Development of a Suite of Luciferase Gene Probes for the Screening and Detection of Marine Bioluminescent Systems and Organisms

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LONG TERM GOALS

The long-term goal of this project is to integrate molecular information regarding diversity and physiology of bioluminescence into the framework of observational technologies to improve the representation and forecasting of bioluminescent events.

OBJECTIVES

The focus of this proposal is:

(a) to conduct laboratory and field trials using luciferase gene probes developed in the lab in conjunction with diversity studies to screen for related, yet novel luciferase genes in the genomes of marine bioluminescent organisms.

(b) to detect *in situ* bioluminescent microorganisms in order to supplement bathyphotometric luminescence measurements with coincident real-time molecular data

(c) to conduct real-time PCR quantification of luciferase mRNA in dinoflagellates under different growth stages

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(d) to use bioluminescent molecular markers to search for and isolate novel microscopic bioluminescent organisms

APPROACH

The initial part of the project developed a diversity protocol to dissect the population structure of *Lingulodinium polyedrum*. Our protocol allowed the amplification of up to six microsatellite loci together with either the complete ITS1-5.8S-ITS2 region or a partial 18S region of the ribosomal gene of *L. polyedrum* from single motile cells and resting cysts. During the past year, a 'universal' oligonucleotide primer set, along with species and genus-specific primers specific to the luciferase gene were developed for the detection of bioluminescent dinoflagellates. These primers amplified luciferase sequences from bioluminescent dinoflagellate cultures and from environmental samples containing bioluminescent dinoflagellate populations. Finally, molecular assays were employed to quantify the level of luciferase transcription in different dinoflagellate species. In parallel, analysis of samples obtained during a recent cruise to the North Atlantic will provide information on the horizontal and vertical distribution of luciferase by processing DNA samples using primers previously developed (Baker et al., in press). Samples were additionally collected for RNA extractions and will be analyzed using a luciferase quantitative PCR (qPCR) assay developed at NOC. Samples were also concentrated for single cell isolation and subsequent PCR.

WORK COMPLETED

Multiplex genotyping of single cells. Two approaches were used to improve the population genetic analysis of phytoplankton: (a) the application of highly polymorphic microsatellite markers, which allow detailed population genetic studies; and (b) the development of methods that enable the direct genetic characterization of single cells as an alternative to clonal cultures. We combined these two approaches in a method that allowed multiplex microsatellite genotyping of single phytoplankton cells, providing a novel tool for high resolution population genetic studies (Frommlet and Iglesias-Rodriguez, in review, *J. Phycology*).

Development of luciferase gene probes. A 'universal' oligonucleotide primer set, along with species and genus-specific primers specific to the luciferase gene were developed for the detection of bioluminescent dinoflagellates. These primers amplified luciferase sequences from bioluminescent dinoflagellate cultures and from environmental samples containing bioluminescent dinoflagellate populations. Novel luciferase sequences were obtained for strains of *Alexandrium cf catenella* (Balech), *Alexandrium fundyense* (Balech) and also from a strain of *Gonyaulax spinifera* (Diesing), which produces bioluminescence undetectable to the naked eye. The phylogeny of partial luciferase sequences revealed 5 significant clades of the dinoflagellate luciferase gene, suggesting divergence between some species and providing clues on their molecular evolution (Baker et al., in press).

Luciferase transcription levels in different dinoflagellate species. Initially, the highly bioluminescent species, *Pyrocystis lunula* has been the focus of this study. RNA was extracted from cultures of *P. lunula* collected at different times through the daily cycle and was reverse transcribed using random hexamers. Quantitative PCR was undertaken using SYBR Green as the detection chemistry for the measurement of luciferase transcription, and was quantified using cRNA transcripts to produce a standard curve.

North Atlantic field study to assess distribution and abundance of luciferase. Samples were collected during the course of the D231 cruise to the North Atlantic, which comprises the 'Extended Ellett Line' (Figure 1). 19 stations were sampled during the course of the cruise (table 1). 78 samples were collected and are currently being processed for DNA and RNA analysis using luciferase primers previously developed (Baker et al., in press).

RESULTS

Multiplex microsatellite genotyping of single cells. Our single cell PCR protocol (Figure 2) allowed the amplification of up to six microsatellite loci together with either the complete ITS1-5.8S-ITS2 region or a partial 18S region of the ribosomal gene of *L. polyedrum* from single motile cells and resting cysts (Figure 3). The numbers of microsatellites used in population genetic studies of phytoplankton species are usually between 3 and 10 loci (e.g. Evans and Hayes 2004, Rynearson and Armbrust 2005, Iglesias-Rodríguez et al. 2006). Our results showed that it is possible to amplify six microsatellite loci in combination with either the ITS or partial 18S rDNA from single cells which demonstrates that commonly used numbers of microsatellite loci in culture based population genetic studies can also be analyzed from single cells.

Evaluation of the multiplex amplification of microsatellite loci. This study represents the first multiplex microsatellite genotyping protocol for single cells of a phytoplankton species and, as far as we are aware, of any microorganism. Garner (2002) has shown that the success rate in amplifying microsatellite loci is negatively correlated with genome size. This negative effect of large genomes on amplification success is probably due to the dilution of available primers by nonspecific binding and a decrease in target to non-target DNA. Given that dinoflagellates, and in particular the species *L. polyedrum*, have very large genomes compared to other phytoplankton species (Spector 1984, Rizzo 2002), we anticipate that similar single cell genotyping protocols could also be developed for other phytoplankton species and also other microorganisms.

Screening for luciferase genes in bioluminescent dinoflagellate populations. Seawater samples from San Luis Obispo Bay, California, U.S.A. were collected autonomously from the California Polytechnic State University pier using 5 L Niskin bottles mounted with a CTD seabird SBE-37 SIP MicroCAT profiler and a bioluminescence bathyphotometer (Herren et al. 2005) (Figure 4). DNA samples were collected for luciferase analysis. Sequence identities at the nucleotide level ranged from 37.3% to 100% across approximately 480 bp. All the *L. polyedrum* strains, with the exception of *L. polyedrum* AF085332 and one sequence amplified from the English Channel were identical. The average sequence identity amongst members of the *Alexandrium* genus was 94.4%. The two *P. lunula* strains were not identical, sharing a 96.4% sequence identity. The lowest nucleotide sequence identity of 37.3% was shared between *Pyrocystis fusiformis* (Blackman) and *Alexandrium tamarense* CCMP 1493. *G. spinifera* CCMP 409 shared the highest sequence identity with *Protoceratium reticulatum* CCMP 1889 of 91.5%.

Phylogeny of luciferase. The phylogenetic tree of the luciferase sequences, based on an alignment of approximately 160 amino acids, revealed 5 main clusters of the dinoflagellate luciferase sequences, a *L. polyedrum* clade, *Pyrocystis* clade, *Alexandrium* clade, *Gonyaulax spinifera* and a *Protoceratium reticulatum* clade (Fig 5). Our data has extended our knowledge on dinoflagellate luciferases, and demonstrates that luciferase is conserved across at least 5 genera, 10 species, and is even conserved in low light emitting dinoflagellates, such as *G. spinifera*. We present molecular tools for assessing the presence of bioluminescent dinoflagellates using universal and species-specific luciferase primers with

applications for the study of bioluminescence in the natural environment. Although bioluminescent dinoflagellate blooms are often clearly visible in the water column, lower cell densities or strains which emit low light, have the potential to go unreported. By having a universal primer set, one can rapidly create a profile of the bioluminescent dinoflagellate community, which is important to assess the population dynamics of dinoflagellates, and to diagnose and predict bioluminescence in the water column.

Quantification of luciferase activity. Work has been undertaken to develop molecular assays to quantify the level of luciferase transcription in different dinoflagellate species. Initially, the highly bioluminescent species *Pyrocystis lunula* has been the focus of this study. RNA was extracted from cultures of *P. lunula* collected at different times through the daily cycle and was reverse transcribed using random hexamers. qPCR was undertaken using SYBR Green as the detection chemistry for the measurement of luciferase transcription, and was quantified using cRNA transcripts to produce a standard curve.

The qPCR assay has to date been analyzed on two strains of *P. lunula* (Figure 6), with the preliminary results showing that transcription levels of luciferase in this species are relatively constant through the daily cycle. This is consistent with previous studies that have employed Northern hybridisation analysis of luciferase mRNA which have indicated that luciferase is constitutively expressed. These new results, however, describe the actual number of transcripts being produced per 200 ng dinoflagellate RNA and will be used to compare different species that are known to have different bioluminescent mechanisms. Specifically, in *P. lunula,* the light emitting organelles, the scintillons, are characterised as migrating from the centre of the cell to the cell periphery in the dark phase, whereas in *L. polyedrum*, the scintillons are known to be degraded at the end of the dark phase. Currently, little is know about gene expression or the bioluminescent mechanisms in *Alexandrium* species, so these will also be investigated and compared with the other species.

These assays will be used to compare different species, but will also be used to investigate how luciferase transcription changes in response to different environmental conditions and also at different developmental stages (e.g., gametes *versus* vegetative cells). The assay will also be applied to environmental samples to see the ‘amount’ of bioluminescence potential there is in a particular water sample. This work will be coupled with investigations of the translation rates of luciferase in different dinoflagellate species. Currently, work is being undertaken to analyse luciferase protein expression using a combination of SDS-PAGE, western blotting and immunological hybridisation with luciferase antibodies.

IMPACT/APPLICATIONS

Changes in the distribution and expression of luciferase will be tested in the field in collaboration with Dr. Mark Moline in October/November 2007. The functional variability of the genes tested will be assessed using samples taken by the REMUS. These samples will then be processed for morphological analysis, culture and genetic screening, so that the target organism(s) may be isolated and characterized.

TRANSITIONS

This ONR grant supplied funds for a luminescent spectrometer with high sensitivity. We have developed a protocol to monitor bioluminescence with very low cell densities down to a single cell. This will be extremely useful for calibrating the molecular signal with measurements of bioluminescence. We are currently using this approach to test differences in bioluminescence between gametes and vegetative cells of *L. polyedrum*.

RELATED PROJECTS

Isolation and characterization of viruses infecting bioluminescent dinoflagellates (MSc project, funded by the University of Southampton). Viruses infecting planktonic organisms are known to be important components of aquatic microbial communities, playing key roles in influencing host community diversity and primary production, impacting upon biogeochemical cycles and also bloom termination. Recent research has identified two dinoflagellates viruses, highlighting the importance of viruses in dinoflagellate communities. None, however, have been identified as infectious to the bioluminescent dinoflagellates. This project aims to isolate viruses from natural samples (Ellet Line cruise) and characterize them using a combination of microbiological and molecular techniques. Student: Ms. Charlie Best.

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PUBLICATIONS

Frommlet, J. and **Iglesias-Rodriguez, M.D**., 2007, Single cell multiplex PCR in Lingulodinium polyedrum: a new approach to assess dinoflagellate population structure, *J. Phycology.*

Baker, A., Robbins, I., Moline, M.A. and **Iglesias-Rodriguez, M.D.**, Oligonucleotide primers for the detection of bioluminescent dinoflagellates reveals novel luciferase sequences and information on the molecular evolution of this gene, *J. Phycol.* [in press].

Conference presentations:

Frommlet, J., Hutter, A., Saunders, G. and **Iglesias-Rodríguez, M.D.**, Photophysiological strategies within the dinoflagellate species *Lingulodinium polyedrum* and the effect of light on the life cycle of this species, 4th European Phycological Congress, Oviedo, Spain, 22-27 July 2007.

Table 1: Stations sampled on the Ellett line during the D321b cruise to the North Atlantic, 2007.

Station Reference	Position	Depths Sampled (M)	Maximum Depth (M)
IB23S	63°19.185 N 20°12.833 W	5, 20, 75, 112	123
IB21S	63°00.123 N 19°91.720 W	5, 20, 75, 125	235
IB16X	61°06.893 N 19°31.023 W	5, 20, 75, 125	2435
IB4	58°29.736 N 16°00.738 W	5, 20, 75, 125	1189
IB3	58°15.367 N 15°20.459 W	5, 20, 75, 125	660
IB2	57°56.997 N 14°34.999 W	5, 20, 75, 125	436
IB1	57°39.882 N 13°53.833 W	5, 45, 75, 133	139
A	57°35.013 N 13°38.432 W	5, 27, 75, 100	130
F	57°30.739 N 12°15.112 W	5, 32, 75, 125	1799
H	57°29.087 N 11°31.928 W	10, 20, 32, 75	2020
I	57°27.800 N 11°19.019 W	7, 22, 47, 77	740
J	57°26.949 N 11°05.358 W	5, 25, 75, 125	575
K	57°23.483 N 10°52.031 W	10, 32, 75, 125	783
N	57°14.248 N 10°02.966 W	5, 32, 75, 125	2099
O	57°09.119 N 09°43.181 W	10, 45, 60, 125	1944
P	57°05.873 N 09°25.016 W	7, 22, 77, 125	1412
Q	57°02.936 N 09°13.432 W	5, 32, 75, 125	339
R	56°59.983 N 08°59.902 W	5, 20, 45, 75	135
9G	56°47.964 N 07°20.831 W	5, 10, 32, 75	112

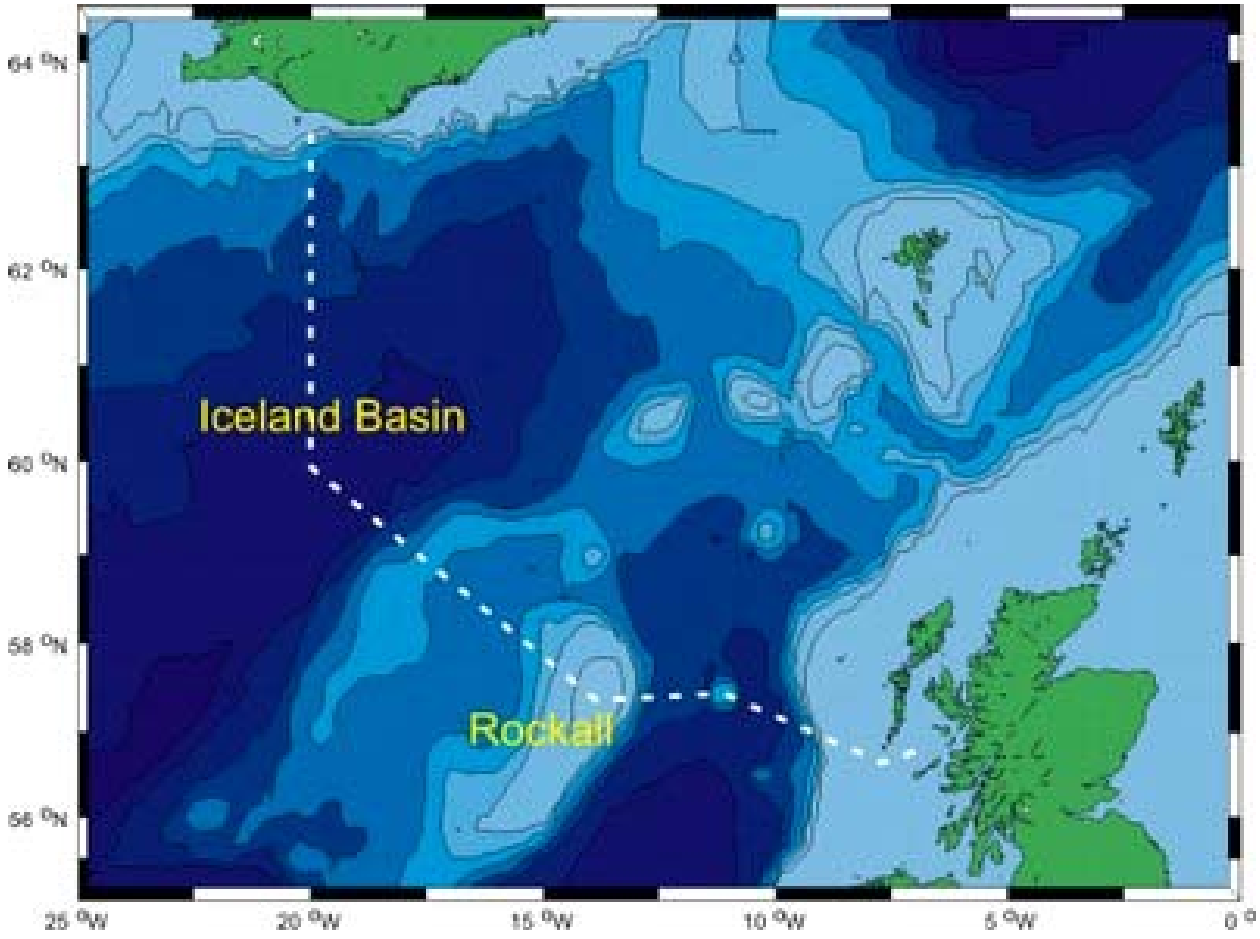

Figure 1. The extended Ellet Line transect surveyed in August 2007

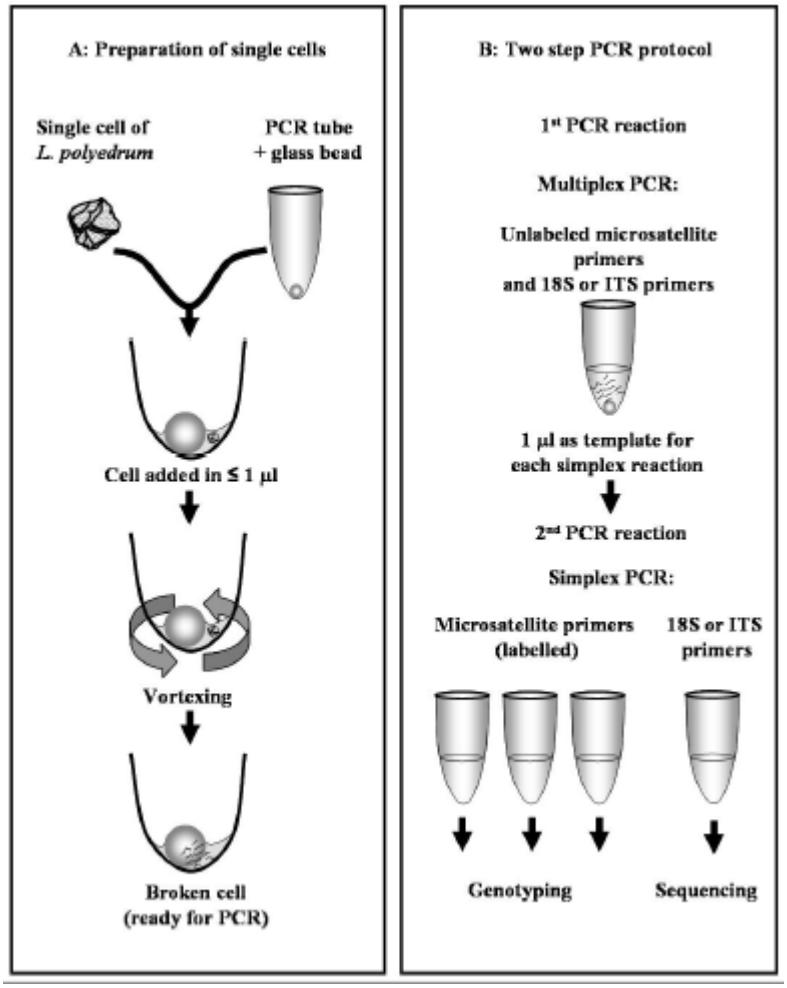

Figure 2. Summary of the multiplex genotyping of single cells method [(A) Preparation of single cells for genetic analysis. (B) Two-step PCR protocol for the amplification of up to six microsatellite loci together with either the ITS or partial 18S rDNA and downstream data analysis.]

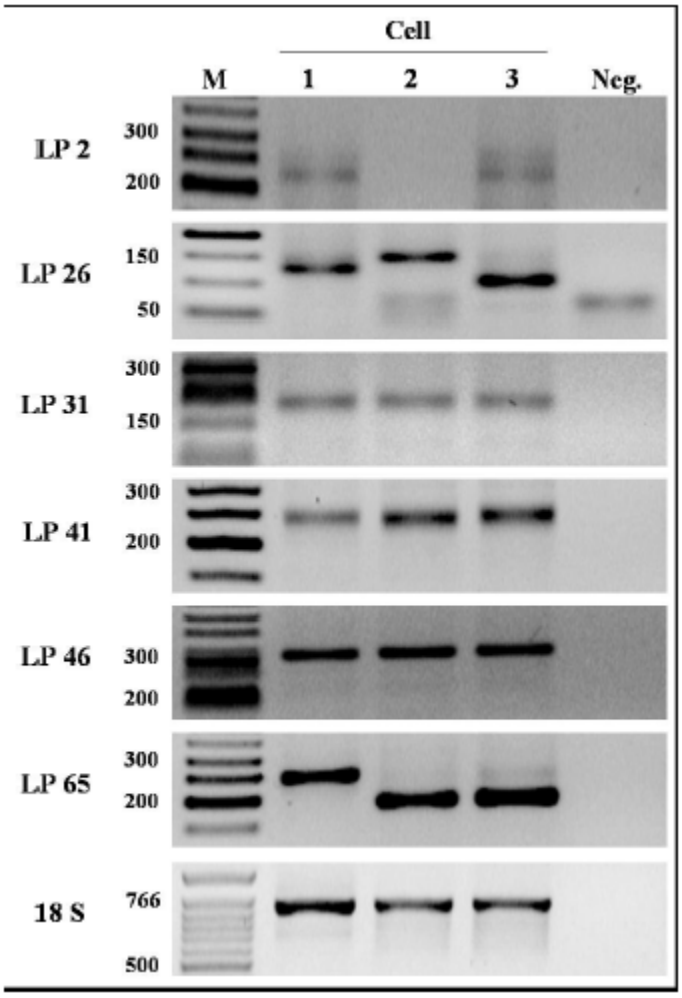

Figure 3. Single cell PCR gel [Gel photographs showing the PCR products obtained from three single cells (lanes1, 2, and 3) following the multiplex amplification of six microsatellite loci (LP2, LP26, LP31, LP41, LP46, and LP65) and a 732 bp product of the 18S rDNA. The cells were isolated from a single preserved water sample collected in La Jolla Bay, California in 2004. The left lane (M) contained a 50 bp size standard. Neg. = Negative Control. The low molecular weight band in the negative control of LP26 was due to primer interactions and not a contamination of the negative control.]

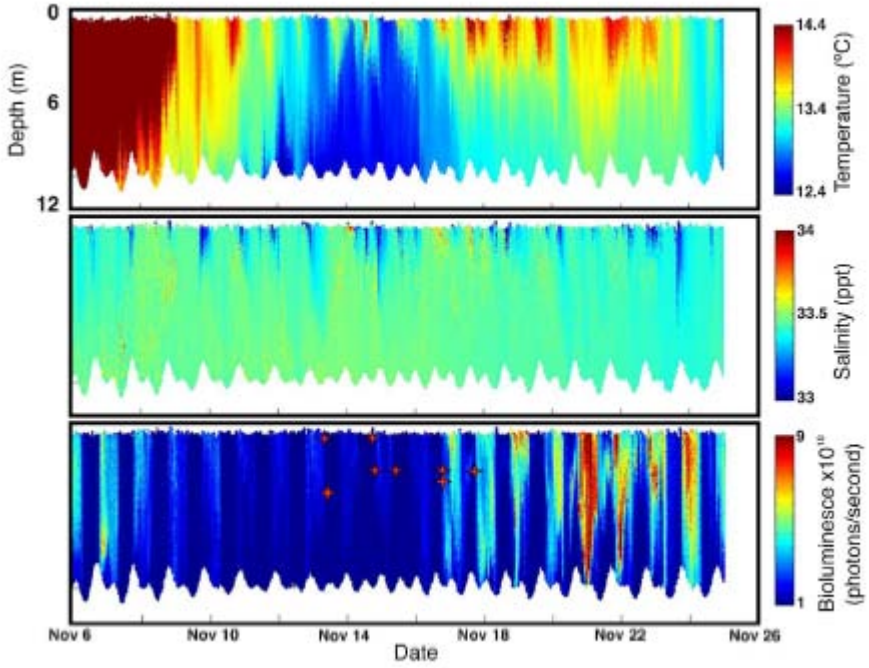

Figure 4. Time series data [Time series of the depth distribution of temperature (top), salinity (middle) and bioluminescence potential (bottom) from San Luis Obispo Bay, California, U.S.A. Red stars in the lower panel indicate the depth and time of sampling of environmental samples. Dynamics show that sampling occurred during a transition from a cold water intrusion onto the shelf to a water mass subjected to stratification by local heating. Bioluminescence intensified during this warmer period.]

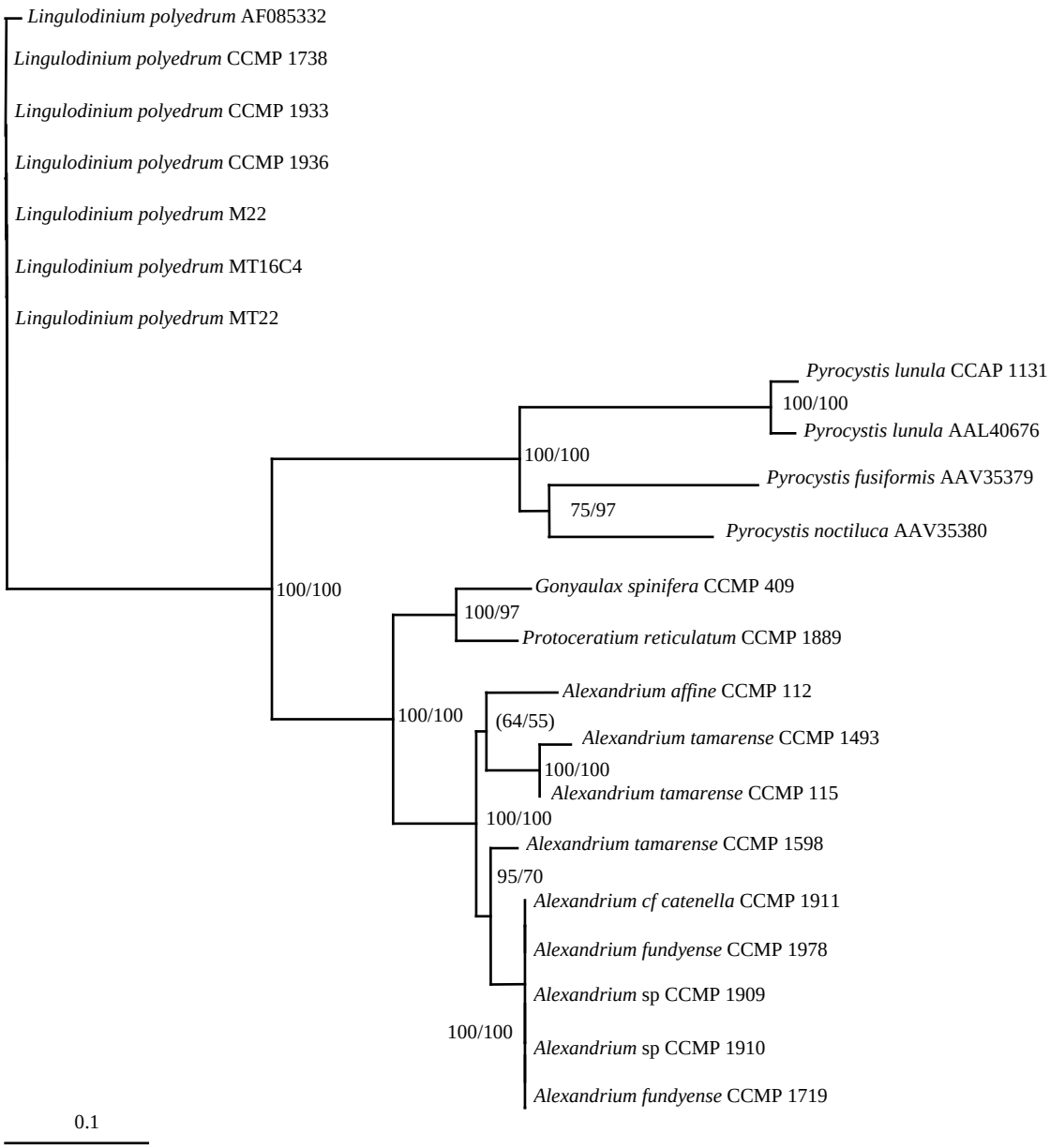

Figure 5. Phylogeny of luciferase. [Unrooted neighbour-joining tree based on an amino acid alignment of partial sequences of the N-terminal region and beginning of the first domain of the luciferase gene using a distance algorithm between luciferase sequences generated in this study and other dinoflagellate sequences from GenBank (Neighbour, in PHYLIP version 3.66). Bootstrap values were retrieved from 100 replicates and are indicated at the nodes (distance matrix and parsimony, respectively). The distance between 2 strains is acquired by adding the lengths of the connecting branches, using the scale which depicts 1 amino acid substitution per 10 amino acid residues.]

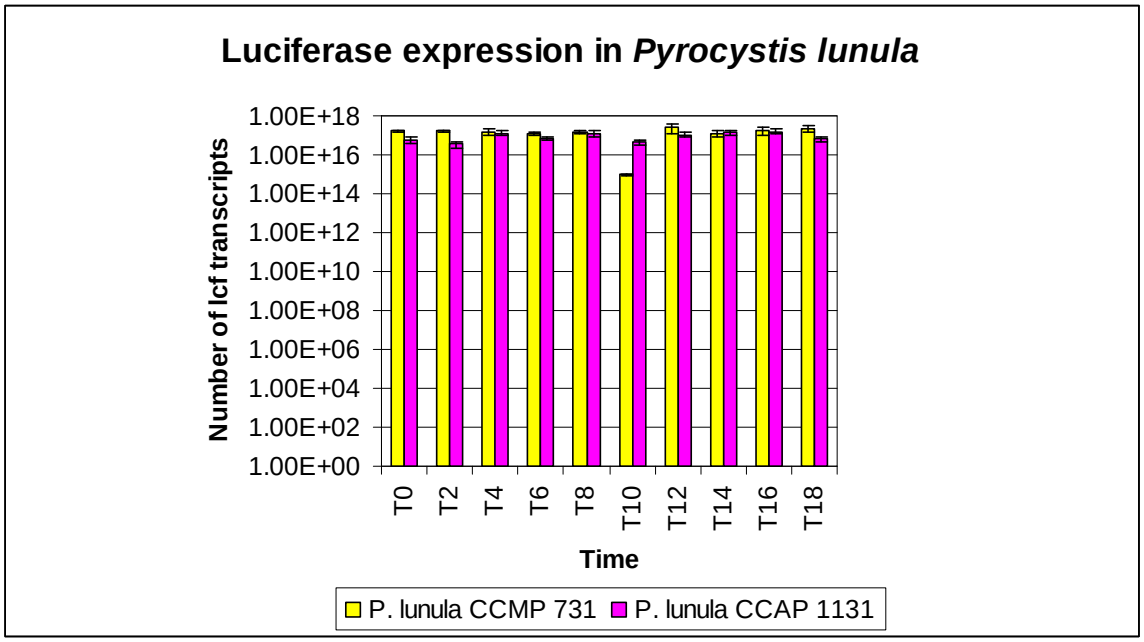

Figure 6. qPCR data. [Number of luciferase transcripts per 200 ng total RNA extracted from Pyrocystis lunula, collected at different times over a daily cycle.]