intensity value of the cells in one well, and then the value was divided by the total number of cells in the well. Using the data on bioluminescence intensity per cell per second of each *Alexandrium* species, several parameters, such as the integrated

Table 1. Information on the isolation and maintenance of the experimental organisms and also *Alexandrium* species in which bioluminescence capability was previously tested or newly tested

Organisms	Strain name	Location	Date	T	S	Media / Prey	Biolu
Alexandrium andersonii	CCMP2222	Gulf of Naples, Italy	Aug 1, 1996	-	-	L1	NewBL
A. minutum	CCMP1888	Laguna Obidos, Portugal	-	-	-	L1	PNB
A. tamutum	ATSH1609	Shiwha, Korea	Sep 9, 2016	25.9	32.6	F/2	NewBL
A. insuetum	CCMP2082	Uchiumi Bay, Japan	Jun 6, 1985	-	-	F/2	NewBL
A. ostenfeldii	NIES-4274	Lake Koyama, Japan	Nov 29, 2016	-	-	F/2	PB
A. pacificum	KSUDinoE4	Kunsan, Korea	Sep 2020	23.5	29.2	F/2	PB
A. affine	AATA1308	Taean, Korea	Aug 14, 2013	21.5	32.2	F/2	PB
A. pohangense	APPH1409	Pohang, Korea	Sep 2014	23.3	31.1	Mp	NewBL
A. fraterculus	AFYS1309	Yeosu, Korea	Sep 6, 2013	23.4	32.8	L1	PB
A. mediterraneum	AMYS1807	Yeosu, Korea	Jul 2018	24.2	30.0	F/2	NewBL
A. tamarense	CCMP115	English Channel, UK	Jun 24, 1957	-	-	F/2	PB
A. pseudogonyaulax	KSUDinoB4	Kunsan, Korea	Sep 2020	23.5	29.2	F/2	NewBL
A. hiranoi	NIES-3611	Muroto Cape, Japan	Apr 29, 2008	-	-	F/2	NewBL
Protoceratium reticulatum	RCC4104	Pacific Ocean, Japan	Aug 30, 2013	-	-	L1	PB
Lingulodinium polyedra	CCMP1931	La Jolla, California, USA	May 1998	-	-	L1	PB

Culture information was obtained from the National Center for Marine Algae and Microbiota (NCMA; USA), Roscoff Culture Collection (RCC; France), and Microbial Culture Collection at the National Institute for Environmental Studies (NIES; Japan).
T, temperature (°C); S, salinity; NewBL, newly tested for bioluminescence in the present study; PNB, previously known to be not bioluminescent; PB, previously known to be bioluminescent; Mp, *Margalefidinium polykrikoides*.

bioluminescence intensity per cell for 200 s and the maximum bioluminescence intensity per cell per second, were calculated as described by Latz and Lee (1995). The mean 200-second-integrated bioluminescence intensity per cell ($BL_{Int200s}$) was calculated by averaging the sums of the bioluminescence intensity per cell per 200 s in four wells. Furthermore, the mean maximum bioluminescence intensity per cell per second (BL_{Max}) was calculated by averaging the highest bioluminescence intensities per cell per second in the four wells. In the present study, relative luminescence unit per cell (RLU $cell^{-1}$) and RLU per cell per second (RLU $cell^{-1}$ s^{-1}) were used as the units of $BL_{Int200s}$ and BL_{Max}, respectively (Lindström et al. 2017).

Sequencing large subunit ribosomal DNA and *lcf*

To obtain the LSU rDNA and *lcf* sequences, a 10 mL aliquot of each dense culture was harvested at 3,667 g (4,000 rpm) for 10 min in a 15 mL conical tube. The gDNA was extracted from the pellet using a Wizard SV Genomic DNA Purification System (Promega).

The amplification reaction mixtures (50 µL in total volume) were as follows: 5 µL of 10× F-StarTaq buffer, 1 µL of 10 mM of dNTP mix, 0.25 µL of 5 U $µL^{-1}$ BioFACT F-Star Taq DNA polymerase (BioFACT Co., Ltd., Daejeon, Korea), 2 µL of 10 µM of each primer, and 1 µL of the extracted gDNA. The primers used to amplify regions of LSU rDNA were D1R (5-ACCCGCTGAATTTAAGCATA-3) (Scholin et al. 1994), 28–1483R (5-GCTACTACCACCAAGATCTGC-3) (Daugbjerg et al. 2000), and LSUB (5-ACGAACGATTTGCACGTCAG-3) (Litaker et al. 2003). The gDNA was amplified in an AllInOneCycler (Bioneer, Daejeon, Korea) under the following conditions: 2 min at 95°C for initial denaturation, 38 cycles of 40 s at 95°C, 30 s at the annealing temperature, and 1 min at 72°C, with a final extension of 5 min at 72°C. The universal *lcf* primers for amplifying the *lcf* of dinoflagellates were used: *Lcf*UniCHF3 (5′-TCCAGGTTGCACGCTTCGA-3′) (Baker et al. 2008) and *Lcf*UniCHR4 (5′-GGGTCTTGTCGCCGTACTCAAA-3′) (Baker et al. 2008). These primer sets (*Lcf*CHF3 and *Lcf*CHR4) targeted the *lcf* N-terminal region, which is more diverse than the central regions (Baker et al. 2008). Polymerase chain reactions (PCRs) were performed under the following conditions: 5 min at 95°C for initial denaturation, 35 cycles of 45 s at 95°C, 30 s at 62°C and 45 s at 68°C, and a final extension at 68°C for 5 min.

The PCR products were purified using an AccuPrep DNA Purification Kit (Bioneer), and sequencing was performed using an ABI 3730XL DNA Analyzer (Applied Biosystems, Foster City, CA, USA).

Phylogenetic analysis

To explore the phylogenetic relationships of the bioluminescent *Alexandrium* species, revealed in the present study or in previous studies, the sequences of the LSU rDNA region of the *Alexandrium* species were aligned using the software MEGA v4 (Tamura et al. 2007), including sequences obtained from GenBank. Maximum likelihood (ML) analysis of the region was conducted using the program RAxML 7.0.3, with the general tIme reversible plus gamma (GTR + GAMMA) model (Stamatakis 2006). Furthermore, 200 independent tree inferences were used to identify the best tree. The ML bootstrap values were determined using 1,000 replicates.

To investigate any differences in the phylogenetic trees based on the LSU rDNA region and *lcf* of the individual *Alexandrium* species, the *lcf* sequences of the six *Alexandrium* species obtained in the present study and the five *Alexandrium* species available in GenBank were aligned. *Protoceratium reticulatum* was added because it formed a clade with *A. pohangense*, and *Gonyaulax spinifera* was added because it was positioned as a base of an *Alexandrium* clade. *Lingulodinium polyedra* was added as an outgroup. ML analysis of the region was conducted as described previously.

Bayesian analysis was conducted using MrBayes v.3.1 (Huelsenbeck and Ronquist 2001, Ronquist and Huelsenbeck 2003) with the default GTR + G + I model to determine the best available model for the data. For all sequence regions, four independent Markov chain Monte Carlo runs were performed simultaneously until the average standard deviation of split frequencies reached <0.01. The trees were sampled every 1,000 generations. To ensure likelihood convergence, the first 1,000 trees were discarded as burn-ins. Five million generations of LSU rDNA and three million generations of the *lcf* of *Alexandrium* species were run in MrBayes, and at least two million generations were saved for each tree reconstruction. The parameters were as follows for assumed nucleotide frequencies of LSU rDNA: substitution rate matrix with A–C substitution = 0.106, A–G substitution = 0.236, A–T substitution = 0.103, C–G substitution = 0.043, C–T substitution = 0.416, G–T substitution = 0.095; the proportion of sites assumed to be invariable = 0.051; and the rate for variable sites assumed to follow a gamma distribution with shape parameter = 1.075. For the assumed nucleotide frequencies of the *lcf*, parameters were: substitution rate matrix with A–C substitution = 0.090, A–G substitution = 0.255, A–T substitution = 0.071, C–G substitution = 0.074, C–T substitution = 0.424, and G–T sub-

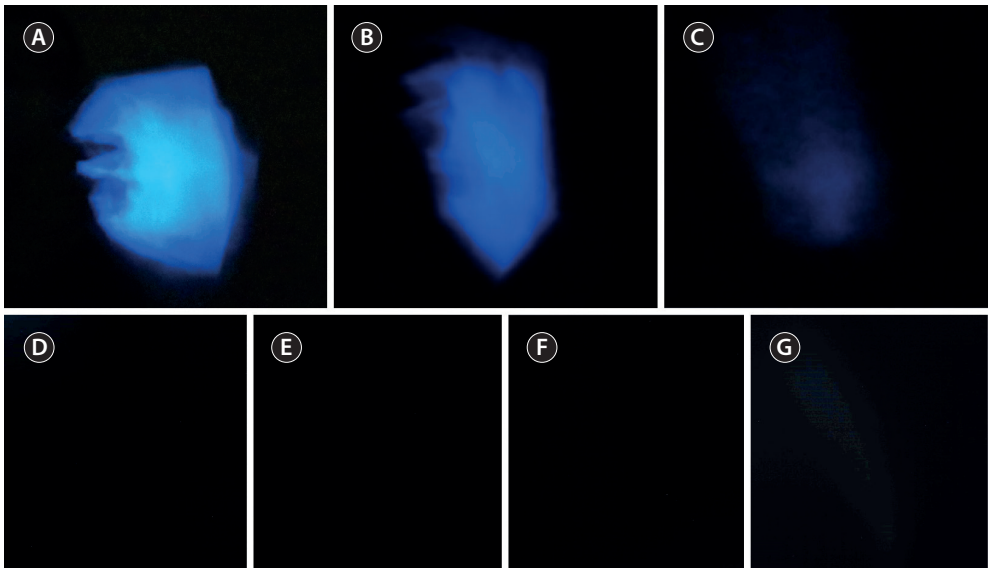

Fig. 1. Photographs of bioluminescence showing the bioluminescent capability of the *Alexandrium* species tested in the present study. A 250 mL culture flask containing a dense culture of each species in the late exponential stage was shaken by hand. (A) *Alexandrium mediterraneum* AMYS1807 (30,000 cells mL^{-1}). (B) *Alexandrium pohangense* APPH1409 (2,000 cells mL^{-1}). (C) *Alexandrium tamutum* ATSH1609 (10,000 cells mL^{-1}). (D) *Alexandrium andersonii* CCMP2222 (7,000 cells mL^{-1}). (E) *Alexandrium hiranoi* NIES-3611 (1,000 cells mL^{-1}). (F) *Alexandrium pseudogonyaulax* KSUDinoB4 (1,000 cells mL^{-1}). (G) *Alexandrium insuetum* CCMP2082 (30,000 cells mL^{-1}).

stitution = 0.086; the proportion of sites assumed to be invariable = 0.391; and the rate for variable sites assumed to follow a gamma distribution with shape parameter = 54.594. Moreover, the assumed nucleotide frequencies of LSU rDNA comprised the sequences of the *Alexandrium* species belonging to the *lcf* phylogeny, substitution rate matrix with A–C substitution = 0.120, A–G substitution = 0.228, A–T substitution = 0.089, C–G substitution = 0.066, C–T substitution = 0.395, and G–T substitution = 0.101; the proportion of sites assumed to be invariable = 0.041; and the rate for variable sites assumed to follow a gamma distribution with shape parameter = 1.353.

Statistical analysis

To test whether the $BL_{Int200s}$ or BL_{Max} of eight *Alexandrium* species differ from one another, univariate analyses were performed. Normality and homogeneity of variance were tested using Shapiro-Wilk's *W* and Levene's tests, respectively. A parametric one-way analysis of variance (ANOVA) was performed. If the data did not satisfy the homogeneity assumption, Welch's one-way ANOVA and the Games-Howell *post-hoc* test were performed (Welch 1947, Games and Howell 1976). When the data did not meet the normality assumption, a nonparametric Kruskal-Wallis test and Mann-Whitney U test with Bonferroni correction ($p < 0.05$) were conducted (Mann and Whitney 1947, Kruskal and Wallis 1952, Dunn 1961). Spearman's rank correlation coefficient was used to examine relationships between the variables equivalent spherical diameter (ESD) of bioluminescence species, maximum swimming speed, maximum growth rate, $BL_{Int200s}$, and BL_{Max}. Statistical analyses were performed using SPSS ver. 25.0 (IBM-SPSS Inc., Armonk, NY, USA).

RESULTS

Bioluminescence capability of *Alexandrium* species

Among the seven *Alexandrium* species tested, *A. mediterraneum* AMYS1807, *A. pohangense* APPH1409, and *A. tamutum* ATSH1609 had bioluminescent capability, whereas *A. andersonii* CCMP2222, *A. hiranoi* NIES-3611, *A. insuetum* CCMP2082, and *A. pseudogonyaulax* KSUDinoB4 did not (Table 2, Fig. 1).

Position of bioluminescent *Alexandrium* species in a phylogenetic tree based on the LSU rDNA sequences

In a phylogenetic tree based on the LSU rDNA sequences, all 10 bioluminescent *Alexandrium* species

were positioned in all four large clades (Fig. 2). Of the 10 species, six (*A. affine*, *A. catenella*, *A. fraterculus*, *A. mediterraneum*, *A. pacificum*, and *A. tamarense*) belonged to a large clade (called bioluminescence clade I, BLClade I), whereas two species (*A. ostenfeldii* and *A. tamutum*) belonged to another large clade (BLClade II). However, *A. pohangense* (BLClade III) and *A. monilatum* (BLClade IV) belonged to other large clades.

Phylogenetic tree based on the *lcf* sequences

In phylogenetic trees based on the *lcf* and LSU rDNA sequences, the positions of nine bioluminescent *Alexandrium* species were the same, with the exception of *A. pohangense* (Fig. 3). In the phylogenetic tree based on the *lcf* sequences, *A. pohangense* formed a separate clade with *Protoceratium reticulatum* (Fig. 3A); however, in the phylogenetic tree based on the LSU rDNA sequences, *A. pohangense* was positioned inside a large *Alexandrium* clade (Fig. 3B).

Bioluminescence intensity of eight *Alexandrium* species

The $BL_{Int200s}$ of eight *Alexandrium* species ranged from 0.02×10^4 to 32.2×10^4 RLU cell^{-1} (Table 2, Fig. 4A); that of *A. ostenfeldii* was the highest, whereas that of *A. tamarense* was the lowest. The $BL_{Int200s}$ values of the eight *Alexandrium* species were significantly different (Welch's ANOVA, $F_{7,\ 9.06} = 67.15$, $p < 0.01$) and were divided into three subsets (Games-Howell *post-hoc* test, $p < 0.05$) (Fig. 4A). The BL_{Max} of the eight *Alexandrium* species ranged from 0.01×10^4 to 10.3×10^4 RLU cell^{-1} s^{-1} (Table 2, Fig. 4B). The BL_{Max} values of the eight *Alexandrium* species were significantly different (Kruskal-Wallis test, $H_7 = 28.80$, $p < 0.01$), and the Mann-Whitney U test with Bonferroni correction ($p < 0.05$) revealed that the BL_{Max} values of the *Alexandrium* species were divided into three subsets (Fig. 4B).

DISCUSSION

Bioluminescence capability

The present study explored the bioluminescence capability of seven *Alexandrium* species (*A. andersonii*, *A. hiranoi*, *A. insuetum*, *A. pseudogonyaulax*, *A. mediterraneum*, *A. pohangense*, and *A. tamutum*). Previously, the bioluminescence capability of nine *Alexandrium* species was explored. Thus, the bioluminescence capability of a total of 16 *Alexandrium* species, almost half the 33 formally described *Alexandrium* species, has now been explored. It is worthwhile to investigate the bioluminescence capability of the remaining 17 *Alexandrium* species.

The present study is the first to examine bioluminescence in *A. mediterraneum*, *A. pohangense*, and *A. tamutum*. Previously, eight *Alexandrium* species were reported to be bioluminescent; thus, a total of 11 *Alexandrium* species, 33% of the 33 formally described *Alexandrium* species, have been revealed to be bioluminescent. Before the present study was conducted, 90% of the tested *Alexandrium* species (eight of nine tested species) were

Table 2. Mean 200-second-integrated bioluminescence intensity per cell ($BL_{Int200s}$, $\times 10^4$ RLU cell^{-1} ± standard error) and mean maximum bioluminescence intensity per cell per second (BL_{Max}, $\times 10^4$ RLU cell^{-1} s^{-1} ± standard error) of the 13 *Alexandrium* species tested in the present study

Species	Strain name	ESD	BC	$BL_{Int200s}$	BL_{Max}
Alexandrium andersonii	CCMP2222	14.9	×	0.00 ± 0.0	
A. minutum	CCMP1888	20.4	×	0.00 ± 0.0	
A. insuetum	CCMP2082	26.4	×	0.00 ± 0.0	
A. pseudogonyaulax	KSUDinoB4	35.4	×	0.00 ± 0.0	
A. hiranoi	NIES-3611	40.0	×	0.00 ± 0.0	
A. tamutum	ATSH1609	22.5	○	1.54 ± 0.1	0.19 ± 0.1
A. ostenfeldii	NIES-4274	29.8	○	32.2 ± 4.5	10.3 ± 1.3
A. pacificum	KSUDinoE4	30.3	○	1.55 ± 0.1	0.34 ± 0.1
A. affine	AATA1308	31.4	○	9.23 ± 2.1	2.80 ± 0.7
A. pohangense	APPH1409	32.0	○	19.2 ± 2.0	2.75 ± 0.5
A. fraterculus	AFYS1309	32.3	○	12.7 ± 2.1	4.53 ± 0.9
A. mediterraneum	AMYS1807	33.0	○	0.70 ± 0.1	0.14 ± 0.0
A. tamarense	CCMP115	34.6	○	0.02 ± 0.0	0.01 ± 0.0

RLU, relative luminescence unit; ESD, equivalent spherical diameter (μm); BC, bioluminescence capability; ○, bioluminescent; ×, not bioluminescent.

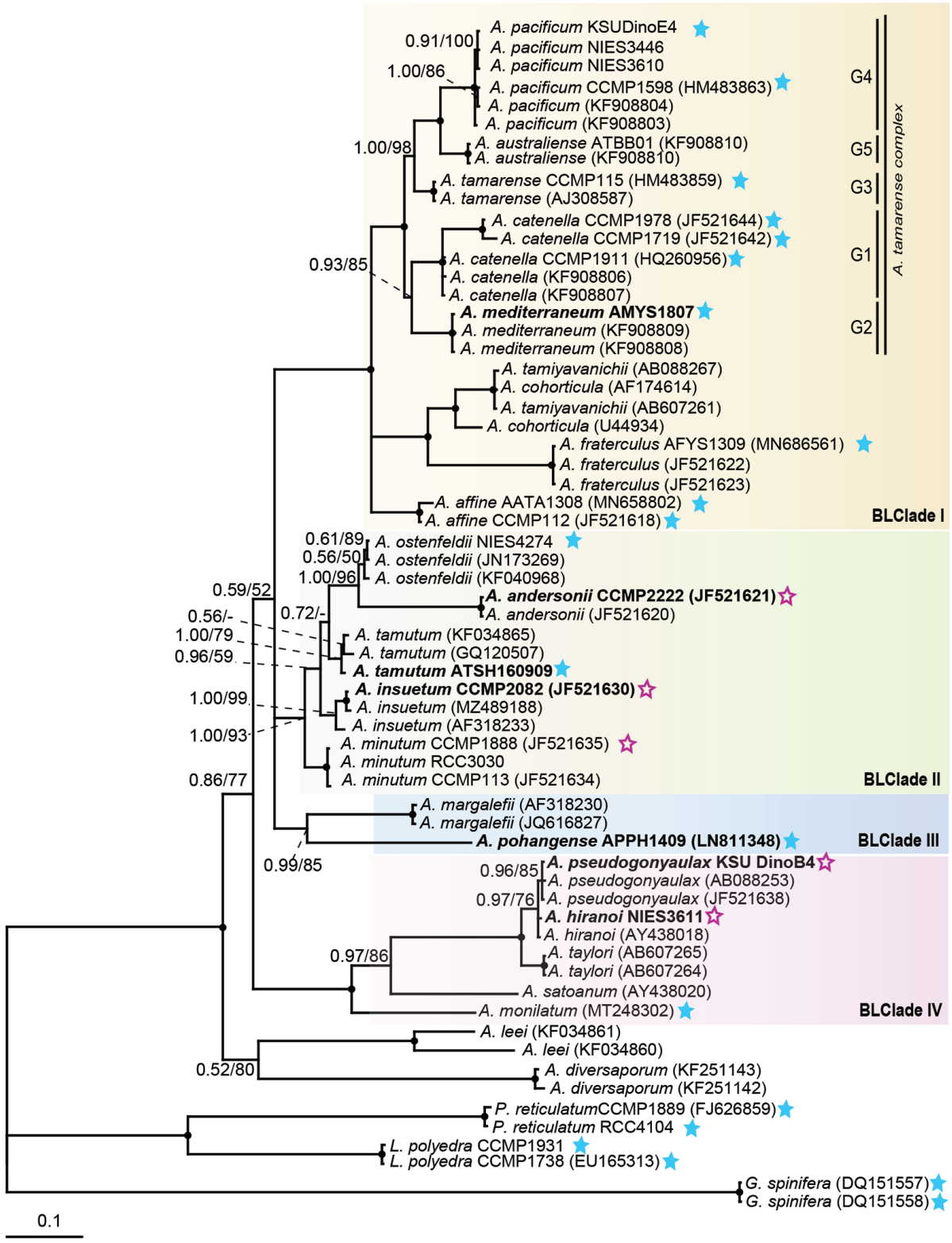

Fig. 2. Consensus Bayesian tree of nuclear large subunit ribosomal DNA region (based on 890 bp aligned position), using the GTR + G + I model. The bioluminescent dinoflagellates *Lingulodinium polyedra*, *Protoceratium reticulatum*, and *Gonyaulax spinifera* were used as the outgroups. The numbers above the branches indicate the Bayesian posterior probability (≥0.5, left) and maximum likelihood bootstrap values (≥50%, right), and solid black circles indicate full support (1.0 and 100%). Bioluminescent strains are indicated by a blue closed star, whereas nonbioluminescent strains are indicated by a purple open star. The strains tested in the present study are written in bold.

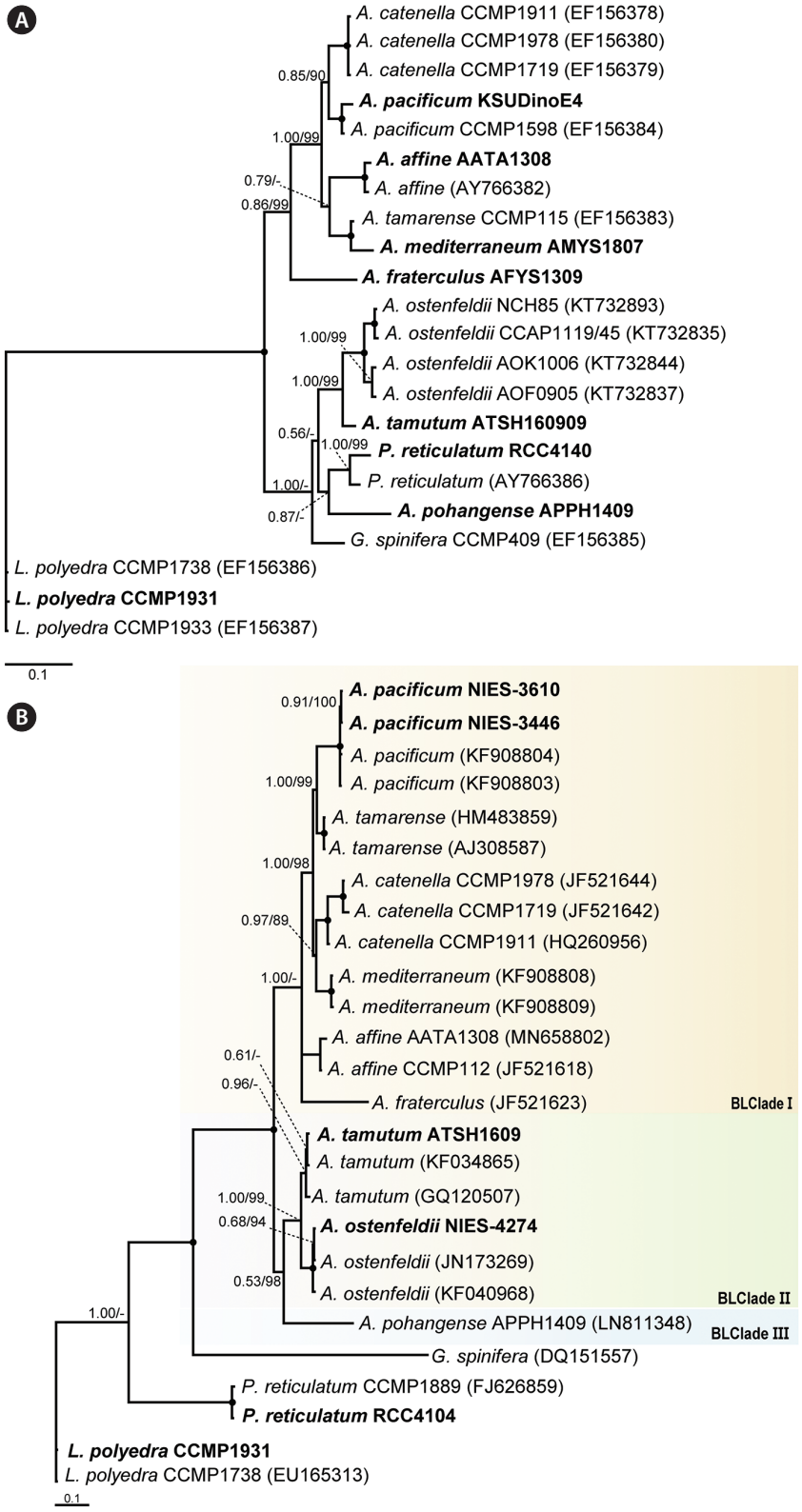

Fig. 3. Consensus Bayesian tree, based on 498 bp aligned position of nuclear luciferase gene (*lcf*) (A) and based on 890 bp aligned position of the large subunit ribosomal DNA (B), using the GTR + G + I model and *Lingulodinium polyedra* as the outgroup. The numbers above the branches indicate the Bayesian posterior probability (≥0.5, left) and maximum likelihood bootstrap values (≥50%, right), and solid black circles indicate full support (1.0 and 100%). The sequences obtained in the present study are in boldface.

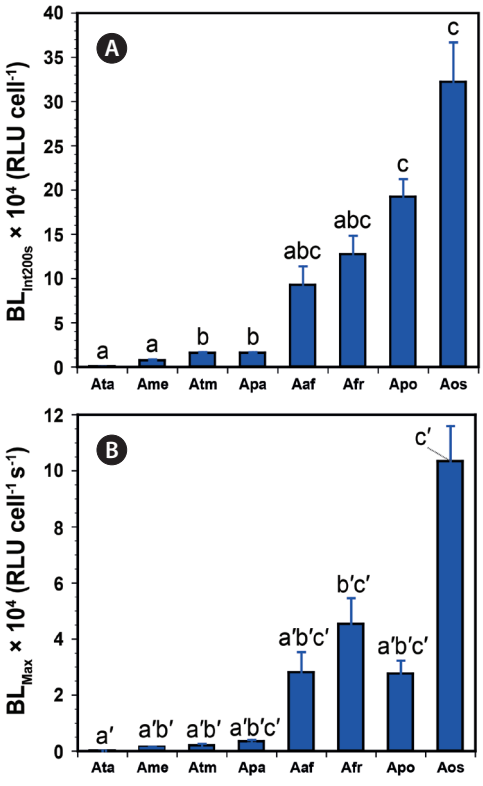

Fig. 4. Bioluminescence intensity of each *Alexandrium* species. (A) The mean 200-second-integrated bioluminescence intensity per cell ($BL_{Int200s}$). Significantly different subsets of the *Alexandrium* species based on Games-Howell *post-hoc* tests ($p < 0.05$) of Welch's ANOVA: (a) *Alexandrium tamarense* (Ata) and *A. mediterraneum* (Ame); (b) *A. tamutum* (Atm) and *A. pacificum* (Apa); (abc) *A. affine* (Aaf) and *A. fraterculus* (Afr); (c) *A. ostenfeldii* (Aos) and *A. pohangense* (Apo). (B) The mean maximum bioluminescence intensity per cell per second (BL_{Max}). Significantly different subsets of *Alexandrium* species based on the Bonferroni *post-hoc* test ($p < 0.05$) of Kruskal-Wallis test: (a′) Ata; (a′b′) Ame and Atm; (a′b′c′) Apa, Aaf, and Apo; (b′c′) Afr; (c′) Aos. RLU, relative luminescence unit. Values represent the treatment means ± standard error (n = 4).

known to be bioluminescent. However, the addition of our data shows that only 69% of tested *Alexandrium* species were bioluminescent. Thus, it is incorrect to assert that as a rule, *Alexandrium* species are bioluminescent; in fact, bioluminescence capability of these organisms is species-dependent.

Position of bioluminescent *Alexandrium* species in a phylogenetic tree based on the LSU rDNA sequences

In a phylogenetic tree based on the LSU rDNA sequences, six of 10 bioluminescent *Alexandrium* species belonged to a large clade (BLClade I), although the 10 bioluminescent *Alexandrium* species spanned four large clades (BLClade I–IV). In BLClade I, all *Alexandrium* species are known to be bioluminescent, except for *A. australiense*, *A. cohorticula*, and *A. tamiyavanichii*, whose bioluminescence capability has not yet been tested. Thus, it is worthwhile to explore the bioluminescence capability of these three species to test the hypothesis that all *Alexandrium* species in BLClade I are bioluminescent.

In BLClade I, *A. affine* AAV1, *A. catenella* CCMP1719, *A. pacificum* CCMP1598, and *A. tamarense* UW2C are known to be toxic, whereas *A. fraterculus* AF0307MIE01 and *A. mediterraneum* SZN01 are known to be nontoxic (Higman et al. 2001, Nguyen-Ngoc 2004, Orr et al. 2011, John et al. 2014, Eckford-Soper et al. 2016, Subong et al. 2017, Blossom et al. 2019). Both bioluminescence and toxicity are tools for anti-predation (Esaias and Curl 1972, Colin and Dam 2003, Bergkvist et al. 2008, Wohlrab et al. 2010, Lindström et al. 2017). Although all six *Alexandrium* species in BLClade I were bioluminescent, the toxicity status of these six species was split (Table 3, Fig. 5). Thus, bioluminescence is likely to be a primary defense tool and toxicity as a supplementary tool in this clade. Furthermore, *A. catenella*, *A. pacificum* CCMP3434, and *A. tamarense* are known to be mixotrophic, whereas *A. affine* CCMP112, *A. fraterculus* AFYS1309, and *A. mediterraneum* CCMP3433 are not mixotrophic (Table 3, Fig. 5) (Jeong et al. 2005, Lee et al. 2016, Lim et al. 2019). Thus, the acquisition of a mixotrophic capability in the six *Alexandrium* species in BLClade I may have occurred later than that of bioluminescence capability. However, further studies are needed to confirm the timing of the acquisition of a mixotrophic and bioluminescence capability in *Alexandrium* species.

Phylogenetic tree based on *lcf* sequences

The positions of nine bioluminescent *Alexandrium* species in the phylogenetic trees based on the *lcf* sequences were almost the same as those in the phylogenetic trees based on the LSU rDNA. This congruence is also shown in internal transcribed spacer and small subunit rDNA phylogenies (Baker et al. 2008, Valiadi et al. 2012, Le Tortorec et al. 2016). Thus, the *lcf* and ribosomal DNA may have coevolved. In the phylogenetic trees based on LSU rDNA sequences, *A. pohangense* was included in BLClade III. However, in the phylogenetic trees based on *lcf* genes, *A. pohangense* formed a clade with *P. reticulatum*, separate from the other *Alexandrium* species, unlike in the phylogenetic trees based on LSU rDNA sequences. The sequences in the *lcf* N-terminal region of *A. pohangense* differed from those of *P. reticulatum* by

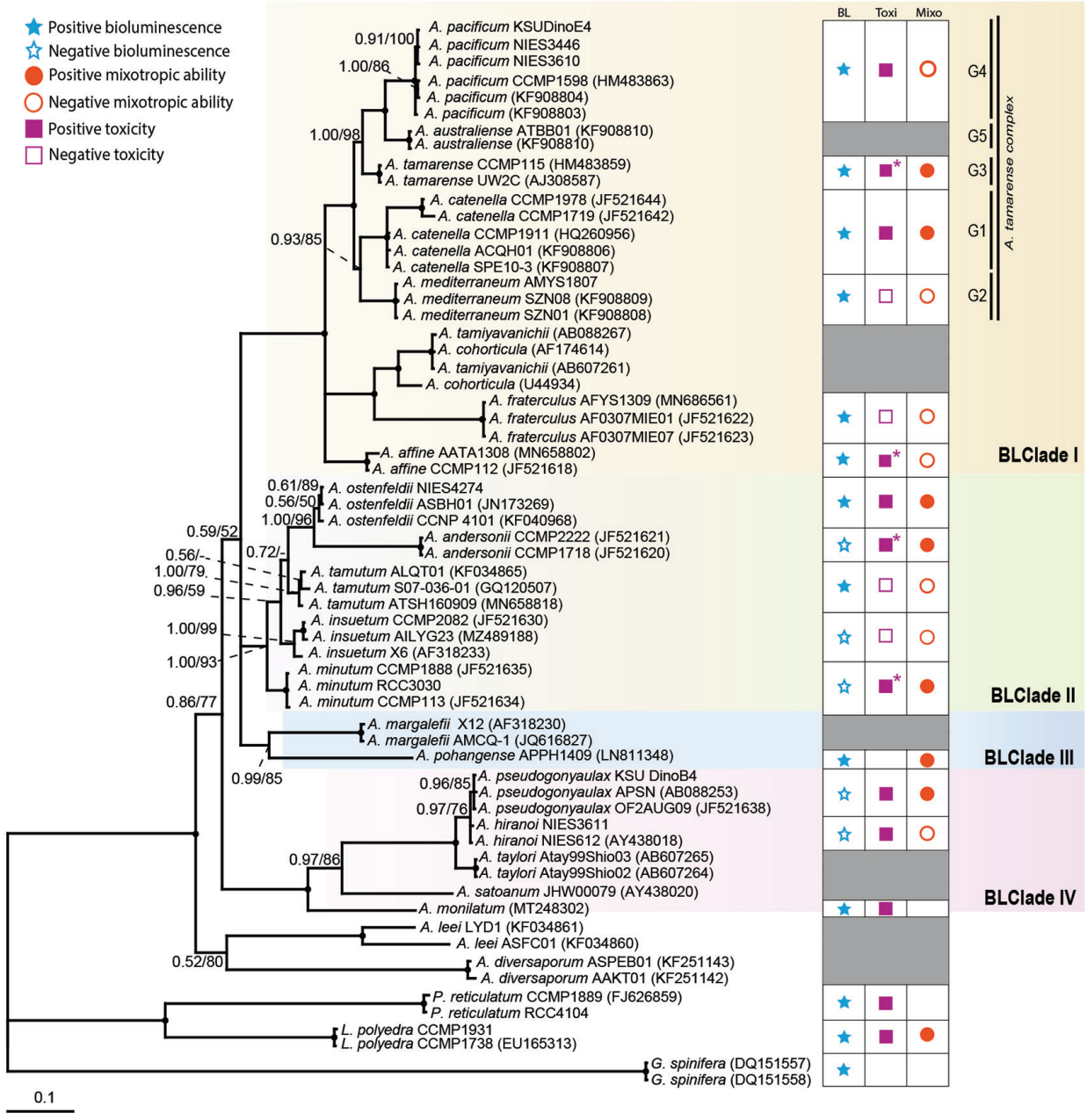

Fig. 5. Comparison of the presence (closed symbols) or absence (open symbols) of bioluminescent capacity (BL), toxicity (Toxi), and mixotrophic ability (Mixo) in the Consensus Bayesian tree of the nuclear large subunit ribosomal DNA region in Fig. 2. Asterisks indicate that some strains are toxic, whereas others are nontoxic (Prakash and Taylor 1966, Murakami et al. 1988, 1998, Jacobson and Anderson 1996, Satake et al. 1997, Draisci et al. 1999, Ciminiello et al. 2000, Higman et al. 2001, MacKenzie et al. 2004, Montresor et al. 2004, Nguyen-Ngoc 2004, Jeong et al. 2005, Lilly et al. 2005, Yoo et al. 2009, Orr et al. 2011, 2013, Blossom et al. 2012, 2017, John et al. 2014, Lim et al. 2015, 2019, Eckford-Soper et al. 2016, Lee et al. 2016, Subong et al. 2017, Murray et al. 2020).

41 bp (9.8%) (GenBank accession No. AY766386), but they differed from the other *Alexandrium* species by 43–81 bp (10.3–19.3%) (GenBank accession numbers listed in Fig. 3). Similarly, Valiadi et al. (2012) reported that the *lcf* in the catalytic domains of *A. monilatum* (included in BLClade IV of the phylogenetic tree based on LSU rDNA) was more similar to that of *L. polyedra* than that of *Alexandrium* species. Thus, it may not be surprising that *A. pohangense* forms a clade with *P. reticulatum*, rather than other *Alexandrium* species.

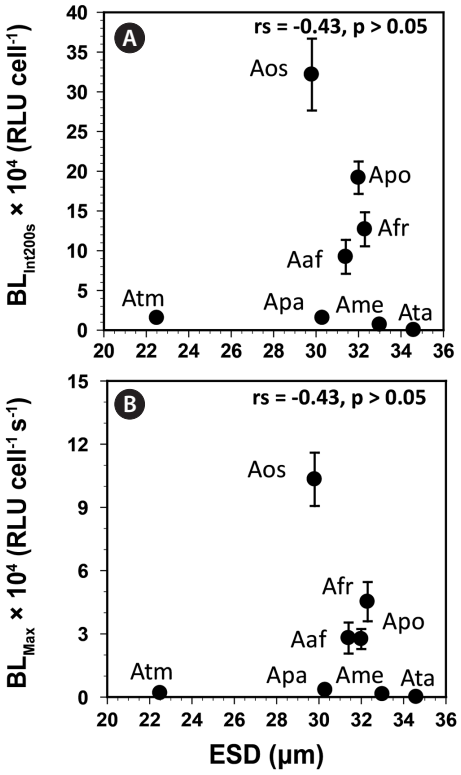

Fig. 6. Mean 200-second-integrated bioluminescence intensity per cell ($BL_{Int200s}$) (A) and mean maximum bioluminescence intensity per cell per second (BL_{Max}) (B) of bioluminescent *Alexandrium* species as a function of cell size (equivalent spherical diameter [ESD], μm), as described in Table 2. The values represent the treatment means ± standard error (n = 4). The Spearman correlation coefficient and p-value were described in the figures. Aaf, *A. affine*; Afr, *A. fraterculus*; Ame, *A. mediterraneum*; Aos, *A. ostenfeldii*; Apa, *A. pacificum*; Apo, *A. pohangense*; Ata, *A. tamarense*; Atm, *A. tamutum*.

Bioluminescence intensity of eight *Alexandrium* species

The results of the present study clearly showed that there were interspecific variations in the bioluminescence intensity of *Alexandrium* species. However, none of the $BL_{Int200s}$ or BL_{Max} values of the *Alexandrium* species investigated in the present study were significantly correlated with the ESD (Fig. 6). Thus, other factors may affect the $BL_{Int200s}$ or BL_{Max} of *Alexandrium* species. In addition, none of the $BL_{Int200s}$ or BL_{Max} values of the *Alexandrium* species investigated in the present study were significantly correlated with the maximum growth rate (Fig. 7). However, when the maximum growth rate of *A. tamarense* was excluded, the BL_{Max} of *Alexandrium* species was negatively correlated with the maximum growth rate (Spearman's rank correlation coefficient; rs = -1.00, $p < 0.01$). In general, the energy gained by a bioluminescent organism is used for reproduction or growth, respiration (swimming and metabolism), excretion, and bioluminescence. Thus, the bioluminescence intensity of bioluminescent organisms may be low if the growth rate is high.

None of the $BL_{Int200s}$ or BL_{Max} values of the *Alexandrium* species investigated in the present study were significantly correlated with the maximum swimming speed (Fig. 8) (Spearman's rank correlation coefficient; rs = -0.01 and -0.12, $p > 0.05$). However, the $BL_{Int200s}$ and BL_{Max} values of *A. ostenfeldii* were considerably greater than those of the other *Alexandrium* species. Therefore, *A. ostenfeldii*

Table 3. Comparison of bioluminescence capability (BC), mixotrophy, and toxicity of *Alexandrium* species

Species	Strain	BC	Mixotrophy	Toxicity	Reference
Alexandrium insuetum	CCMP2082	×	×	×	Orr et al. (2013), Lim et al. (2019)
A. minutum	CCMP1888	×	○	○	Jeong et al. (2005), Orr et al. (2011)
A. andersonii	CCMP2222	×	○	×	Orr et al. (2011), Lee et al. (2016)
A. pseudogonyaulax	KSUDinoB4	×	○[a]	○[a]	Murakami et al. (1988), Blossom et al. (2012, 2017)
A. hiranoi	NIES-3611	×	×[a]	○	Murakami et al. (1998), Blossom et al. (2012)
A. acatenella		○	-	○[a]	Prakash and Taylor (1966), Esaias and Curl (1972)
A. affine	CCMP112	○	×	×	Orr et al. (2011), Lee et al. (2016)
A. fraterculus	AFYS1309	○	×	×[a]	MacKenzie et al. (2004), Lee et al. (2016)
A. catenella	CCMP1719	○	○[a]	○	Jeong et al. (2005), Orr et al. (2013), Lim et al. (2019)
A. mediterraneum	AMYS1807	○	×[a]	×[a]	John et al. (2014), Lim et al. (2019)
A. monilatum		○	-	○[a]	Sweeney (1963), MacKenzie et al. (2004)
A. tamarense	CCMP115	○	○[a]	×	Jeong et al. (2005), Orr et al. (2013), Lim et al. (2019)
A. pacificum	KSUDinoE4	○	×[a]	○[a]	John et al. (2014), Lim et al. (2019), Murray et al. (2020)
A. tamutum	ATSH1609	○	×[a]	×[a]	Montresor et al. (2004), Lim et al. (2019)
A. ostenfeldii	NIES-4274	○	○[a]	○[a]	Jacobson and Anderson (1996), Lilly et al. (2005)
A. pohangense	APPH1409	○	○	-	Lim et al. (2015)

The data were obtained from the present study and from the literature.
○, presence of bioluminescent capability, mixotrophic ability, or toxicity; ×, absence of bioluminescent capability, mixotrophic ability, or toxicity.
[a]The strains used for testing mixotrophic ability and toxicity differed from the strain used for testing bioluminescence.

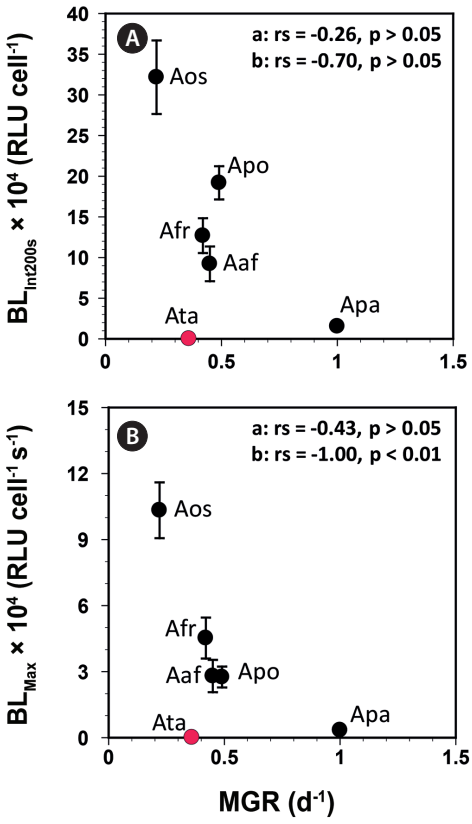

Fig. 7. Mean 200-second-integrated bioluminescence intensity per cell ($BL_{Int200s}$) (A) and the mean maximum bioluminescence intensity per cell per second (BL_{Max}) (B) of the bioluminescent *Alexandrium* species as a function of maximum growth rate (MGR; d^{-1}). Values represent the treatment means ± standard error (n = 4). a: the Spearman correlation coefficient and p-value of all species. b: the Spearman correlation coefficient and p-value of all species except for *A. tamarense* (red circle). Aaf, *A. affine*; Afr, *A. fraterculus*; Aos, *A. ostenfeldii*; Apa, *A. pacificum*; Apo, *A. pohangense*; Ata, *A. tamarense*. MGR data were obtained from Gu (2011), Laabir et al. (2011), Lim et al. (2015), Bill et al. (2016), and Lee et al. (2019).

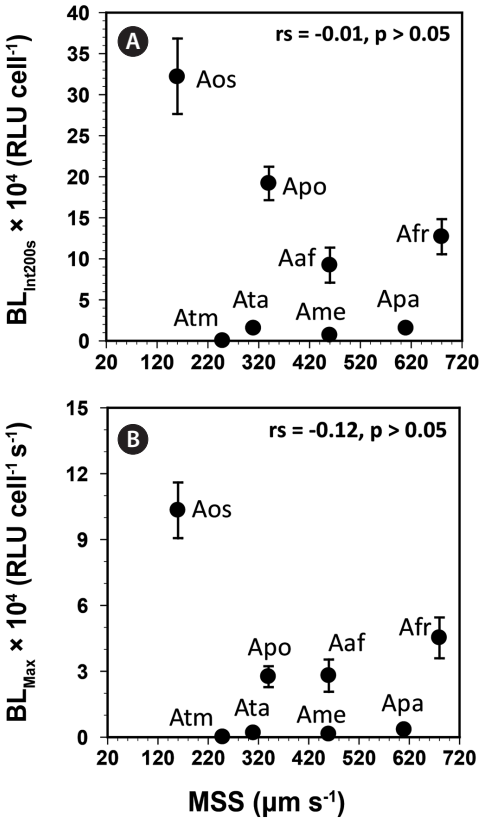

Fig. 8. Mean 200-second-integrated bioluminescence intensity per cell ($BL_{Int200s}$) (A) and the mean maximum bioluminescence intensity per cell per second (BL_{Max}) (B) of the bioluminescent *Alexandrium* species as a function of maximum swimming speed (MSS; μm s^{-1}). Values represent the treatment means ± standard error (n = 4). The Spearman correlation coefficient and p-value were described in the figures. Aaf, *A. affine*; Afr, *A. fraterculus*; Ame, *A. mediterraneum*; Aos, *A. ostenfeldii*; Apa, *A. pacificum*; Apo, *A. pohangense*; Ata, *A. tamarense*; Atm, *A. tamutum*. MSS data were obtained from Lewis et al. (2006), Lim et al. (2015, 2018), Lee et al. (2016), and Kang et al. (2018).

may spend less energy on swimming and may demonstrate notably higher $BL_{Int200s}$ or BL_{Max} values than other *Alexandrium* species.

The present study enhanced our knowledge of bioluminescence in *Alexandrium*. It is the first to report bioluminescence capability in *A. mediterraneum*, *A. pohangense*, and *A. tamutum* and to report lack of bioluminescence capability in *A. andersonii*, *A. hiranoi*, *A. insuetum*, and *A. pseudogonyaulax*. Furthermore, this is the first study to report the bioluminescence intensity of more than three species in one genus and to explore factors affecting differences in bioluminescence intensity. Moreover, our data revealed that phylogenetic trees based on the LSU rDNA and *lcf* sequences of *Alexandrium* species were consistent except for *A. pohangense*. The bioluminescence capabilities of 17 *Alexandrium* species have not yet been explored and provide a rich opportunity for further studies of bioluminescence in *Alexandrium* species.

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CONFLICTS OF INTEREST

The authors declare that they have no potential conflicts of interest.

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