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# Molecular and biochemical basis for the loss of bioluminescence in the dinoflagellate *Noctiluca scintillans* along the west coast of the USA

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| Abstract: | The globally distributed heterotrophic dinoflagellate *Noctiluca scintillans* (Macartney) Kofoid & Swezy is well known for its dense blooms and prominent displays of bioluminescence. Intriguingly, along the west coast of the USA its blooms are not bioluminescent. We investigated the basis for this regional loss of bioluminescence using molecular, cellular and biochemical analyses of isolates from different geographic regions. Several prominent differences were identified in the non-bioluminescent strains: (1) the fused luciferase and luciferin binding protein gene (*lcf/lbp*) was present but its transcripts were undetectable; (2) *lcf/lbp* contained multiple potentially deleterious mutations; (3) the substrate luciferin was absent, based on the lack of luciferin blue autofluorescence and the absence of luciferin-derived metabolites; (4) although the cells possessed scintillons, the organelles that contain the luminescent chemistry, electron microscopy revealed additional scintillon-like organelles with an atypical internal structure; (5) cells isolated from the California coast were 43% smaller than bioluminescent cells from the Gulf of Mexico. Phylogenetic analyses based on large subunit rDNA did not show divergence of the non-bioluminescent population in relation to bioluminescent *N. scintillans* from the Pacific Ocean and Arabian Sea. This study demonstrates that gene silencing and the lack of the luciferin substrate have resulted in the loss of an important dinoflagellate functional trait over large spatial scales in the ocean. As the |

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# Molecular and biochemical basis for the loss of bioluminescence in the dinoflagellate *Noctiluca scintillans* along the west coast of the USA

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## Abstract

The globally distributed heterotrophic dinoflagellate *Noctiluca scintillans* (Macartney) Kofoid & Swezy is well known for its dense blooms and prominent displays of bioluminescence. Intriguingly, along the west coast of the USA its blooms are not bioluminescent. We investigated the basis for this regional loss of bioluminescence using molecular, cellular and biochemical analyses of isolates from different geographic regions. Several prominent differences were identified in the non-bioluminescent strains: (1) the fused luciferase and luciferin binding protein gene (*lcf/lbp*) was present but its transcripts were undetectable; (2) *lcf/lbp* contained multiple potentially deleterious mutations; (3) the substrate luciferin was absent, based on the lack of luciferin blue autofluorescence and the absence of luciferin-derived metabolites; (4) although the cells possessed scintillons, the organelles that contain the luminescent chemistry, electron microscopy revealed additional scintillon-like organelles with an atypical internal structure; (5) cells isolated from the California coast were 43% smaller than bioluminescent cells from the Gulf of Mexico. Phylogenetic analyses based on large subunit rDNA did not show divergence of the non-bioluminescent population in relation to bioluminescent *N. scintillans* from the Pacific Ocean and Arabian Sea. This study demonstrates that gene silencing and the lack of the luciferin substrate have resulted in the loss of an important dinoflagellate functional trait over large spatial scales in the ocean. As the bioluminescence system of dinoflagellates is well characterized, non-bioluminescent *N. scintillans* provide an ideal model to explore the evolutionary and ecological mechanisms that lead to intraspecific functional divergence in natural dinoflagellate populations.

## Introduction

Since first being described by Henry Baker in 1753 as "animalcules" (Harvey 1957), the globally distributed marine dinoflagellate *Noctiluca scintillans* – whose Latin name means "glowing bright by night" and has the common name "sea sparkle" – is known for its brightly bioluminescent blooms with abundances as high as $10^6$ cells $L^{-1}$ (Staples 1966; Daniel et al. 1979; Mohamed and Mesaad 2007; Kopuz et al. 2014). *N. scintillans* plays important roles in food webs (Fock and Greve 2002; Yilmaz et al. 2005) as a food source (Sulkin et al. 1998; Vargas and Madin 2004; Zhang et al. 2017a), a voracious predator of phytoplankton and zooplankton (Kimor 1979; Buskey 1995; Kiørboe and Titelman 1998; Nakamura 1998b; Johnson and Shanks 2003; Zhang et al. 2016; Stauffer et al. 2017), an important competitor of zooplankton for phytoplankton prey (Umani et al. 2004; Yilmaz et al. 2005), a contributor to the recycling of organic material through the ingestion of fecal pellets (Kiørboe 2003) and the excretion of inorganic nutrients that become available to primary producers (Zhang et al. 2017b).

Although the genus *Noctiluca* Suriray comprises only one globally-distributed species, *N. scintillans*, there are regional varieties with distinct characteristics. The "green" *Noctiluca* variety, endemic to waters of southeast Asia and the Arabian Sea region (Harrison et al. 2011), contains the photosynthetic green flagellate symbiont *Pedinomonas noctilucae* (e.g. Elbrachter and Qi 1998) within its vacuoles, which provides about 70% of its energy requirements (Sweeney 1978). Otherwise, the "red" *Noctiluca* variety, which lacks the green endosymbiont, is widely distributed in temperate and subtropical waters (Harrison et al. 2011); its orange-red color is due to carotenoid pigments obtained from food prey or synthesized *de novo* (Balch and Haxo 1984).

Whereas all other dinoflagellates harbor separate luciferase (LCF) and luciferin binding protein (LBP), *Noctiluca scintillans* contains a fused LCF-LBP, encoded by the *lcf/lbp* gene. This is likely to be the ancestral gene arrangement for dinoflagellates, since, according to phylotranscriptome analyses (Janouskovec et al. 2017), *N. scintillans* represents a basal taxon among dinoflagellates, having a typical dinokaryotic nucleus only during the gamete life stage (Hansen et al. 2004) and lacking mitochondrial mRNA editing that is typical of phylogenetically more recent species (Chang 1960; Sweeney 1963). It is thought that *lcf/lbp* split into two separate genes in more recent dinoflagellates (Liu and Hastings 2007; Valiadi and Iglesias-Rodriguez 2014), potentially to allow for individual regulation of each protein (Valiadi and Iglesias-Rodriguez 2013).

Light emission by dinoflagellate cells originates from scintillons, organelles distributed throughout the peripheral cytoplasm that contain luciferin and LCF. *Noctiluca scintillans* cells have around $10^4$ scintillons of 0.5-1.5 µm in diameter (Eckert 1966; Eckert and Reynolds 1967), within a cytoplasmic layer that can be as thin as 0.11 µm (Nawata and Sibaoka 1979). Thus, scintillons project into the vacuolar space that occupies a large proportion of the *N. scintillans* cell volume and contains sap of high acidity (Nawata and Sibaoka 1976). Light flashes are triggered by mechanical stimulation of the cell (Nicol 1958), prompting a propagating action potential across the vacuole membrane (Eckert 1965a; Eckert 1965b; Eckert and Sibaoka 1968) that opens voltage gated proton channels (Rodriguez et al. 2017). The subsequent flux of protons from the vacuole into the scintillons causes a decrease in pH that dissociates luciferin from LBP and activates luciferase, resulting in the oxidation of luciferin which releases energy in the form of visible light (Fogel and Hastings 1972; Nawata and Sibaoka 1979).

Dinoflagellate bioluminescence acts as a predator defense behavior to reduce grazing (Esaias and Curl 1972; White 1979). Flash responses to predator contact serve to startle predators (Buskey et al. 1983; Buskey and Swift 1985) and to act as a light alarm to attract secondary visual predators of the dinoflagellate predators (Morin 1983; Mensinger and Case 1992; Abrahams and Townsend 1993; Fleisher and Case 1995; Cusick and Widder 2013). Cells also increase their bioluminescence when chemical cues from predators are present (Lindström et al. 2017). Despite the ecological significance of dinoflagellate bioluminescence, some species have bioluminescent and non-bioluminescent strains (Valiadi et al. 2012). Characterizing patterns in the expression of *lcf* and biosynthesis of luciferin will aid in understanding the evolution of dinoflagellate bioluminescence and in identifying environmental conditions that favor the maintenance or loss of light production.

The aim of this study was to investigate the molecular, cellular and biochemical basis for the lack of bioluminescence in *N. scintillans* from the west coast of the USA. This red form of *N. scintillans* differs from those of other regions by its smaller size (Eckert and Findlay 1962; Balch and Haxo 1984; Tada et al. 2000; Liu and Hastings 2007) and lack of bioluminescence (Chang 1960; Esaias 1973; Dewey 1976; Sulkin et al. 1998; Hoppenrath and Leander 2010). In comparison to a bioluminescent variety from the Gulf of Mexico, we assessed cell size, existence of scintillons, presence of luciferin and its derived metabolites and the presence, sequence, and expression of *lcf/lbp*. Our results confirmed the lack of luciferin fluorescence, but surprisingly showed that scintillons are still present in the cells. Furthermore, *lcf* is present in the genome but its expression is repressed. Therefore, the bioluminescence system of *N. scintillans* from the west coast of the USA has become non-functional. However, phylogenetic analyses of large subunit (LSU) rDNA sequences showed that the loss of bioluminescence has not resulted in a divergent

112 population, or a different species. We therefore propose that this is a good model for further
113 studies on the eco-evolutionary and oceanographic processes driving functional divergence in
114 natural plankton populations.
115

116 **Materials**

117 **Cell culture and bioluminescence testsstests**

118 Strains of *N. scintillans* were obtained from three locations off the west coast of the USA (LJ –
119 La Jolla, CA; SC – Santa Cruz, CA; SPMC – Shannon Point Marine Center, Puget Sound, WA),
120 as well as the Gulf of Mexico (GM – Port Aransas, TX) (Figure 1, Table 1). As cell cultures
121 remain viable for a limited time (Sato et al. 1998), in some cases it was necessary to re-establish
122 cultures from the same location. Cells were cultured in filter-sterilized (0.22-µm membrane
123 filter, Steritop, Millipore, UK) or Whatman GF/F (GE Healthcare Bio-Sciences, Pittsburgh, PA
124 USA) filtered seawater supplemented with appropriate amounts of prey culture. Subculturing
125 was conducted every 10-14 days. To prevent overgrowth of the prey, a low irradiance of 15
126 µmol $m^{-2}$ $s^{-1}$ was used. The phytoplankton prey species *Dunaliella tertiolecta* Butcher CCMP
127 1320 (Chlorophyceae) and *Prorocentrum micans* Ehrenberg CCMP 691 (Dinophyceae) were
128 cultured in f/2 (Guillard and Ryther 1962) and L1 (Guillard and Hargraves 1993) seawater
129 media, respectively, without silicate. The dinoflagellate *Pyrocystis lunula* (Schütt) Schütt, used
130 for luciferin extractions, was cultured in half strength f/2 medium. All cultures were maintained
131 at 19-20°C with a 12:12 h light:dark cycle.
132 The bioluminescence of *N. scintillans* strains was assessed visually. When no
133 bioluminescence was observed, dense cultures were tested using a luminometer as described in

Valiadi et al. (2012). Cell size was measured as equivalent spherical diameter using a Beckman Multisizer 3 with 1000-µm aperture tube. Cell cultures were first sieved through 100 µm Nitex mesh and then resuspended in GF/F filtered seawater prior to measurement. The instrument noise level was 36 µm, well below the expected average cell size of non-bioluminescent cells (approx. 250 µm).

**Nucleic acid extraction and reverse transcription**

*Noctiluca scintillans* cells were harvested at stationary phase when the cultures were dense and most of the prey cells had been consumed. Approximately 400 mL of culture were filtered gently onto 25-mm diameter, 5-µm pore size Nuclepore polycarbonate membranes (Whatman, U.K.) and stored at -80 ºC prior to processing. The filter-bound cells were disrupted using a micropestle while still frozen in liquid nitrogen and DNA extraction was performed as described previously (Valiadi et al. 2012). RNA was isolated using the Nucleospin RNA II kit (Macherey-Nagel, Germany) which includes an on-column DNase digestion step. The quantity and purity of the isolated DNA and RNA were assessed using a Nanodrop spectrophotometer (ND-3000, Nanodrop, USA). The RNA was reverse transcribed using the High Capacity RNA-to-cDNA kit (Applied Biosystems, USA), which utilizes random primers. A control reaction was included for each RNA sample, omitting the reverse transcriptase enzyme (-RT reaction). The PCR quality of the DNA and cDNA preparations, and the absence of genomic DNA carryover contamination in the latter, was assessed by amplification of the small subunit (SSU) rRNA gene using specific primers (Lin et al. 2006).

**PCR**

The oligonucleotide primers used in this study and their gene targets are detailed in Table S1. Detection of *lcf* was achieved using previously described “universal” primers for this gene

(Valiadi et al. 2012), with either DNA or cDNA as the template. Primers to amplify and sequence nearly the entire *lcf/lbp* open reading frame (ORF) were designed using the program Primer3 (http://frodo.wi.mit.edu/primer3/) and the published *N. scintillans lcf/lbp* gene sequence (accession no. JF838193; (Liu and Hastings 2007). The 25 µL reactions consisted of 0.1 µM of each primer, 250 µM of each dNTP, 1 x standard PCR buffer, 0.5 U GoTaq DNA polymerase (Promega, UK) and 50-100 ng of DNA or cDNA template. PCR amplification was achieved using the following thermocycle: 5 min at 95 °C for initial denaturation, followed by 30 cycles of 95 °C for 45 s, 55 °C for 30 s, 68 °C for 3 min, and a final extension at 68 °C for 10 min.

The LSU rDNA was amplified using specific primers designed to prevent amplification of rDNA from the *P. micans* prey in strain SPMC 136. These PCRs were conducted using the high fidelity and high yield Advantage 2 Polymerase mix (Clontech, USA). Each 25 µL reaction contained 0.02 µM of each primer, 200 µM of each dNTP, 1 x Advantage 2 PCR buffer and 1 x Advantage 2 polymerase mix. PCR amplification was achieved using the following thermocycle: 3 min at 95 ºC for initial denaturation followed by 30 cycles at 95 ºC for 20 s, 55 ºC for 30 s, 72 ºC for 2 min and a final extension at 72 ºC for 10 min.

**Sequence and phylogenetic analyses**

The *lcf/lbp* genes of *N. scintillans* with differing bioluminescence abilities were compared by cloning amplicons from the genomic DNA of two non-bioluminescent strains, LJ and SPMC, and the bioluminescent strain GM. LSU rDNA sequences were compared to determine the phylogenetic relationship between non-bioluminescent *N. scintillans* and bioluminescent strains from other ocean regions. Three fragment clones were sequenced for each LSU rDNA amplicon. DNA sequences were trimmed to remove vector and primer sequences and assembled using the CAP3 sequence assembly program (http://doua.prabi.fr/software/cap3). For both genes, the

BLASTn tool (NCBI) was used to confirm that the correct gene had been amplified and to retrieve similar sequences from the GenBank database for phylogenetic analyses. To help understand the variability of these genes in natural populations we also searched for *N. scintillans lcf/lbp* and LSU rDNA sequences in the TaraOceans metagenome datasets using the Ocean Gene Atlas platform (http://tara-oceans.mio.osupytheas.fr/ocean-gene-atlas/) to help understand the variability of *N. scintillans* genes in natural populations. Nucleotide sequence alignments were carried out using ClustalW (Thompson et al. 1994) implemented in MEGA v7.0 (Kumar et al. 2016) with manual refinement. Where multiple sequences from a single strain were identical, only one was retained as a reference in further analyses.

For LSU rDNA, Bayesian phylogenetic analyses were performed using MrBayes 3.2.6 (Ronquist et al. 2012) following the selection of the most appropriate model of evolution (TIM1+G) by jModelTest 2.1.9 (Darriba et al. 2012), based on the Bayesian information criterion (BIC). Analyses were performed in two independent runs of 5,000,000 generations and four Markov chains, with sampling every 100 generations resulting in 50,000 trees and a burn-in of 25%. The final phylogenetic tree was generated using FigTree 1.4.4 (http://tree.bio.ed.ac.uk/software/figtree).

**Confocal laser scanning microscopy**

Cells were collected individually during the mid-dark phase using a plastic Pasteur pipette and placed on a glass slide with a raised circular well, constructed using double sided tape, that could accommodate the large *N. scintillans* cells. The cells were immobilized by adding a drop of viscous Protoslow quieting solution (Blades Biological Ltd, UK). Imaging of the cells was performed using a Leica TCS SP5 laser scanning confocal microscope (Leica Microsystems Inc. USA). Optical slices of 0.5 µm were obtained for part of the cell, stopping when the focal

distance of the microscope would cause the objective to contact the raised coverslip. Luciferin autofluorescence was induced by a UV laser with an excitation wavelength of 405 nm and recorded and imaged at 416-520 nm.

**Transmission electron microscopy**

Cultures of the bioluminescent *N. scintillans* strain GM and the non-bioluminescent strain SPMC136 were cooled on ice and then fixed with 2% glutaraldehyde. Fixed cells were concentrated in small baskets made of a 40-µm plankton net. To avoid further disturbance to the cells, all the following steps until embedding in Spurr resin were performed in these baskets. The cells were washed free of glutaraldehyde using 0.1 M PIPES buffer containing 1% NaCl (added to raise the osmolarity of the buffer). They were then post-fixed in 1% osmium tetroxide in the same buffer for 1 h at room temperature. After rinsing in distilled water, the cells were stained with 2% aqueous uranyl acetate for 20 min followed by standard dehydration in an ethanol series and a final 10-min wash in acetonitrile, before embedding overnight in Spurr resin. Individual specimens were placed in separate polymerizing molds, then fresh Spurr resin was added and left to polymerise for 24 h at 60°C. Sectioning was performed with an ultramicrotome (Reichert-Jung Ultracut E) and the sections were mounted on uncoated copper grids and stained with lead citrate. Grids were viewed and photographed with a Hitachi H-7000 electron microscope.

**Luciferin and luciferase extractions**

Luciferin was extracted following established methods (Nakamura et al. 1989). Approximately 4-10 x $10^3$ cells of *N. scintillans* GM, 21-108 x $10^3$ cells of *N. scintillans* LJ, or 500-950 x $10^3$ cells of *P. lunula* were collected by vacuum filtration onto Whatman GF/F paper, and the filter paper resuspended in 10 mL of pre-heated, anaerobic extraction buffer (5 mM potassium phosphate, 5 mM 2-mercaptoethanol, pH 8.0) under an argon atmosphere in a 50 mL conical

vial. The samples were vortexed for 5 s, boiled for 1 min, and then cooled on ice, all while under an argon atmosphere, followed by centrifugation for 20 min at 20,000 × *g* at 4°C. The supernatant was passed through a 0.2 µm filter and directly analyzed on an Agilent 1290 infinity liquid chromatography system with a diode array UV/Vis detector and Agilent 6500 series Q-TOF mass spectrometer with an Electrospray Ionization source using only ultrahigh purity nitrogen (99.999%) as drying gas. A Phenomenex Luna 5 µm C18(2) 100 Å column (150 × 4.6 mm, 5 µm particle size) was employed. Mobile phase: A: 0.1% formic acid in water, B: 0.1 % formic acid in acetonitrile. Gradient: 5% B for 5 min, ramp to 100% B in 10 min, 100% B for 5 min, ramp to 5% B in 1 min, 5% B for 3 min. Flow rate: 0.7 mL/min. Source parameters: Drying gas: 11 liters per min, 300 °C; N: Nebulizer:35 psig; C: Capillary:3000 V; F: Fragmentor:100 V; S: Skimmer:65 V; OCT 1 RF Vpp: 750. Tandem MS (Collision Induced Dissociation) parameters: Isolation width: Narrow; Collision energy: 20 V.

In an attempt to detect bona fide dinoflagellate luciferin in *N. scintillans*, we adopted several approaches aimed at suppressing potential luciferin-LBP binding and oxidation of the luciferin. We extracted using isopropanol: water to unfold LBP. We attempted to avoid oxidation through extended anaerobic incubation of *N. scintillans* followed by anaerobic extraction. We performed direct LC-MS analysis of *N. scintillans* luciferase extracts as well as anaerobic ultrafiltration at pH 6.0, to encourage release from LBP, followed by LC-MS analysis.

Luciferase extracts were prepared according to established protocols (Schmitter et al. 1976). Approximately 7-33 x $10^3$ cells of *N. scintillans* were collected using vacuum filtration on Whatman GF/F filter paper, which was plunged into ice-cold buffer containing 50 mM Tris-HCl, 10 mM EDTA, and 5 mM 2-mercaptoethanol. After vortexing for 30 s, cells were disrupted in a glass homogenizer, cell debris was removed by collecting the supernatant after centrifuging at

5000 x *g* for 10 min; following high speed centrifuging at 27,000 x *g* for 15 min, the supernatant was stored at ˚4˚C or -˚80˚C until testing.

**Extract cross-reactions**

To measure bioluminescence cross-reactions, 10 µL volumes of ice-cold *N. scintillans* luciferase extract and *P. lunula* luciferin extract were spotted in different places at the bottom of a test tube, which was placed in a Sirius luminometer (Berthold Detection Systems). The reaction was initiated by injection of 250 µL of 5 mM phosphate buffer pH 6.0 with 1 min light measurement. The blank, representing background, consisted of buffer injection alone. The presented mean values (± standard deviations) are from a representative experiment performed on 14 May 2018.

## Results

### Geographic distribution of bioluminescent and non-bioluminescent *N. scintillans*

The environmental ranges of the bioluminescent and non-bioluminescent varieties of *N. scintillans* in the northeastern Pacific Ocean do not appear to overlap, as the west coast of the USA is the only known region where non-bioluminescent *N. scintillans* are present. The observed lack of mechanically stimulated bioluminescence in strains isolated from the coasts of California and estuarine waters of Washington (Figure 1, Table I) was confirmed using a luminometer and low pH treatment (Valiadi et al. 2012) to activate the luminescent chemistry independently of mechanotransduction. Furthermore, numerous isolates of *N. scintillans* from California have been tested over the years and have invariably been found to lack bioluminescence (Table I).

**Morphological differences**

Cell size was the most obvious morphological difference between isolates; the non-bioluminescent cells were approximately half the size of bioluminescent cells. Bioluminescent *N. scintillans* GM2017 cells had an equivalent spherical diameter of 468 ± 49 µm ($N$ = 1126 cells), while that for the non-bioluminescent strain LJ2017 was 266 ± 37 µm ($N$ = 2872) (Figure S1), representing a 43% smaller size. Otherwise the strains were morphologically indistinguishable except for their ability to produce light when mechanically disturbed.

**Detection and partial characterization of luciferase gene remnants**

We tested one bioluminescent and two non-bioluminescent strains of *N. scintillans* for the presence and expression of *lcf* using "universal" PCR primers for dinoflagellate *lcf* (Valiadi et al. 2012). Fragments of the correct size (~ 270 bp) were amplified from genomic DNA isolated from all strains (Figure S2).

Sequencing of a large region of the *lcf*/*lbp* open reading frame revealed that the non-bioluminescent strains contain *lcf*/*lbp* pseudogenes with multiple mutations (Figure S3). These are mainly deletions ranging from 2 to 96 bp. The highest number of mutations was found in strain SPMC originating from Washington (USA), where sequences of several *loci* contained deletions of at least 36 bp. In comparison, none of the sequences of the *lcf*/*lbp* ORF from bioluminescent *N. scintillans* examined in this study showed any deleterious mutations. Sequence alignments with *lcf*/*lbp* of another bioluminescent isolate from the Gulf of Mexico examined previously (Valiadi and Iglesias-Rodriguez 2014), and bioluminescent environmental *N. scintillans* samples from the southwest Atlantic (Valiadi et al. 2014), showed that while bioluminescent *N. scintillans lcf/lbp* exhibit deletions, these have a maximum size of 3 bp. No *N.*

*scintillans lcf/lbp* sequences were present in the TaraOceans metagenome from the northeast Pacific area.

Reverse Transcription-PCR only detected *lcf* in cDNA derived from RNA isolated from the bioluminescent strain (Table I, Figure S2). The successful amplification of the dinoflagellate SSU rDNA fragment from the cDNAs of the two non-bioluminescent strains, LJ2000 and SPMC136, verified the quality of the cDNA and therefore the negative result obtained using the *lcf* primers. Therefore, while the *lcf* gene was present in the genomes of both bioluminescent and non-bioluminescent strains, its transcript was undetectable in cells of the latter.

**Identification of scintillons and luciferin by microscopy**

The presence of scintillons is indicated by blue autofluorescence of dinoflagellate luciferin in response to ultraviolet light excitation (Johnson et al. 1985; Fritz et al. 1990). All *N. scintillans* strains were examined by confocal laser scanning microscopy to determine the presence and cellular location of luciferin. Cells of the bioluminescent strain exhibited blue fluorescent sources around the cell periphery (Figure S4), consistent with the presence of luciferin in scintillons. The number of scintillons in each cell was sufficiently high that due to their small size the fluorescence merged into a nearly continuous glow around the cell periphery. As scintillons are small in comparison to the large cell size it was not possible to determine their abundance from the confocal microscope images. Cells of the non-bioluminescent strains LJ2000 and SPMC136 did not exhibit any blue autofluorescence, suggesting that they lack luciferin.

The presence of scintillons in the cells of the bioluminescent strain GM and the non-bioluminescent strain SPMC136 was further investigated using transmission electron microscopy (Figure 2). The bioluminescent strain GM cells contained smooth membrane-bound electrondense organelles of 0.7 to 0.9 µm size, around the cell periphery near the cell wall,

identical in size and appearance to scintillons (Nicolas et al. 1985; Nicolas et al. 1987). These occurred in clusters with, or near, trichocysts. Organelles of equivalent electron density, morphology, size and similar in appearance to scintillons of the bioluminescent strain were also observed in cells of the non-bioluminescent strain SPMC136. Additionally, there were several structures of smaller size that also occurred near the cell covering and were surrounded by a membrane that resembled scintillons. However, their contents were granular, often with a very regular pattern. These scintillon-like structures were unique to the non-bioluminescent strain.

**Luciferin and luciferase extracts**

As previously reported by Liu et al. (2007), cross-reactions of luciferin ('hot') and luciferase ('cold') extracts confirmed that *Pyrocystis lunula* luciferin reacted strongly with *N. scintillans* GM2017 luciferase ($3.9 \pm 0.2 \times 10^7$ RLU $s^{-1}$, N = 3), and that the luciferase extract also contained co-extracted endogenous luciferin, based on light emission without the addition of *P. lunula* luciferin ($3.3 \pm 0.4 \times 10^5$ RLU $s^{-1}$, N = 3). The luciferase extract of the non-bioluminescent strain LJ2017 displayed weak but nonzero luciferase activity in the presence of *P. lunula* luciferin ($1.1 \pm 0.1 \times 10^4$ RLU $s^{-1}$, N = 3). However, unlike the strain GM extract it did not emit light of its own accord ($23.7 \pm 4.0$ RLU $s^{-1}$, N = 3; similar to the blank), indicating the absence of luciferin in strain LJ2017.

We employed Liquid Chromatography-Mass Spectrometry (LC-MS) to examine the presence of luciferin metabolites in *N. scintillans*. While *P. lunula* luciferin can cross-react with luciferases from other dinoflagellates (Schmitter et al. 1976; Liu and Hastings 2007), it is unknown whether the endogenous luciferins are identical. Despite considerable efforts, we were unable to detect luciferin in its reduced (native) form in bioluminescent *N. scintillans* strain

GM2017. We confirmed that this was not due to a methodological failure by successful detection of reduced luciferin in *P. lunula* extracts, provided ultrapure nitrogen was used as drying gas in the mass spectrometer source. As the presence of endogenous luciferin in strain GM2017 is corroborated by microscopy, luminometry, and the observation that luciferase extracted from this strain exhibits luminescence even in the absence of added luciferin, we suspect that the reduced luciferin is either tightly bound to the LBP domain of the LCF-LBP protein, or enzymatically or non-enzymatically oxidized during the luciferin extraction procedure. These results suggest that alternative methods are required to detect native luciferin in LBP-containing dinoflagellates.

Nevertheless, we were able to detect enzymatically oxidized luciferin (oxyluciferin) and spontaneously air-oxidized luciferin in aqueous extracts of strain GM2017, as indicated by exact masses, tandem MS fragmentation patterns and UV-Vis absorbance maxima consistent with those from previous reports (Figure 3, S5) (Nakamura et al. 1989). The presence of these luciferin-derived metabolites suggests that bioluminescent *N. scintillans* GM2017 may harbor the same luciferin as *P. lunula*, which supports our suggestion that the reduced form was not detected due to it being bound to LBP. In contrast, no luciferin metabolites were detected in the non-bioluminescent *N. scintillans* strain LJ2017.

**Phylogenetic analyses based on LSU rDNA**

Bayesian phylogenetic analyses of *N. scintillans* based on a 595-bp alignment of partial LSU rDNA sequences did not resolve non-bioluminescent *N. scintillans* as a separate group. Rather, they clustered with sequences from isolates originating from the South China Sea and were not significantly different from other isolates originating from other Chinese seas or from the Arabian Sea; different clone sequences from most isolates included in the analysis were intermingled (Figure 4). Both these regions contain bioluminescent *N. scintillans*: the red variety

in China and the green variety in the Arabian Sea. The Gulf of Mexico bioluminescent strain, which was the only representative from the Atlantic Ocean, was distinct from all other sequences.

To better characterize the high intra-strain variability of LSU rDNA in natural *N. scintillans* populations, we retrieved 5 *N. scintillans* LSU rDNA Unigenes of approximately 400 bp in length from the TaraOceans dataset. None of these originated from within our study region in the coastal waters of the northeast Pacific, but rather showed the highest abundances in the open northeast and southeast Pacific Ocean, Arabian Sea and Mediterranean Sea. Nevertheless, we included these sequences in a new alignment and performed a cluster analysis based on genetic distance. This truncated dataset contained only 185 base positions, where all sequences overlapped. Despite its limited phylogenetic resolution, the analysis showed that the TaraOceans dataset clustered in a significantly different group, unrelated to any other *N. scintillans* LSU rDNA sequences known to date (Figure S6).

## Discussion

The globally-distributed dinoflagellate *N. scintillans*, historically well-known for its bright bioluminescence (Harvey 1957), has non-bioluminescent populations along the west coast of the USA (Chang 1960; Eckert and Findlay 1962; Sweeney 1963; Esaias 1973; Dewey 1976; Balch and Haxo 1984; Sulkin et al. 1998; this study). The results of this study indicate that the bioluminescence system of these non-bioluminescent *N. scintillans* populations has been "silenced" and that the lack of bioluminescence is due to two factors: a mutated *lcf* with undetectable expression and the lack of luciferin.

**Non-bioluminescent *N. scintillans* cells do not express lcf**

The presence of *lcf* genes in the genome of non-bioluminescent *N. scintillans* suggests that they were bioluminescent in the past, but that the bioluminescence system has been silenced at the molecular level by numerous deletions of more than 36 bp, suggesting that this gene has lost its coding potential. Dinoflagellate gene families contain multiple copies with variable sequences; for bioluminescence genes this variability may be as high as 12% base differences (Valiadi and Iglesias-Rodriguez 2014). The presence of long stretches of deletions in the *lcf* genes of non-bioluminescent strains of normally bioluminescent dinoflagellate species has been reported previously (Valiadi et al. 2012). Pseudogenes containing frameshift deletions were also found in the *lcf* genes of bioluminescent *N. scintillans* isolates and environmental samples (Valiadi and Iglesias-Rodriguez 2014; Valiadi et al. 2014), although the maximum deletion was only 3 bp. Thus, extensive deletions in *N. scintillans lcf/lbp* appear to be confined to the non-bioluminescent strains.

While there is no clear relationship between the presence of pseudogenes and genetic function in dinoflagellates, our observation of large-scale deletions in the *lcf/lbp* genes of non-bioluminescent *N. scintillans* correlate with loss of function. The slight luciferase activity detected in one of the non-bioluminescent strains tested suggests that some of the gene copies may still be functional, and therefore the loss of bioluminescence may not be complete. The genetic mechanisms that transiently or permanently switch off genes in dinoflagellates, including those coding for bioluminescence, are unknown. The mutations present in *lcf* of non-bioluminescent *N. scintillans* may cause the lack of gene expression or be a result of it due to the lack of selection for the protein structure and function. Deep sequencing of *lcf/lbp loci* will be informative in understanding the functional genomic silencing process within dinoflagellates.

**Non-bioluminescent *N. scintillans* cells lack luciferin**

Luciferins are known to be widespread in food webs, being transferred from prey to predator, where they can accumulate in tissue even in non-bioluminescent animals (Shimomura 1987; Shimomura 2006). Thus, bioluminescent organisms may either synthesize luciferin or obtain it through their diet. For example, manipulations that deplete stores of luciferin in laboratory-maintained animals have demonstrated that the lophograstrid *Gnathophausia ingens* Dohrn requires a dietary source of coelenterazine (Frank et al. 1984), and the midshipman fish *Porichthys notatus* Girard requires cipridinid ostracod luciferin (Tsuji et al. 1972; Barnes et al. 1973). Populations of *P. notatus* found north of San Francisco Bay (Strum 1969b; Warner and Case 1980; Thompson and Tsuji 1989) are non-bioluminescent due to the lack of cipridinid luciferin (Tsuji et al. 1972; Barnes et al. 1973), presumably because the ostracod prey serving as the dietary source of luciferin is restricted to southern waters (Kornicker and Baker 1977). Despite their lack of bioluminescence, the photophores of the northern *P. notatus* are structurally identical to those of bioluminescent fish (Strum 1969a; Strum 1969b), and these populations exist in large numbers, suggesting that the lack of bioluminescence is a recent event that has not had a deleterious effect on species physiology or survival.

The absence of luciferin metabolites in non-bioluminescent *N. scintillans* cells, as well as the lack of fluorescent particles, supports earlier evidence that they lack luciferin (Eckert and Reynolds 1967). Similarly, non-bioluminescent strains of other bioluminescent dinoflagellates do not display luciferin fluorescence (Johnson et al. 1985). Dinoflagellate luciferin is structurally related to chlorophyll (Dunlap et al. 1981) and is thought to be synthesized *de novo* in photosynthetic dinoflagellates as part of the chlorophyll biosynthetic pathway (Wu et al. 2003). The origin of luciferin in heterotrophic dinoflagellates is unknown. Although non-

bioluminescent *N. scintillans* in southern California feed on bioluminescent dinoflagellate prey (Torrey 1902; Stauffer et al. 2017; Busch et al. 2019), this does not restore bioluminescence under laboratory conditions (M. Latz, personal obs.), suggesting that *N. scintillans* does not obtain luciferin from its diet. Furthermore, the bioluminescence of another heterotrophic dinoflagellate, *Protoperidinium crassipes* (Kofoid) Balech, persists when maintained on a diet composed solely of rice flour (Yamaguchi and Horiguchi 2008). As *N. scintillans* possesses a plastid tetrapyrrole pathway consistent with the endogenous production of luciferin (Janouskovec et al. 2017), the possibility of *de novo* synthesis of luciferin in *N. scintillans* and other non-photosynthetic dinoflagellates requires further investigation.

**Significance of non-bioluminescent *N. scintillans* cell architecture**

Despite lacking both luciferin and LCF, non-bioluminescent *N. scintillans* cells contain organelles that are morphologically similar in size and appearance to the scintillons of bioluminescent dinoflagellates (Nicolas et al. 1985; Nicolas et al. 1987). Similarly, non-bioluminescent northern west coast USA populations of the midshipman fish *P. notatus* contain photophores that are morphologically identical to those of bioluminescent southern populations (Strum 1969a; Strum 1969b), despite lacking luciferin. As only bioluminescence-related proteins have been found in scintillons (Desjardins and Morse 1993), the chemical composition of the smooth electron dense contents of scintillons from non-bioluminescent cells is unclear. Further investigation of the chemical composition of scintillons as well as the newly described scintillon-like organelles is required in order to establish the origin and role of the latter in non-bioluminescent cells.

Strains of the non-bioluminescent *N. scintillans* that originate from the west coast of the USA differ morphologically from bioluminescent cells from other locations only by their smaller

size. The diameter of bioluminescent cells measured in this study averaged 468 µm, which is within the range of 300-900 µm previously reported for other locations (Nawata and Sibaoka 1976; Nakamura 1998a; Tada et al. 2000; Liu and Hastings 2007; Mohamed and Mesaad 2007). In comparison, the average diameter of non-bioluminescent cells was 266 µm, which is within the range of 100-400 µm (Dewey 1976; Balch and Haxo 1984; Sulkin et al. 1998; Stauffer et al. 2017) reported for *N. scintillans* collected along the west coast of the USA. Similarly, cells of non-bioluminescent strains of the dinoflagellate *P. lunula* were found to be 50% smaller than cells of bioluminescent strains (Swift et al. 1973). However, this correlation between dinoflagellate cell size and bioluminescence ability is not universal in intraspecific comparisons, as non-bioluminescent *Alexandrium catenella* (Whedon & Kofoid) Balech cells are of similar size to bioluminescent cells (Schmidt et al. 1978). Factors that can regulate cell size include top-down control through size-selective predation, bottom-up control through environmental conditions and food supply, and genetic control related to changes in gene expression associated with the lack of bioluminescence. Although the cell size of bioluminescent *N. scintillans* varies with growth conditions, being inversely proportional to growth rate for cultured cells (Buskey 1995; Kiørboe and Titelman 1998), and water temperature for field populations (Tada et al. 2000; Yilmaz et al. 2005), the factors responsible for the smaller size of non-bioluminescent cells are unknown.

On an intraspecific level, the bioluminescence emission per cell of dinoflagellates is proportional to cell size (Buskey 1995); larger cells may benefit more from the predator protection of bioluminescence than would smaller cells if their brighter light emission is more effective in predator defense. However, the role of bioluminescence in reducing predation on *N. scintillans* is unknown. The predators of *N. scintillans* are difficult to determine because the

fragile nature of the cells does not allow them to persist in gut contents. Although they are considered too large to be grazed upon by most zooplankton (Yilmaz et al. 2005), known predators of vegetative cells include large copepods (Petipa 1960), crab larvae (Lehto et al. 1998; Sulkin et al. 1998) and gelatinous zooplankton (Daan 1989; Fock and Greve 2002; Vargas and Madin 2004; Gomes et al. 2014), and progametes are consumed by ciliates (Zhang et al. 2017a). Both non-bioluminescent and bioluminescent isolates of *N. scintillans* are capable of high population growth rates (Buskey 1995; Nakamura 1998b; Busch et al. 2019), which may be effective in overcoming predation pressure, negating the need for bioluminescence as a predator deterrent.

If gelatinous zooplankton do exert major predation pressure on *N. scintillans* along the west coast of the USA, there would be no ecological advantage in producing bioluminescence, which would be ineffective in deterring grazing by these nonvisual predators. However, gelatinous zooplankton prey on bioluminescent *N. scintillans* in other regions, and blooms of the bioluminescent dinoflagellate *Lingulodinium polyedra* (F.Stein) J.D.Dodge can immediately precede those of the non-bioluminescent *N. scintillans* in Californian waters (Sweeney 1975; Hayward et al. 1995; Gregorio and Pieper 2000; John et al. 2003). These observations suggest that the factors that regulate light production in *N. scintillans* and *L. polyedra* differ and may reflect a different physiological or ecological niche for each species.

**Phylogenetic and functional variation in dinoflagellates**

Phylogenetic analyses of LSU rDNA sequences did not resolve *N. scintillans* according to bioluminescence function, but rather grouped non-bioluminescent strains from the northwest USA with bioluminescent Pacific strains from China and the Arabian Sea. Only the bioluminescent strain from the Gulf of Mexico (southern USA, Atlantic Ocean) was clearly

different. The LSU and ITS regions of rDNA have been used to distinguish species complexes with distinct functional groups in other dinoflagellates like *Alexandrium*, based on thresholds of genetic distance (p-distance 0.02 for ITS) (Litaker et al. 2007). Our phylogenetic analyses agree with previous work comparing red *N. scintillans* populations from various Chinese Seas and an American population from the Gulf of Mexico (Pan et al. 2016), and the green *N. scintillans* from the Arabian Sea (Wang et al. 2016); despite significant functional differentiation these "varieties" appear to belong to the same species. Intraspecific functional variation is very common in dinoflagellates, particularly in toxin production in morphologically indistinguishable but phylogenetically distinct species of *Alexandrium* (Lilly et al. 2007; John et al. 2014) and in regionally distinct varieties of *Pyrodinium bahamense* L.Plate (Steidinger et al. 1980; Azanza 1997). Our findings show that loss of gene function and biochemical pathways are important in creating functional diversity within dinoflagellate species and may be relevant to other important functions like toxicity. We do note, however, that LSU rDNA sequences retrieved from the TaraOceans datasets are different from all others previously sequenced, even when comparing the same geographic region, suggesting some bias in the sequences obtained by PCR from cultured isolates. A robust phylogeographic study of *N. scintillans*, particularly in the context of functional diversity, should employ alternative PCR-independent methods, or a different phylogenetic marker.

**Potential ecological drivers of functional divergence in *N. scintillans***

Non-bioluminescent *N. scintillans* forms isolated blooms over a large area along the entire west coast of the USA. Several observations have confirmed that *N. scintillans* occurring in this region is exclusively non-bioluminescent. We speculate that the California Current, a dominant oceanographic feature of the US west coast (reviewed by Wyrtki 1967), may define the southern

519 part of the geographic range of non-bioluminescent *N. scintillans* populations. The California
520 Current is part of the North Pacific Gyre (Sverdrup et al. 1942), which transfers waters eastwards
521 from the North Pacific as part of the Subarctic Current to the Washington coast, at an
522 interannually varying latitude of approximately 45-50°N (Sydeman et al. 2011) (Figure 1). This
523 current bifurcates, with part of it flowing northward into the Gulf of Alaska, while the other part
524 flows southward towards the equator as the California Current, and then veers west when it
525 reaches the southern tip of the Baja California coast, becoming the North Equatorial Current. The
526 cell isolates in this study from La Jolla, California, are possibly at the southern-most end of the
527 non-bioluminescent *N. scintillans* range, and within the California Current. In contrast, the
528 bioluminescent variety is found in the hydrographically disconnected water of Baja California
529 (Lapota and Losee 1984) and further south off the Galapagos and Cocos Islands (Staples 1966).
530 There has been only one report of low numbers of bioluminescent *N. scintillans* cells in
531 Californian waters (Herren et al. 2004), suggesting that if these cells are transported to this area
532 or are present in the background community, they are outnumbered by the non-bioluminescent
533 variety. Thus, these functionally-distinct varieties of *N. scintillans* may occupy distinct
534 environmental niches. Geographic separation and local ecological adaptation of non-
535 bioluminescent *N. scintillans* in California may be augmented by the closed circulation in coastal
536 bays due to upwelling-associated nearshore fronts (Barth et al. 2000; Di Lorenzo 2003; Lynn et
537 al. 2003).

538 The northern and western extent of non-bioluminescent *N. scintillans* is unknown. The
539 SPMC strain used here originates from Puget Sound in Washington (approximately 50°N)
540 which, depending on the year, may be aligned with or sit north of the Subarctic Current
541 bifurcation and hence in changing proximity to the location of California Current formation

542 (Sydeman et al. 2011). The non-bioluminescent population does not extend to the Chuckchi Sea,
543 where blooms of *N. scintillans* off the north coast of Alaska (McInnes et al. 2015) are
544 bioluminescent (Staples 1966; Tibbs 1967). The northwest Pacific harbors blooms of the red
545 variety of *N. scintillans*, while in tropical waters further south (e.g., Indonesia), the
546 bioluminescent green variety is present (Harrison et al. 2011). Furthermore, although the
547 bioluminescence characteristics of red *N. scintillans* blooms in Japan, Korea and China have not
548 been studied, there are published and anecdotal reports of *N. scintillans* bioluminescence in this
549 region (Haneda 1955; Nawata and Sibaoka 1979; Sato and Hayashi 1998; Han et al. 2012). A
550 large-scale ecological and (phylo)genetic study on the different morphological and functional
551 varieties of *N. scintillans* could provide significant insight into the ecological niche of
552 dinoflagellate bioluminescence and mechanisms of evolutionary functional adaptation in
553 dinoflagellates.
554

555 **Conclusions**

556 This study of non-bioluminescent *Noctiluca scintillans* represents the most complete
557 examination to date of the molecular, biochemical and cellular basis for the intraspecific
558 functional diversity that is often observed in dinoflagellates, as well as other plankton. We show
559 that bioluminescence, an important predator defense strategy in dinoflagellates, can be
560 "silenced" at the molecular level over large spatial scales in the ocean, perhaps related to distinct
561 oceanographic provinces. As *N. scintillans* is well-known for its bioluminescence globally, the
562 loss of this property along the northeast Pacific coast suggests that the environmental factors that
563 influence light production in dinoflagellates differ between the large heterotrophic *N. scintillans*

564 and smaller photosynthetic species that flourish in the same waters. The ecological significance
565 of the loss of bioluminescence in this heterotrophic dinoflagellate requires further investigation.
566

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**FIGURE LEGENDS**

Figure 1. Map of the USA showing major ocean currents along the western seaboard where non-bioluminescent *Noctiluca scintillans* is found. The geographic origins (collection sites) of the three strains used in this study are marked: LJ – La Jolla (non-bioluminescent), California; SPMC – Shannon Point Marine Center, Washington (non-bioluminescent); GM – Gulf of Mexico (bioluminescent). Information on currents is re-drawn from Sverdrup et al. (Sverdrup et al. 1942).

Figure 2. Transmission electron micrographs of *Noctiluca scintillans* cell sections. Upper left panel – detail of the cell periphery of bioluminescent *N. scintillans* GM with a scintillon surrounded by membrane and trichocysts along the cell wall. Lower left panel – slice of the cell

871 surface of *N. scintillans* GM with a large number of scintillons. Upper right panel – detail of the
872 cell periphery of the non-bioluminescent *N. scintillans* strain SPMC136 with a scintillon
873 surrounded by a membrane near the cell wall and an additional smaller scintillon-like structure
874 with a granular content. Lower right panel – scintillon-like structure at higher magnification
875 showing detail of the internal granular structure. S = scintillons, S? = scintillon-like structure
876 with lower density contents, T = trichocyst, CW = cell covering, C = cytoplasm.
877
878 Figure 3. LC-MS analysis of dinoflagellate luciferin and its metabolites in *Noctiluca scintillans*
879 and *Pyrocystis lunula*. Charts show the summed extracted ion chromatograms for the m/z values
880 of luciferin, oxyluciferin and air-oxidized luciferin (m/z of 589.3, 603.3 and 605.3, respectively).
881
882 Figure 4. Bayesian phylogenetic tree based on a nucleotide alignment of partial LSU (large
883 subunit) rDNA sequences. Gaps were excluded in the analysis. Values shown at major nodes are
884 posterior probabilities; values lower than 0.7 are excluded. Sequences from GenBank are
885 labelled with the accession number, isolate and clone information. The geographic origins of *N.*
886 *scintillans* sequences are indicated: CA & WA – California and Washington; CS – Chinese Seas;
887 AS – Arabian Sea; GM – Gulf of Mexico. The branch length scale shows 0.3 substitutions per 10
888 sites.

Table I. *Noctiluca scintillans* cultures originating from collections in the USA. Bioluminescence (BL) was assayed by stirring (Jin et al. 2013) or manual agitation. Molecular, cellular and biochemical tests on a subset of these cultures: Luciferin fluorescence – the presence of blue autofluorescence; Luciferin metabolites – the presence of luciferin derived metabolites based on LC-MS analysis; *Lcf* in gDNA – presence of luciferase gene in genomic DNA; *Lcf* in cDNA – presence of the luciferase gene in cDNA reverse transcribed from mRNA; Scintillons – presence of scintillons in electron micrographs.

| **Collection date** | **Origin** | **BL** | **Time BL tested after collection (months)** | **ID** | **Luciferin fluorescence** | **Luciferin metabolites** | ***Lcf* in gDNA** | ***Lcf* in cDNA** | **Scintillons** |
|---|---|---|---|---|---|---|---|---|---|
| 30 Nov 1998 | Gulf of Mexico, Port Aransas, TX | + | 0.3 | GM | + | | + | + | + |
| 20 Feb 2000 | Scripps Pier, La Jolla, CA | – | 2, 6 | | | | | | |
| 21 Jan 2009 | Fidalgo Island, WA | – | 14 | SPMC136 | – | | + | – | + |
| 30 Aug 2010 | Scripps Pier, La Jolla, CA | – | 0 | LJ | | | + | – | + |
| 21 Nov 2011 | Scripps Pier, La Jolla, CA | – | 1 | | | | | | |
| 22 Mar 2012 | Scripps Pier, La Jolla, CA | – | 2 | | | | | | |
| 3 Jul 2012 | Santa Cruz, CA | – | 0.3 | | | | | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| 10 Jul 2012 | Scripps Pier, La Jolla, CA | – | 0.3 | | | |
| 12 Apr 2017 | Gulf of Mexico, Port Aransas, TX | + | 0.5, 13 | GM2017 | + | + |
| 9 May 2017 | Scripps Pier, La Jolla, CA | – | 0 | LJ2017 | – | – |
| 26 Apr 2018 | Santa Cruz, CA | – | 0.5 | | | |

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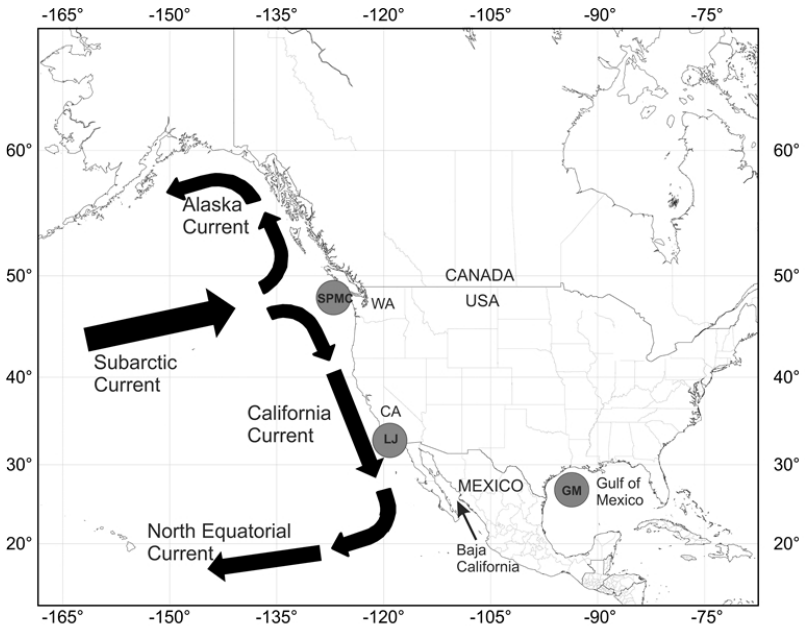


Figure 1

254x190mm (96 x 96 DPI)

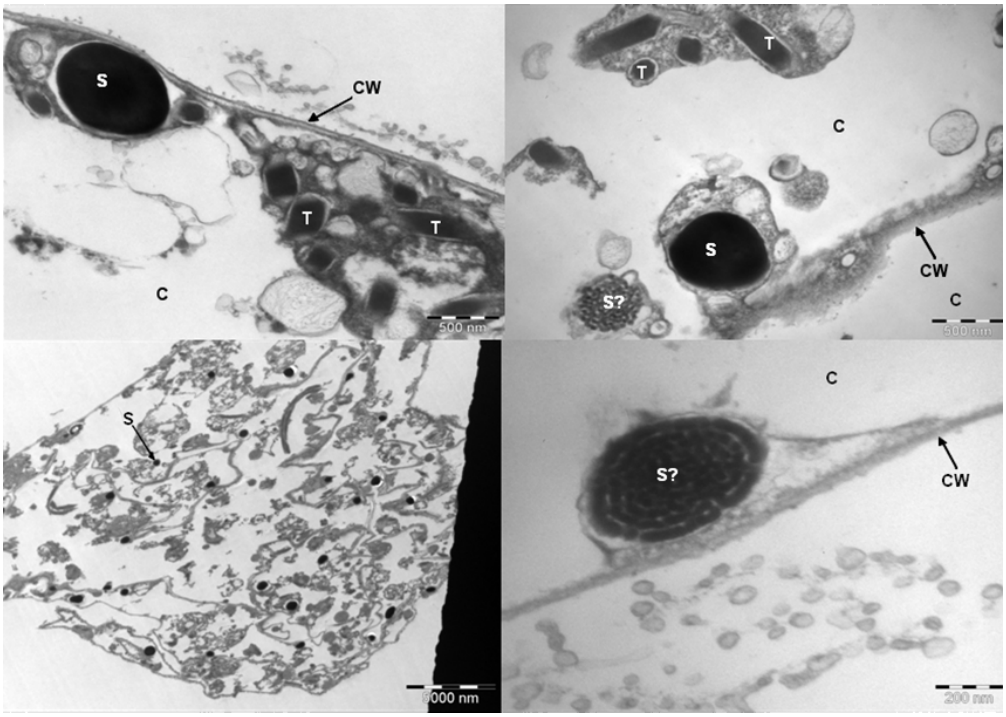


Figure 2

228x165mm (95 x 93 DPI)

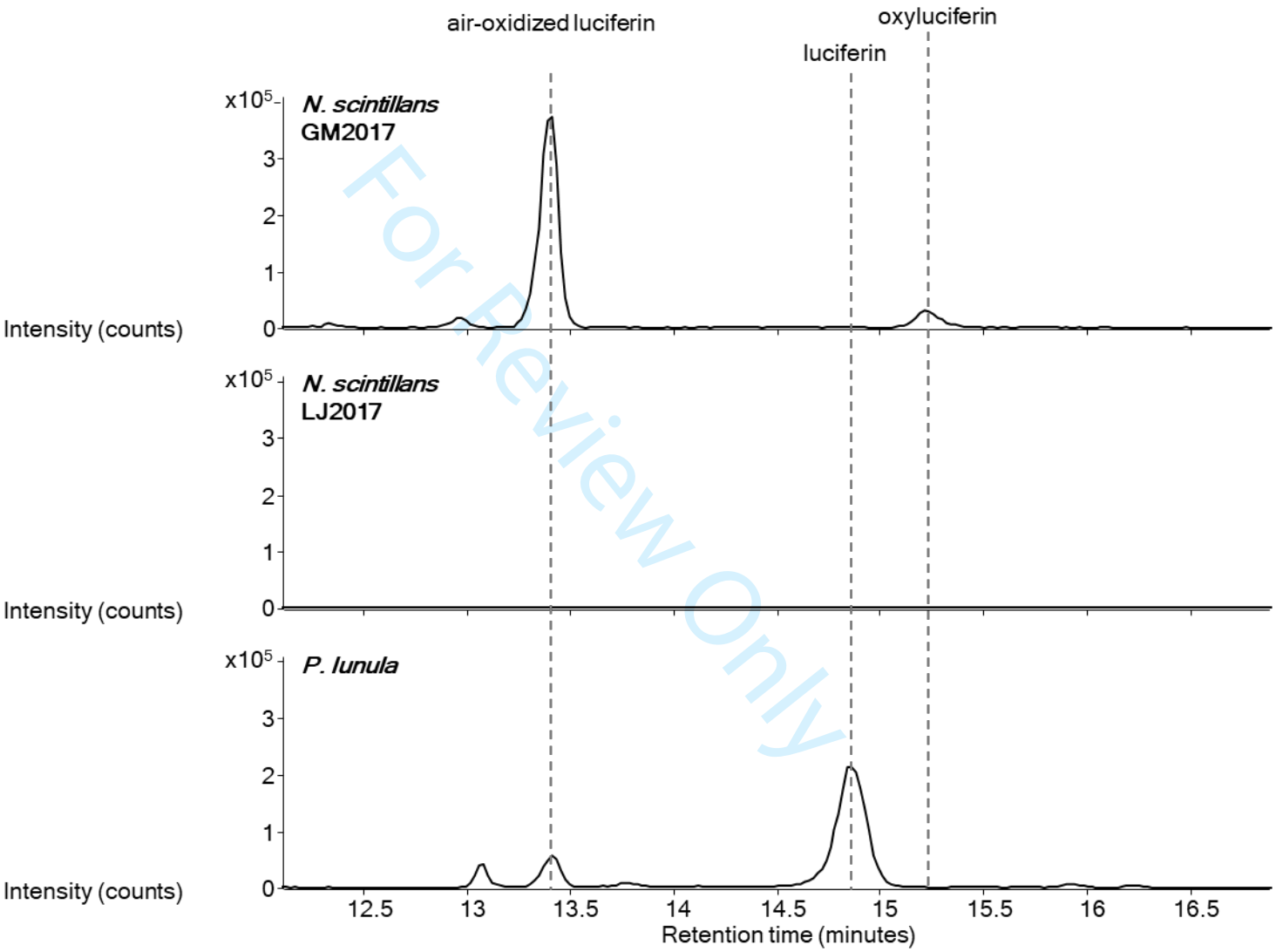

Figure 4

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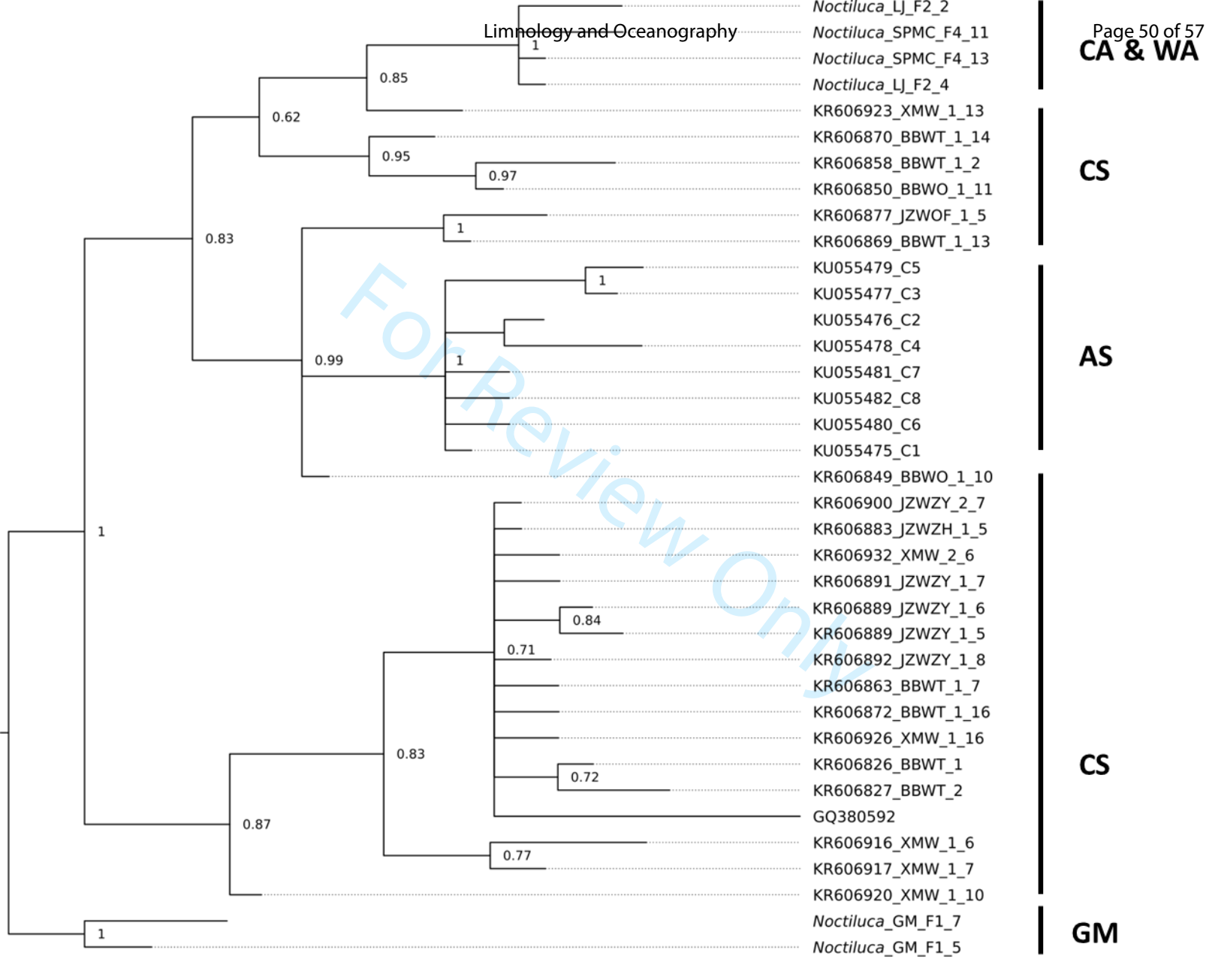
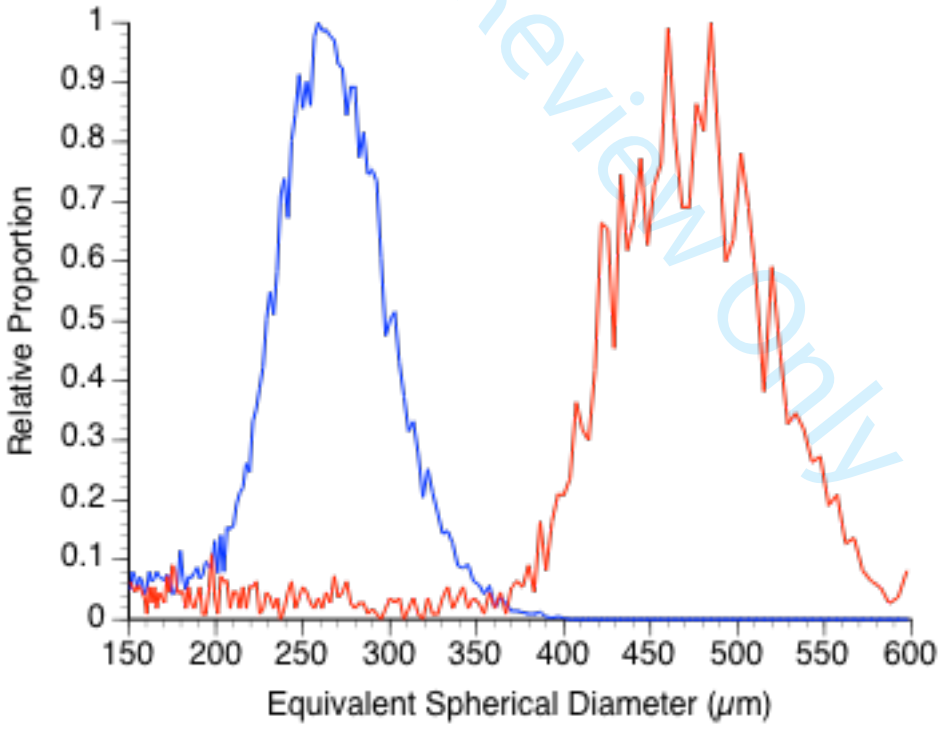


**Supporting Information**

# Molecular and biochemical basis for the loss of bioluminescence in the dinoflagellate *Noctiluca scintillans* along the west coast of the USA

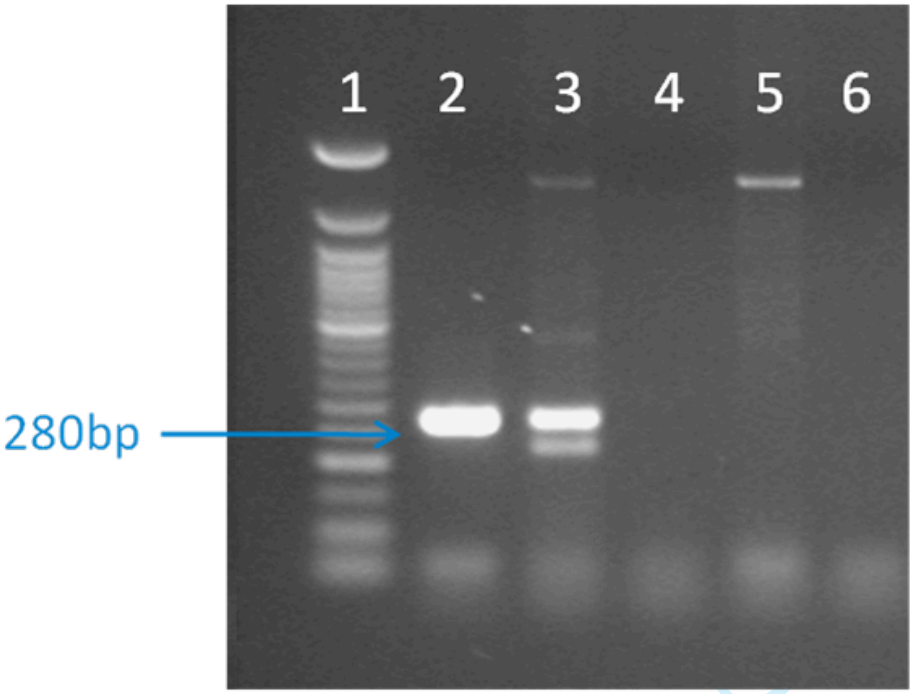
Martha Valiadi, Tristan de Rond, Ana Amorim, John R. Gittins, Chrysoula Gubili, Bradley S. Moore, M. Debora Iglesias-Rodriguez, and Michael I. Latz

Figure S1. Cell size distribution for the bioluminescent *Noctiluca scintillans* strain GM2017 (red) and smaller non-bioluminescent strain LJ2017 (blue).



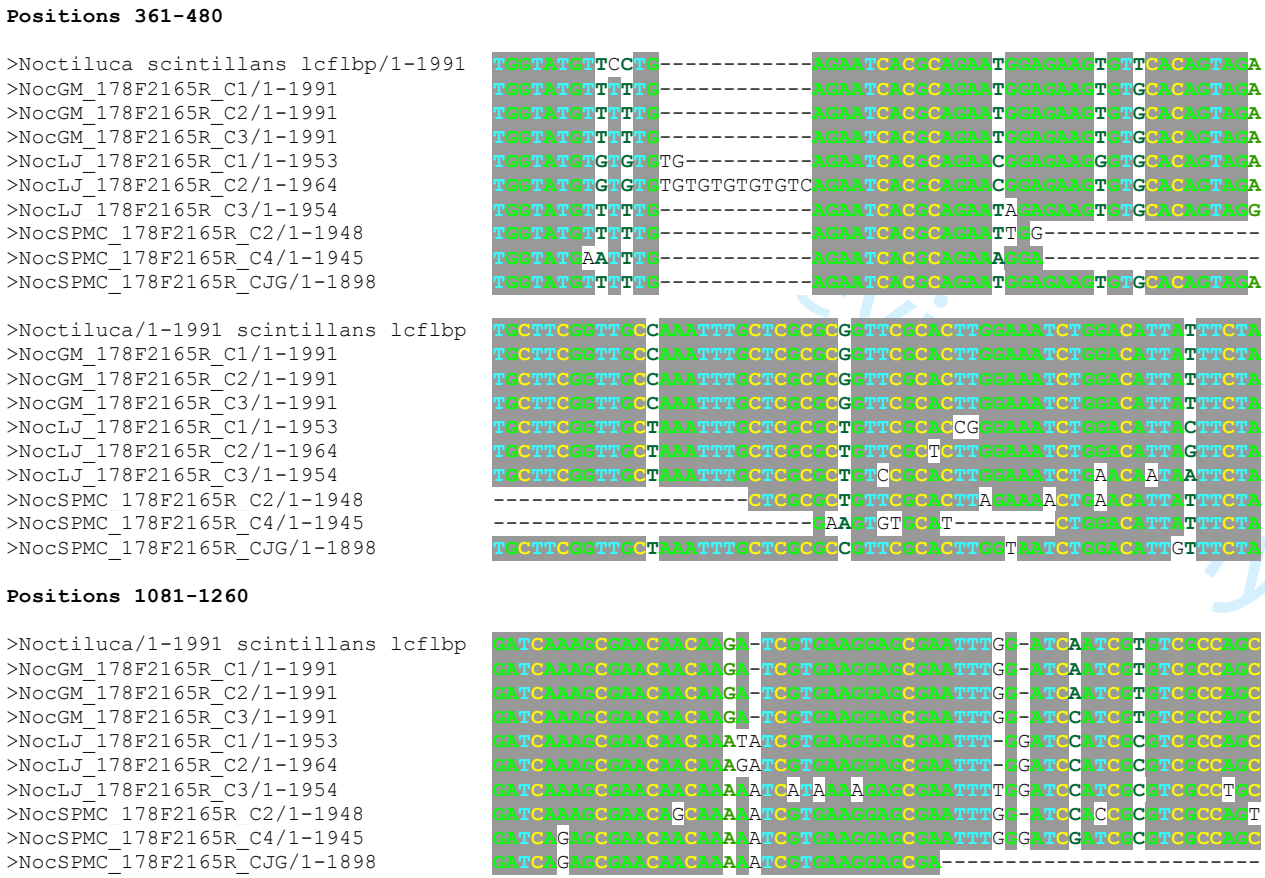
**Supporting Information**

Figure S2. Gel photograph showing the results of PCR for the detection of the *N. scintillans lcf* gene. Lanes: 1) 50-bp DNA marker; 2) Positive control strain GM, cDNA; 3) Non-bioluminescent strain SMPC136, DNA; 4) Strain SMPC136, cDNA; 5) Non-bioluminescent prey *Prorocentrum micans*, DNA; 6) No template negative control. The 280-bp *lcf* band amplified by primers DinoLcfF4/DinoLcfR2 is indicated by a blue arrow.

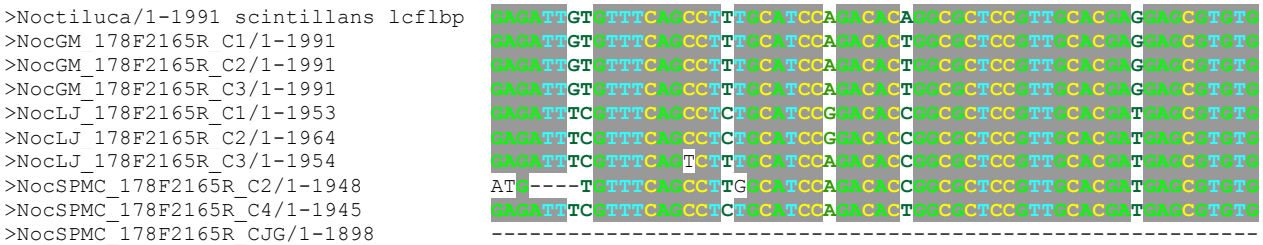


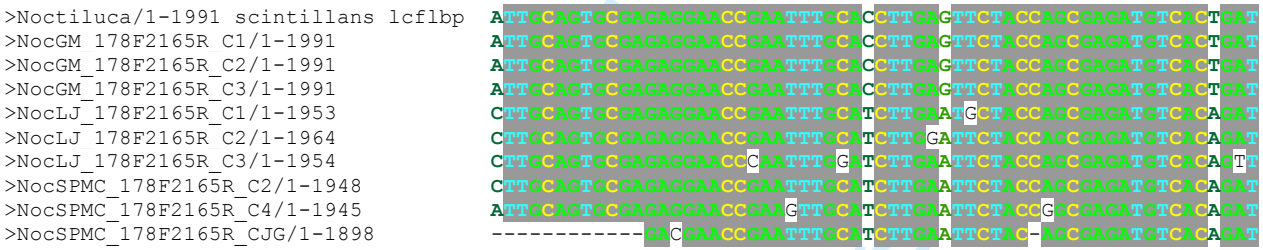
## Supporting Information

Figure S3. Alignment of partial *lcf*/*lbp* gene sequences amplified from different *N. scintillans* strains. Number preceded by C are clone identifiers. The length of the sequence obtained from each clone is given after the hyphen.

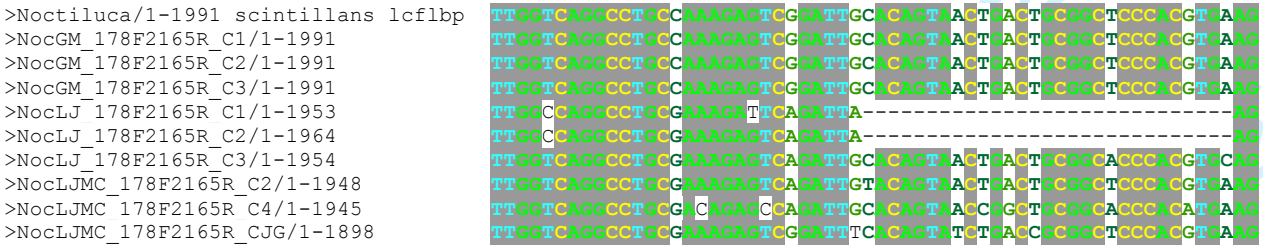


# Supporting Information



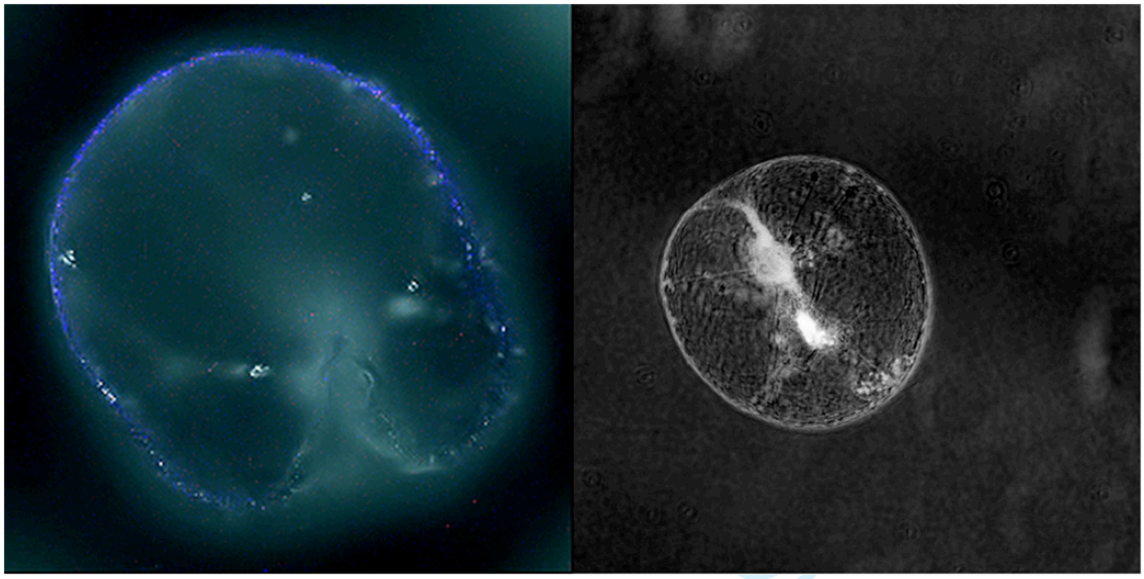




**Positions 1861-1920**



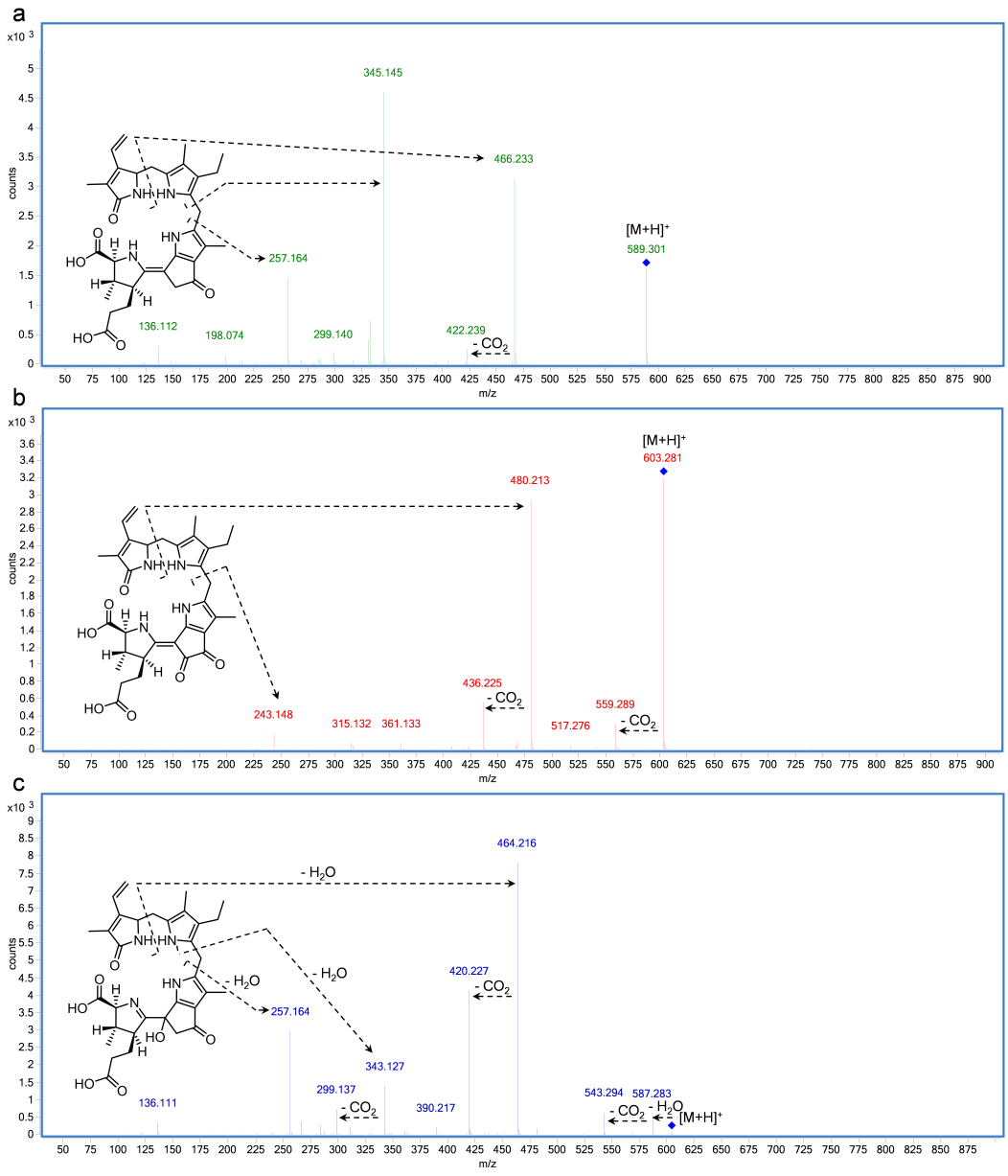
**Supporting Information**

Figure S4. Confocal laser scanning microscope images of bioluminescent and non-bioluminescent cells of *Noctiluca scintillans*, illuminated with ultraviolet light for luciferin fluorescence and red light for cell outlines. (Left) A bioluminescent cell exhibits blue fluorescence indicating the presence of luciferin within the scintillons, which are located in the cell periphery. (Right) A non-bioluminescent cell does not exhibit blue fluorescence, indicating the absence of luciferin. The cells are photographed at the same magnification to show the true size difference: the maximum width of the bioluminescent and non-bioluminescent cells shown are 440 μm and 200 μm, respectively.



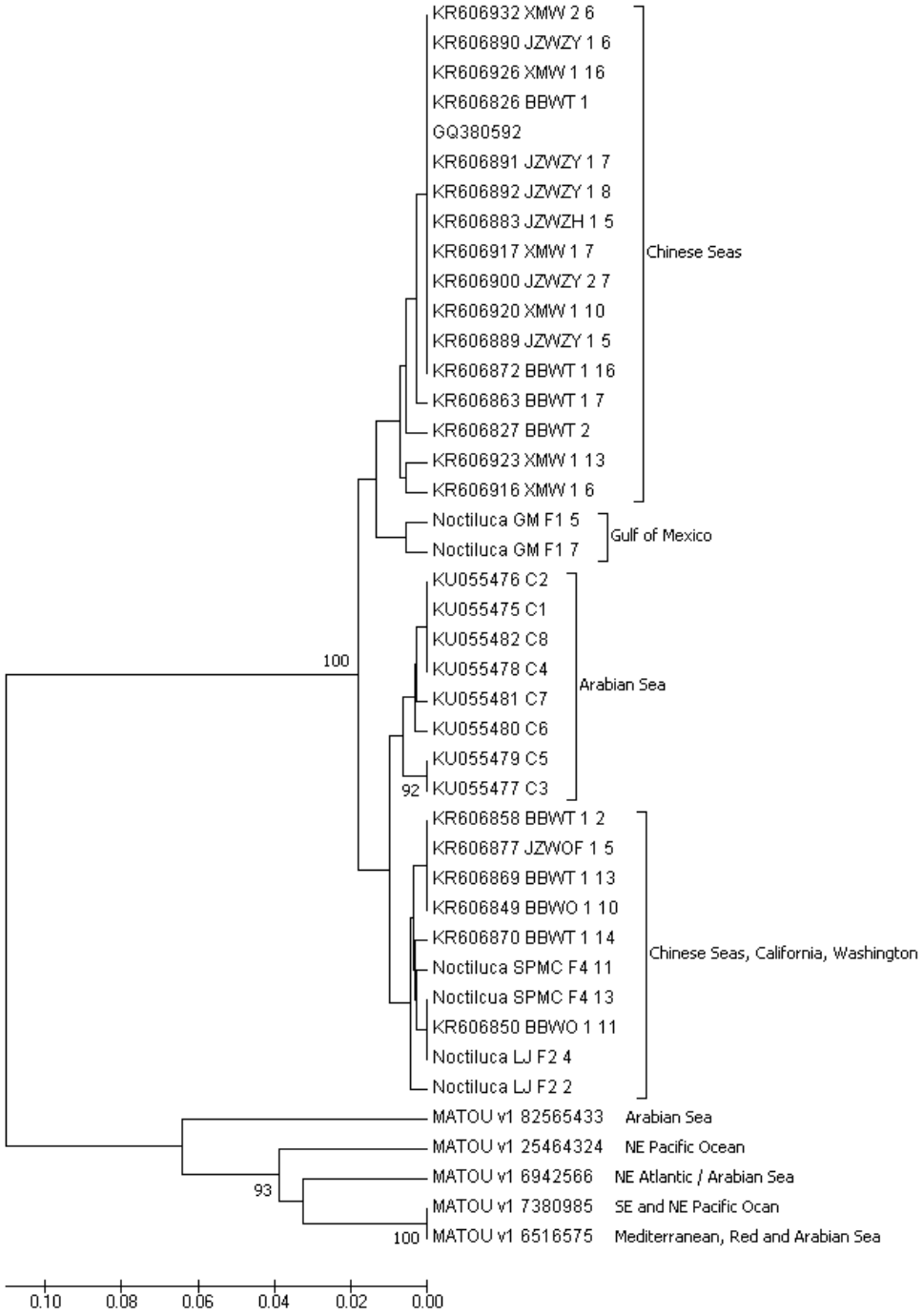
## Supporting Information

Figure S5. Characterization of dinoflagellate luciferin and its metabolites based on Tandem MS fragmentation spectrum (Collision Induced Dissociation, collision energy = 20 V) and UV/Visin-line with LC (mobile phase is water/acetonitrile 0.1% formic acid). (a) *Pyrocystis lunula* luciferin. $\lambda_{max}$ = 374 nm. HR-ESI-MS expected for $[M+H]^+$: 589.302, observed: 589.301. (b) *Noctiluca scintillans* GM oxyluciferin. $\lambda_{max}$ = 354 nm. HR-ESI-MS expected for [M+H]+: 603.281, observed: 603.280. (c) *Noctiluca scintillans* GM non-enzymatic oxidation product. $\lambda_{max}$ = 290 nm (shoulder). HR-ESI-MS expected for [M+H]+: 605.297, observed: 605.296.



**Supporting Information**

Figure S6. Dendrogram of *Noctiluca scintillans* LSU rDNA sequences, based on an alignment of 185 positions. Similarities are inferred using the UPGMA method based on p-distance. The scale shows the number of base differences per site. Values at the nodes are bootstrap values based on 100 replicates. TaraOceans Unigenes (MATOU – Marine Atlas of Tara Oceans Unigenes) are labeled with the geographic area in which they were most abundant.



**Supporting Information**

Table S1. PCR primers used in this study. Lower case letters indicate a degenerate region. Numbers in NocORF and ribosomal gene primer names indicate the nucleotide position of the respective gene at the 5′ binding site of the primer. Dino = Dinoflagellate; Noc = *N. scintillans*.

| Primer name | Gene target | Sequence (5′-3′) | Reference |
|---|---|---|---|
| DinoLcfF4 | Dino *lcf* | CGGCTACGTGCCCaaracnaaycc | Valiadi et al. (2012) |
| DinoLcfR2 | Dino *lcf* | CACCAGGGGCTCGtaraartartg | Valiadi et al. (2012) |
| NocORF178F | Noc *lcf*/*lbp* | ACCGAAGCTGTTTTGGATTG | Present study |
| NocORF2165R | Noc *lcf*/*lbp* | ACGTCATGTCCTTCCTCCAC | Present study |
| NocLSU54F | Noc LSU rDNA | ATGGCGAATGAATTGGG | Present study |
| NocLSU1021R | Noc LSU rDNA | CACGTTCCCGATTCCTCTAA | Present study |