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BIOLUMINESCENCE IN DINOFLAGELLATES – DIVERSITY, MOLECULAR PHYLOGENY AND FIELD ECOLOGY

by

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ABSTRACT

Marine dinoflagellates are an ecologically important group of protists within the plankton, performing key process such as photosynthesis, heterotrophy and toxin production. Some dinoflagellates are also capable of producing bioluminescence and they are the most abundant protists that produce light in the surface waters of the oceans. This study employed molecular tools to investigate the identity of bioluminescent species, the genetic basis, diversity and functional regulation of bioluminescence, and the distribution of marine bioluminescent dinoflagellate populations.

Using "universal" and taxon specific PCR primers designed to amplify the luciferase gene (*lcf*), the distribution of this gene within dinoflagellates was found to be taxon specific and the first *lcf* sequences obtained from four genetically distant genera revealed a previously unknown high diversity of this gene. The luciferin binding protein gene (*lbp*) was detected for the first time in three genera of ecologically important gonyaulacoid dinoflagellates, showing that this understudied gene is common in dinoflagellate bioluminescence systems. Phylogenetic analyses of both *lcf* and *lbp* provided new insight on species divergence within the toxic genus *Alexandrium* and pointed out important pitfalls in using protein coding genes for phylogenetic studies. The *lcf* PCR primers were employed in the first field study using a molecular approach to detect natural populations of bioluminescent dinoflagellates, showing that this approach outperforms optical bioluminescence measurements and that their distribution and composition is intimately tied to hydrographic patterns that create distinct environmental zones. The *lcf* PCR primers were also employed to study the regional variation in bioluminescence of *Noctiluca scintillans*, showing for the first time that the environment maintains or eliminates bioluminescence, by finding that a hydrographically isolated non bioluminescent variety in the west coast of the USA has "switched off" its bioluminescence system while diverging to possibly become a different species. Also, a novel *lbp* was discovered in *N. scintillans* which improved the current model for the evolution of bioluminescence genes in dinoflagellates.

This thesis is the first study to employ molecular tools in a comprehensive and multifaceted investigation of dinoflagellate bioluminescence and has contributed significantly to building a fundamental understanding of this remarkable phenomenon.

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Finally, a very big thank you to my partner Sebastian Steigenberger, my family and friends for their immense love and support!

DECLARATION OF AUTHORSHIP

I, Martha Valiadi declare that the thesis entitled 'Bioluminescence in dinoflagellates – diversity, molecular phylogeny and field ecology' and the work presented in the thesis are both my own, and have been generated by me as the result of my own original research. I confirm that:

- this work was done wholly or mainly while in candidature for a research degree at this University;
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Chapter 1

INTRODUCTION

1.1 General introduction

Microorganisms are a key component of marine pelagic ecosystems and a driving force of global biogeochemical cycles. Among these, phytoplankton are responsible for approximately 40% of the global annual primary production (Field et al. 1998). The organic matter constituting the phytoplankton sinks to the oceans' interior, removing carbon from the atmosphere and upper ocean in a process known as the biological carbon pump, the strength of which is controlled by grazing pressure and organic matter remineralisation activities by heterotrophic microorganisms (Falkowski et al. 1998, Ducklow et al. 2001). Thus, marine microbial ecosystems and the factors that govern the interactions of the organisms within them, have a fundamental importance on a global scale.

The marine environment contains an immense diversity of microbes that have evolved to perform equally diverse functions in their respective environments and ecological niches. The interactions between the functional characteristics of species or groups of individuals determine the net structure and function of the entire community (Strom 2008, Fuhrman 2009). Deciphering these interactions is central to understanding the structure of life and its implications on a global scale. However, this requires a holistic knowledge of the metabolic capabilities and functional roles of each species (Strom 2008) and how these may vary among the individuals within each species (Tillmann et al. 2009, Alpermann et al. 2010) or at distinct life cycle stages (reviewed by von Dassow and Montresor 2011). Historically, research has focused on functional groups defined by their morphology (e.g. cell wall type), without considering the diversity of functional roles within these groups. More recently, population level studies have revealed the enormous genetic (e.g. Iglesias-Rodríguez et al. 2006, Lowe et al. 2010) and phenotypic diversity (Burkholder and Glibert 2006, Lakeman et al. 2009) that can occur within a given species, questioning the validity and usefulness of the morphospecies

concept in microbial biology. With the enormous complexity of marine microbial ecosystems being increasingly recognised, delineating the roles of particular organisms requires information about all aspects of their biology, including metabolic capabilities, tolerance to physical conditions as well as interactions with other organisms.

One of the most ecologically complex groups of organisms in marine ecosystems are the dinoflagellates. They are a large group of aquatic protists that have developed a remarkably diverse array of unique genomic features, morphologies, nutritional types, behaviours and ecological roles through an up to 500My long (Moldowan and Jacobson 2000) and complex evolution (Hackett et al. 2004). Because of the ability of many dinoflagellate species to produce toxins and form harmful algal blooms (HABs) they have received considerable attention from researchers investigating their morphological and genetic relatedness, nutritional preferences, life cycles (Smayda 1997, Glibert et al. 2005) and more recently their strategies of eliminating predators (Tillmann and John 2002). A remarkable feature of the dinoflagellates is that some species of the group are bioluminescent. Despite bioluminescence being present in a number of ecologically important bloom forming species (Poupin et al. 1999) and its potential role as a defence against predators (e.g. Abrahams and Townsend 1993), this fascinating phenomenon has received little attention. This project therefore focused on developing a basic but multifaceted understanding of bioluminescence in dinoflagellates.

The work presented here covers a large number of distinct topics and therefore the chapters 2 to 5 are structured as stand-alone research articles, each with a relatively concise introduction. Chapter 1 provides the reader with a more general and detailed introduction to various aspects of dinoflagellate biology and the genetic, functional and regulatory aspects of their bioluminescence systems that provide the background rationale leading to the aims and objectives of this project.

1.2 Dinoflagellates

1.2.1 General characteristics

The vast majority of modern dinoflagellate species are marine and occur as free living planktonic swimming cells (Gomez 2005), although a few species are globally important symbionts or parasites. Dinoflagellates currently number more than 2000 species (Guiry 2011) with sizes ranging from 2 μm to 2 mm, and with new species being constantly described or re-described after molecular analyses. Additionally, environmental barcoding has highlighted the previously unknown high diversity of dinoflagellates in nature, representing both species that are difficult to culture as well as the contribution of still unidentified species within the picoplanktonic (< 3 μm) size class (Lin et al. 2009, Stern et al. 2010).

About half of the planktonic dinoflagellates are photosynthetic, but with heterotrophic capabilities being recognised in an increasing number of species, they are best termed as mixotrophs (Stoecker 1999, Jeong et al. 2005c); strictly autotrophic species probably do not exist. Nevertheless, photosynthetic species constitute one of the three major eukaryotic phytoplankton groups in the oceans and they are important primary producers in coastal regions where they regularly form blooms. The other half are heterotrophic feeding on other phytoplankton, microzooplankton or other dinoflagellates and can sometimes exert considerable pressure on other microbes (Jeong et al. 2005a, Sherr and Sherr 2008). Thus, dinoflagellates fall within the phytoplankton and microzooplankton, challenging traditional 'black box' concepts of functional groups in biological oceanography.

1.2.2 Group identifiers

Genetic characteristics

Molecular and microscopic studies have revealed several features that are unique to dinoflagellates and therefore serve as identifiers of the group. These are a unique 'dinokaryotic' nucleus with permanently condensed chromosomes that lacks typical eukaryotic histone proteins in at least one life stage, unique mitosis (reviewed by Hackett et al. 2004), mRNA maturation by spliced leader trans splicing (Zhang et al. 2007b) and

mitochondrial mRNA editing (Lin et al. 2002, Zhang and Lin 2008). In addition, their genomes are among the largest in nature with sizes up to ~75 times that of humans (Hackett et al. 2004), being composed of numerous and mostly non identical copies of genes that typically lack introns and are arranged in tandem arrays among large amounts of non coding DNA (Lin 2011).

Morphology and taxonomy

The taxonomy of dinoflagellates has been described by biologists based on the free living forms and by geologists based on fossilized resting cysts that are produced by ~15% of species (Matsuoka and Fukuyo 2003) and that remain in sediments dated from the Triassic onwards (~200Mya). Since Fensome et al. (1993) produced an in depth effort to reconcile the biological and geological schemes, molecular phylogenetic studies have regularly challenged designated morphospecies and described new ones (e.g. Lilly et al. 2007).

Morphologically, dinoflagellates are distinguished by two dissimilar flagella which differ in their positions in dinokont and desmokont forms; in dinokonts both flagella lie in grooves of the cell and one of them forms a “belt” around the cell, but in desmokonts the flagella are not associated with grooves. This type of morphology is exhibited in at least one life cycle stage. The cell wall contains vesicles that may be filled with cellulose, giving cells a plate like armour, the theca, whose intricate patterns are regularly used for taxonomic identification of species. The morphologies of vegetative dinoflagellate cells are highly diverse, including both thecate and athecate forms of all sorts of shapes and sizes that may be motile or immotile; thecate cells may also have horns. Additionally, their morphology can change significantly over the daily cycle (Pizay et al. 2009) or with environmental conditions (Zirbel et al. 2000) and therefore the usefulness of morphological characters in dinoflagellate taxonomy is questionable. Despite dinoflagellate systematics still being in development, some major taxonomic groups are recognised. Some of the taxa that will be specifically dealt with in this project are summarised in Table 1 with a brief description.

Table 1.1 General characteristics of some major dinoflagellate taxonomic orders.

Order	Example genus	Characteristics
Gonyaulacales	*Alexandrium*	Thecate dinokont, mixotrophic, some species toxic, form blooms
Gymnodiniales	*Gymnodinium*	Athecate dinokont, mixotrophic and heterotrophic, some species toxic, form blooms
Noctilucales	*Noctiluca*	Athecate large vacuolated cell; heterotrophic
Peridiniales	*Protoperidinium*	Thecate dinokont, mixotrophic and heterotrophic
Prorocentrales	*Prorocentrum*	Thecate desmokont, mixotrophic, some species toxic

1.2.3 Life cycles

Dinoflagellate life cycles vary significantly among species (reviewed by Coats 2002). It is generally thought that asexual reproduction is prevalent during periods of growth. However, under adverse conditions such as nutrient limitation, sexual reproduction may occur. Dinoflagellates are normally haploid, except for the genera *Pyrocystis* (Seo and Fritz 2006) and *Noctiluca* (Fukuda and Endoh 2006), and so the main life cycle information comes from haploid species. Sexual reproduction is initiated with the formation of gametes. These gametes fuse to form a diploid planozygote which in turn may form a cyst, some of which are resting cysts (see section 1.2.2). These resting cysts sink to the sediment where they lay dormant (sometimes for a mandatory period of time) until favourable conditions return, enabling swift re-emergence of the population (Anderson 1997).

1.2.4 Origins of plastids

In addition to the diverse morphologies displayed by dinoflagellates, the pigmentation in photosynthetic species is equally diverse. This is a result of multiple losses and replacements of plastids (photosynthetic organelles) gained through an unprecedented number of secondary and tertiary endosymbiotic events, the latter being unique to dinoflagellates (reviewed by Keeling 2010). Photosynthetic species contain chlorophyll a and c_2, beta-carotene and the characteristic dinoflagellate carotenoid peridinin as well as dinoxanthin and diadinoxanthin (Prezelin 1987). Some species have acquired fucoxanthin from diatoms and haptophytes which has replaced the peridinin (Bhattacharya and Medlin 1995, Tengs et al. 2000). Furthermore, some heterotrophic species are currently in early stages of endosymbiosis where

they can retain chloroplasts obtained from their prey but only for limited periods of time (Keeling 2010).

1.2.5 Molecular phylogenies

The origins and processes that have resulted in this high biological complexity have been the subject of many molecular phylogenetic investigations, which have largely focused on understanding the development of morphological characters, plastid transfers and development of toxin production pathways. Phylogenies based on ribosomal genes (rDNA) unequivocally place the dinoflagellates within the 'Alveolata' as a sister group to ciliates and apicomplexans (Cavalier-Smith 1998). However, within the dinoflagellate group phylogenies have regularly yielded conflicting results, often associated with poor statistical support particularly in the basal lineages. This depends on the genes used for the phylogenetic reconstruction, particularly their degree of conservation and heterogeneity of evolutionary rates (see introduction of Hoppenrath and Leander 2010). Typically, phylogenies can be based on sequences of the highly conserved small subunit (SSU/18S) rDNA, the more variable large subunit (LSU/28S) rDNA, the hypervariable internal transcribed spacers (ITS) of the rDNA, and on protein coding genes. Multigene phylogenies are becoming increasingly common (Zhang et al. 2005, Zhang et al. 2007a, Hoppenrath and Leander 2010) but taxon coverage is as yet incomplete and therefore does not allow for large scale comparisons across the whole dinoflagellate group. The obstacles in dinoflagellate molecular systematics are highlighted in the highly debated position of *Noctiluca scintillans* which is either placed within basal lineages based on rDNA and some protein coding genes (Fukuda and Endoh 2008, Ki 2010) or as a derived lineage based on other protein coding genes (Hoppenrath and Leander 2010).

1.2.6 Harmful algal blooms

Dinoflagellates are notorious in coastal areas for their ability to form harmful algal blooms (HABs), leading to adverse effects caused mainly by toxins but also due to anoxia or simple water discoloration. These blooms are formed by a large variety of primarily photosynthetic dinoflagellates and these collectively account for ~75% of all known HAB species (Smayda 1997). Dinoflagellate HABs have large socioeconomic impacts as they can cause extensive fish kills and human illnesses through the consumption of shellfish contaminated with various

dinoflagellate toxins leading to paralytic, diarrhetic and neurotoxic shellfish poisoning (PSP, DSP and NSP, respectively). Evidence suggests that the magnitude and frequency of HABs have been increasing globally since the 1980s (Hallegraeff 1993). Hence, much of the research on dinoflagellate genomics, physiology, functional traits and ecology has focused on HAB species.

1.2.7 Intraspecific functional diversity

As partially sexually reproducing organisms, dinoflagellate morphospecies are known to exhibit considerable genotypic variation (Nagai et al. 2007, Frommlet and Iglesias-Rodriguez 2008, Masseret et al. 2009, Lowe et al. 2010), similar to other phytoplankton where this can sometimes be associated with spatial isolation (Iglesias-Rodriguez et al. 2002, Rynearson and Virginia Armbrust 2004, Casteleyn et al. 2010). Similarly, phytoplankton exhibit intraspecific differences in growth rates and "housekeeping" processes such as nutrient uptake (e.g. Carpenter 1971, Doblin et al. 2000). More recently, research in dinoflagellates has revealed intraspecific differences in non "housekeeping" (i.e. those whose absence does not cause immediate death) functional traits. Much research has been centred on toxin production and other lytic chemicals involved in allelopathy in HAB species, particularly in the genus *Alexandrium*. These processes differ among individuals not only in their magnitude but even in their presence, although their relationship to intraspecific genetic variation remains unclear. For example, the *Alexandrium tamarense* species complex was found to contain five phylogenetic species that were not morphologically distinct of which two were toxic and three were non toxic (Lilly et al. 2007). On the other hand, allelochemical and PSP toxin production in *A. tamarense* was not found to correlate to genotype, and was interpreted as an absence of selection pressure on these traits at the time of sampling (Alpermann et al. 2010). Thus, a major challenge that remains in dinoflagellate ecology is to link the extensive intraspecific genotypic diversity among and within species subpopulations to phenotypic diversity in functional traits that are likely to be a result of the balance between genetic exchange via sexual reproduction, physiological plasticity and differentiation due to spatiotemporal isolation. Disregarding intraspecific differences may even mask the ecological differences between individuals showing or lacking a particular trait within a population and consequently mask the ecological role of that trait as a whole. Information about intraspecific differences is therefore critical to better understand species population dynamics and their effect on the

wider community. There is therefore a need to identify molecular level processes that result in functional diversity within species.

1.2.8 Ecology

Dinoflagellates usually bloom from late spring until late summer and they exhibit a range of strategies to cope with the wide range of environments that they inhabit (Smayda 1997, Smayda and Reynolds 2003). It is difficult to generalise about the specific environmental preferences and tolerances of dinoflagellates as they differ considerably between species. Therefore, descriptions about dinoflagellate ecological traits have to be made cautiously, keeping in mind that any features are likely to primarily apply to the best studied model species.

A key feature for the development of a dinoflagellate bloom is the stratification of the water column as dinoflagellates do not tolerate intense turbulence, unlike diatoms (Smayda 1997). Under these conditions, surface waters often become depleted of inorganic nutrients but dinoflagellates can thrive and outcompete other phytoplankton as they are well adapted to utilising organic nutrients (Oh et al. 2002, Lee and Kim 2007), migrating (usually at night) to deeper nutrient rich waters when the base of the surface mixed layer is shallow enough to reach (Ji and Franks 2007) and they can also consume a wide variety of other protists and bacteria (Jeong et al. 2005a, Jeong et al. 2005b, Jeong et al. 2005c). The life cycle can also play a significant role in the initiation of blooms and survival of a population, particularly if it forms resting cysts (Anderson 1997).

The processes that catalyse the initiation, maintenance and termination of dinoflagellate blooms are only beginning to emerge as new metabolic pathways and functional properties are discovered. This is highlighted best by the large majority (up to75% in some cases) of expressed genes whose function remains unknown; this is more pronounced in dinoflagellates compared to other phytoplankton groups (John et al. 2004). For example, non photosynthetic utilisation of light by xanthorhodopsin was recently recognised to be widespread in both photosynthetic and heterotrophic dinoflagellates (Lin et al. 2010, Slamovits et al. 2011) and additionally, allelopathy was identified as a potentially important survival strategy (Tillmann and John 2002, Tillmann et al. 2008). The physiological and ecological capabilities of dinoflagellates are still largely unknown and without doubt there are several discoveries still to

be made, which leads to another understudied and remarkable property of dinoflagellates, that of bioluminescence.

1.3 Dinoflagellate bioluminescence

1.3.1 Bioluminescence in the sea

Bioluminescence, the biological production of light, is a widespread phenomenon in nature occurring mainly in marine environments. Here, bioluminescence occurs in members of planktonic bacteria and protozoa, many invertebrates and vertebrates with specialized light producing organs that harbour symbiotic bioluminescent bacteria (reviewed by Haddock et al. 2010). Marine bioluminescence is mainly of blue colour, tuned to the wavelengths that travel furthest through the water. Light is produced by a chemical reaction in the presence of oxygen involving a substrate termed ‘luciferin’, and an enzyme termed ‘luciferase’. Despite the high energetic cost thought to be associated with the production of these components as well as their mobilisation within the cell in order to generate light, in a remarkable example of convergent evolution bioluminescence is estimated to have evolved independently at least forty times (Haddock et al. 2010, Widder 2010). This has lead to the chemistries of bioluminescent reactions being as diverse and unrelated as the organisms that produce them (Hastings 1983, Wilson and Hastings 1998, Widder 2010).

Dinoflagellates are the main eukaryotic bioluminescent protists and include the only photosynthetic organisms that are capable of bioluminescence (Widder 2001, Haddock et al. 2010). In surface waters of the oceans dinoflagellates are responsible for most of the bioluminescence observed (Tett 1971). Particularly when their populations are dense, disturbance of the water during the night causes bright blue bioluminescent displays that have been reported since at least 500 BC (Harvey 1957) and are now known to occur globally (Lynch 1978). Considering the ecological importance of dinoflagellates and their prominence as producers of bioluminescence in the sea, very little research has been conducted on this topic. The following sections introduce the reader to the basic aspects of dinoflagellate bioluminescence including the major gaps in the knowledge that this project aimed to fill.

1.3.2 Bioluminescent dinoflagellate species

Bioluminescent species reportedly occur in 17 dinoflagellate genera (compiled by Poupin et al. 1999). Several bioluminescent species are cosmopolitan in both coastal and open ocean regions and include important heterotrophs (e.g. *Noctiluca* and *Protoperidinium*) and toxic (e.g. *Alexandrium*) or generally harmful species (e.g. *Noctiluca, Lingulodinium, and Ceratium*). The genera reported to include bioluminescent species are summarised in Table 2. As is generally the case with bioluminescent organisms, the ability to produce light is exhibited by phylogenetically disparate dinoflagellate taxa. Several genera consist of both bioluminescent and non bioluminescent species and even within species there is variation among clonal strains (Swift et al. 1973, Loeblich and Loeblich 1975, Schmidt et al. 1978). Furthermore, bioluminescent capabilities are known to vary regionally in *N. scintillans* (Sweeney 1963).

Conflicting reports of bioluminescence in cultures or in mixed field populations have led to considerable confusion as to which dinoflagellates species are bioluminescent, limiting studies that rely on microscopy to identify the species responsible for measured bioluminescence or to establish their distribution and seasonality using archive datasets. Additionally, this confusion precludes investigations on the diversity and evolutionary relationships of the organisms that are capable of light production. It is thus important to clarify which organisms really do have light emitting capabilities and at what molecular level is intraspecific variation in bioluminescence controlled.

1.3.3 Light production in the cell

The bioluminescent system of dinoflagellates is unique from both a cellular and molecular perspective. The production of light occurs in cellular organelles called scintillons (DeSa and Hastings 1968) which contain luciferin, the luciferase enzyme (LCF) and, in some species, a luciferin binding protein (LBP) (Schmitter et al. 1976, Johnson et al. 1985, Knaust et al. 1998, Akimoto et al. 2004). Scintillons are dense vesicles approximately 0.5 μm in diameter that sometimes protrude into the vacuole (Johnson et al. 1985, Nicolas et al. 1987) and are abundant (av. 400 per cell in *Lingulodinium polyedrum*) (DeSa and Hastings 1968) in the periphery of the cell during the hours of darkness (Fritz et al. 1990, Seo and Fritz 2000). Light

Table 1.2 Genera with reported bioluminescent species (according to Poupin et al., 1998), with their taxonomic affiliation as defined by Fensome et al. (1993) and an indication of the proportion of species within the genus that they represent; number of species are the ones currently taxonomically accepted according to Guiry (2011). Question marks indicate that the reports are disputed.

Order Family	Genus	No. of reported BL species	Total no. of species in genus
Gonyaulacales			
Ceratiaceae	*Ceratium*	8	63
Goniodomaceae	*Alexandrium*	4	31
	Pyrodinium	1	1
Cladopixidaceae	*Peridiniella*	1	3
Ceratocoryaceae	*Ceratocorys*	1	13
Gonyaulaceae	*Gonyaulax*	11	74
	Lingulodinium	1	2
Pyrocystaceae	*Pyrocystis*	1	16
Pyrophacaceae	*Fragilidium*	4	5
	Pyrophacus	1	4
Gymnodiniales			
Gymnodiniacae	*Akashiwo*?	1	1
	Gymnodinium?	1	245
	Polykrikos	1	6
Noctilucales	*Noctiluca*	1	1
Peridiniales			
Peridiniaceae	*Glenodinium*	1	22
Proroperidiniaceae	*Protoperidinium*	41	287
Prorocentrales	*Prorocentrum*?	1	63

is primarily produced by mechanical stimulation due to shear stress for example upon contact with grazers or by breaking waves (Esaias and Curl 1972, Sweeney 1987). The cell membrane deformation results in an action potential across the vacuole membrane of the cell caused by Ca^{2+} ions released from intracellular stores (von Dassow and Latz 2002). This action potential leads to an influx of protons from the acidic vacuole into the scintillons, decreasing the pH from 8 to 6 (Figure 1.1) and causing the LCF to take on its active conformation. In species that contain LBP, this protein binds the luciferin, protecting it from autoxidation at alkaline pH, and releases it by means of conformational change at pH below 7, where LCF also becomes active. Luciferin is oxidised by LCF to form oxyluciferin via an unknown process which

results in the emission of photons (reviewed by Shimomura 2006) in the form of a brief flash of blue light (approximately 475nm wavelength).

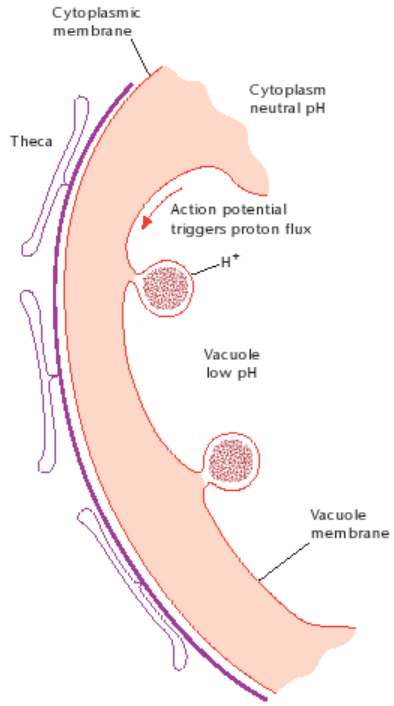

Figure 1.1 Representation of scintillon structure and location within the cell and the proposed mechanism for flash triggering (Knaust et al. 1998).

1.3.4 Molecular composition and evolution of the bioluminescence system

Luciferin

The structure of the luciferin molecule has only been characterised in *Pyrocystis lunula*, where it is a linear tetrapyrrole similar to chlorophyll a (Dunlap et al. 1980, Nakamura et al. 1989) (Figure 1.2). The molecule exhibits a characteristic blue fluorescence (475nm) when irradiated with UV light, a feature used to visualise scintillons in cells by epifluorescence microscopy. However, the fluorescence of the molecule ceases when it has been oxidised either by LCF or by air. Topalov and Kishi (2001) suggested that luciferin is a product of chlorophyll degradation by means of photooxidation, even though fluorescent luciferin is absent in *L. polyedrum* during the day and only becomes abundant during the night (Akimoto et al. 2004).

It is generally assumed that all dinoflagellate luciferins are the same as in *P. lunula* because its luciferin has been shown to cross react with the LCF of all other bioluminescent dinoflagellates species (Hamman and Seliger 1972, Schmitter et al. 1976). As the LCF of the heterotrophic species *N. scintillans* also cross reacts with *P. lunula* luciferin, it has been suggested that this organism's luciferin is derived from chlorophyll obtained through the diet (Liu and Hastings 2007). However, a recent study on the heterotrophic dinoflagellate *Protoperidinium crassipes* showed that bioluminescence persisted after the cells were cultured for one year on rice flour, and so luciferin could not have been synthesised from chlorophyll (Yamaguchi and Horiguchi 2008). Thus, the identity of luciferin in different dinoflagellate species remains an open question.

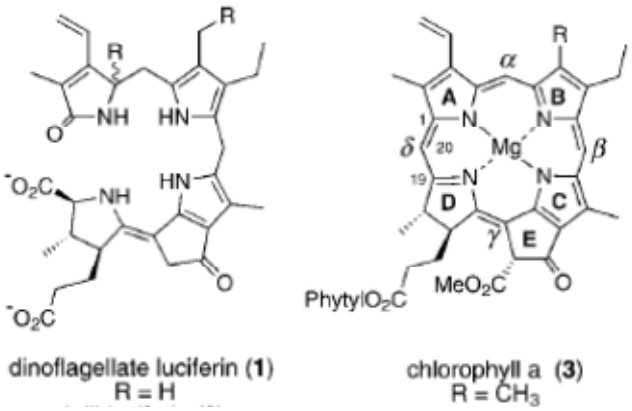

Figure 1.2 Structure of unoxidised *Pyrocystis lunula* luciferin (left) and chlorophyll a, showing the similarities of the two molecules (Topalov and Kishi 2001).

Luciferases and luciferin binding proteins

The complex evolution of dinoflagellate bioluminescence systems has been revealed through the study of the dinoflagellate luciferase gene (*lcf*) that has been fully sequenced in seven genetically closely related photosynthetic dinoflagellates of the Gonyaulacales genera *Alexandrium*, *Lingulodinium*, *Protoceratium* and *Pyrocystis* (Li and Hastings 1998, Liu et al. 2004) and in the heterotrophic dinoflagellate species *Noctiluca scintillans* (Liu and Hastings 2007). The typical gene organisation is summarised in Figure 1.3.

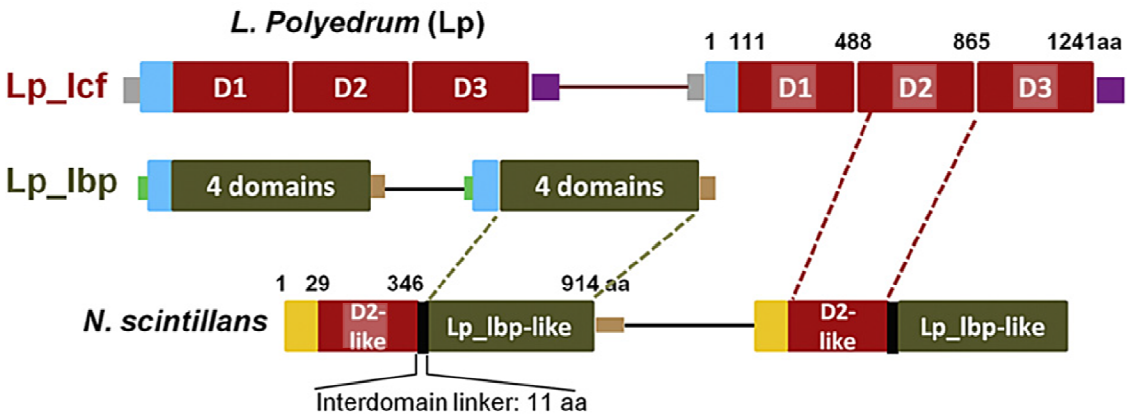

Figure 1.3 Organisation and differences between luciferase (lcf; red) and luciferin binding protein (lbp; green) genes in *Lingulodinium polyedrum* and *Noctiluca scintillans*. Blue and yellow boxes are N-terminal gene regions in the two organisms. Thinner boxes of different colours are the untranslated regions in the two genes and thin lines are intergenic regions. Numbers above domains correspond to amino acid (aa) positions. Regions encoding catalytic sites are indicated by lighter shades in each domain (on numbered gene copies). Figure modified after Lin (2011).

In photosynthetic species *lcf* is composed of three tandemly repeated domains (D1, D2 and D3) each consisting of a central region that is highly conserved at the amino acid level among domains and species and encodes a catalytically active site (Li et al. 1997). This region is flanked by more variable N- and C-terminal regions with roles in the pH response and activity of the enzyme, respectively (Li et al. 2001, Suzuki-Ogoh et al. 2008). Indeed, four histidine residues in the domain N-terminal regions are thought to induce a conformational change that exposes the otherwise folded catalytic sites (Schultz et al. 2005). An N-terminal gene region which shows similarities to glutathione-S-transferase at the amino acid level, precedes the three catalytic domains in all studied species but has an unknown function (Li and Hastings 1998, Liu et al. 2004). As with nearly all dinoflagellate protein coding genes, multiple non identical copies in tandem arrangement are present in each organism. Additionally, three isoforms of *lcf* are known to exist in *P. lunula* and one of them represents the only *lcf* known to contain an intron (Bae and Hastings 1994, Li and Hastings 1998, Okamoto et al. 2001). There are also differences in synonymous substitution rates, being uniform across each

domain of *P. lunula* but close to zero in the central domain regions in *L. polyedrum*; again the significance of these differences among genes is unknown (Okamoto et al. 2001).

Some bioluminescent dinoflagellates are also known to contain LBP. The presence of the LBP gene (*lbp*) is known in *Lingulodinium polyedrum* (Fogel and Hastings 1971, Schmitter et al. 1976), *N. scintillans* (Liu and Hastings 2007) and it has also been found in the transcriptomes of three *Alexandrium* species (Hackett et al. 2005, Erdner and Anderson 2006, Uribe et al. 2008). In fact, the only genus confirmed to lack expressed LBP is *Pyrocystis*. Therefore, while dinoflagellate bioluminescence systems occur as two distinct types (with or without LBP), the prevalence of either is unknown. The only photosynthetic species with well characterised *lbp* is *L. polyedrum.* Its *lbp* occurs as a gene family with two distinct variants showing approximately 86% identity (Lee et al. 1993, Machabée et al. 1994), each composed of 4 repeat domains with little similarity between them, unlike the *lcf* (Liu and Hastings 2007). An N-terminal gene region similar to that in *lcf* precedes the four domains and similarly to *L. polyedrum lcf* (Li and Hastings 1998), the *lbp* of this organism also lacks introns and typical transcription promoters (Lee et al. 1993), in agreement with its translational control through the circadian cycle (Morse et al. 1989b). The physical structure of LBP has not been characterised yet and so it is unclear how exactly it binds the luciferin, but it is thought to exist as a dimer that binds one luciferin molecule (Morse et al. 1989b) and to release it by conformational change below pH 7.

The bioluminescence gene of *N. scintillans* is unique as the *lcf* consists of only one domain which is shorter than that of the photosynthetic species and which is attached to *lbp* as a single hybrid gene (Liu and Hastings 2007). Furthermore, it does not contain the N-terminal gene region found in the photosynthetic species. Despite these differences, the functionally relevant central part of the *lcf* domain retains ~60% sequence similarity at the amino acid level to each of the three domains of *L. polyedrum*; this is reduced to ~40% in the *lbp* domain. The origin of this hybrid gene is difficult to interpret (Liu and Hastings 2007) and its significance is actively debated (Fukuda and Endoh 2008, Hoppenrath and Leander 2010). The triplication of the catalytic domains in the seven studied photosynthetic dinoflagellates is thought to have occurred in their common ancestor before their divergence, as corresponding domains of different species are more similar to each other, than are the three domains within each organism (Liu et al. 2004). If *N. scintillans* is a primitive dinoflagellate as suggested by many phylogenies, then the *lcf*/*lbp* could have undergone fission giving rise to the separate genes.

However, if it is a more recently derived lineage, the separate *lcf* and *lbp* could have fused in this species, with reduction of *lcf* to a single domain (Liu and Hastings 2007). The order of these events is important in understanding the evolution of these proteins in relation to different selection pressures that may require differential regulation pathways of these genes.

1.3.5 Factors influencing bioluminescence

Bioluminescence exhibits a diel rhythm controlled by an endogenous circadian clock in all dinoflagellates tested so far, except *N. scintillans* (Sweeney 1987, Buskey et al. 1992). This makes bioluminescence almost undetectable in the day and brightest at night when it is most effective. The regulation of circadian rhythms in bioluminescence has been investigated in depth in two species where they have been found to vary significantly. In *L. polyedrum* the quantity of LCF and LBP is regulated translationally (Morse et al. 1989a, Knaust et al. 1998, Mittag et al. 1998). The LCF, LBP, luciferin and the scintillons themselves are destroyed at dawn and then begin to be resynthesised at dusk; they peak in quantity approximately four hours into the night when they reach concentrations/ numbers that are 10 times higher than in the day (Dunlap and Hastings 1981, Johnson et al. 1984, Fritz et al. 1990, Akimoto et al. 2004). In contrast, *P. lunula* retains the scintillons and their contents (Knaust et al. 1998, Okamoto et al. 2001) but relocates them interchangeably with chloroplasts to control bioluminescence intensity, placing the scintillons at the periphery of the cell during the night but near the centre of the cell during the day to prevent their stimulation (Seo and Fritz 2000). Bioluminescence is also photoinhibited in dinoflagellates, particularly by blue light, which diminishes their sensitivity to mechanical stimulation (Esaias et al. 1973, Hamman et al. 1981, Sullivan and Swift 1994, Li et al. 1996) and which has been interpreted as additional evidence of the tuning of bioluminescence to the times when it is required and minimising unnecessary energy expenditure.

Further to the diel regulation of bioluminescence, the intensity of flashes can also be affected by the physiological status of the cells and environmental factors. Experiments with starved heterotrophic species (Buskey et al. 1992, Buskey et al. 1994, Li et al. 1996) and nutrient depleted cultures of photosynthetic species (Esaias et al. 1973) have all shown that bioluminescence diminished under these conditions and that the bioluminescence system then became even more sensitive to photoinhibition (Li et al. 1996). On the other hand,

bioluminescence can be photoenhanced, whereby the light emitted at night is proportional to the total amount of light received in the previous day (Sweeney et al. 1959, Sullivan and Swift 1995). While it is clear that bioluminescence output can be affected by the time of day, light and health there seem to be considerable differences between the flash characteristics of different species that have repeatedly been confirmed. The duration of a flash ranges from 80 ms in *N. scintillans* (Eckert, 1996), 130-150 ms in *L. polyedrum* (Latz and Lee 1995) to 500 ms in *Pyrocystis fusiformis* (Widder and Case 1981). The brightness of the light emitted differs considerably among species, ranging from 10^7 photons per cell in *Gonyaulax excavata* (synonym *Alexandrium tamarense*) (Schmidt et al. 1978) to 10^9 photons per cell in *Pyrocystis noctiluca* (Cussatlegras and Le Gal 2007). Reasons for this are unclear but could relate to the number of scintillons, volume of scintillons, amount of available luciferin substrate, the number of scintillons stimulated to flash which can be as few as 5% in *N. scintillans* (Eckert 1966) or simply variation in the sensitivity of each species to mechanical stimulation (Latz et al. 1994). Therefore, species bioluminescence signatures (or their lack) reflect a complex interaction of species specific response, time, physiology and environmental conditions.

1.3.6 Ecology of dinoflagellate bioluminescence

The functions of bioluminescence have mainly been studied in deep sea megafauna and in bacteria, where it has been shown to play a range of important roles in different bioluminescent organisms including oxygen defence, predator avoidance, camouflage and courtship (reviewed by Haddock et al. 2010, Widder 2010). However, the function of bioluminescence in dinoflagellates has been less extensively assessed than in other organisms and only theoretical concepts exist, supported by limited experimental evidence. In dinoflagellates, bioluminescence is proposed to act as defence against predators. The most widely accepted theory is that bioluminescence acts as a 'burglar alarm' (Burkenroad 1943): when a flash is stimulated by contact with a grazer, it will attract a higher level predator that will then consume the grazer. Indeed, laboratory experiments with *L. polyedrum* (Abrahams & Townsend, 1993) and *P. fusiformis* (Fleisher & Case, 1995) have shown that the mortality of grazers was increased and feeding of higher level predators was facilitated, in the presence of bioluminescent dinoflagellates. However, such studies have never been conducted *in situ* or even with species that co-occur in nature. Furthermore, behavioural adaptations could protect grazers from being consumed. For example, some copepods exhibit an escape response when

they are startled by flashes of light (Buskey et al. 1983, Buskey et al. 1987). Therefore, the function of dinoflagellate bioluminescence as a defence mechanism remains only theoretical. Additionally, it does not explain how bioluminescence arose and evolved in these organisms, why at certain times of night many species flash spontaneously (Sweeney and Hastings 1957, Biggley et al. 1969, Bozin and Filimonov 1985, Latz and Lee 1995) , why their genes are among those expressed the highest (Tanikawa et al. 2004, Hackett et al. 2005, Erdner and Anderson 2006, Uribe et al. 2008) and why they are upregulated under limitation by phosphate (Erdner and Anderson 2006).

The limited understanding of the ecological significance of bioluminescence in dinoflagellates stems from the lack of *in situ* studies on these organisms. Planktonic bioluminescence has only been explored in the open ocean by measuring total bioluminescence of both dinoflagellates and zooplankton using bathyphotometers. Several major field studies have attempted to relate overall bioluminescence to other measured hydrographic parameters or to chlorophyll (Swift et al. 1985, Neilson et al. 1995, Ondercin et al. 1995, Shulman et al. 2011). However, because optical bioluminescence measurements are indiscriminatory to the array of organisms that produce light and because there are temporal changes of the contributions of dinoflagellates and zooplankton to the total photon budget, these studies have largely failed to meet their aims. Furthermore, dinoflagellate bioluminescence signatures can be affected by several parameters as outlined in the previous section and are thus difficult to use in estimating the abundance of bioluminescent dinoflagellate cells. Therefore, in order to explore the little known distribution and composition of bioluminescent dinoflagellate populations in the sea, an alternative approach to optical measurements is needed.

1.4 Aims and objectives

Research into the dinoflagellate bioluminescence is still in its infancy and several questions surround many aspects of this phenomenon. The aim of the present project was to employ molecular tools, specifically polymerase chain reaction (PCR), to gain a basic understanding of several key aspects of dinoflagellate bioluminescence: the identity of bioluminescent species, the genetic composition of their bioluminescence systems, the diversity and evolution of bioluminescence related genes, the level of gene regulation that results in intraspecific

differences of light production at local and regional scales, and the distribution of these organisms in the sea. The objectives were as follows:

1. To develop PCR primers for the amplification of *lcf* from all key bioluminescent dinoflagellate taxa (Chapter 2).
2. To employ these primers to identify bioluminescent species, intraspecific differences in bioluminescence and the diversity of *lcf* across genetically close and distant species (Chapter 2).
3. To develop PCR primers for amplification of *lbp* to reveal the composition of dinoflagellate bioluminescence systems (Chapter 3).
4. To employ the *lcf* PCR primers as a tool to detect natural bioluminescent dinoflagellate populations (Chapter 4).
5. To employ the *lcf* PCR primers to investigate the causes of the regional phenotypic variation in the bioluminescence capability of *N. scintillans* (Chapter 5).

A concluding discussion of the findings and limitations of the presented work as well as suggestions for future research is given in Chapter 6.

Chapter 2

DISTRIBUTION AND GENETIC DIVERSITY OF THE LUCIFERASE GENE WITHIN MARINE DINOFLAGELLATES

Abstract

Dinoflagellates are the most abundant protists that produce bioluminescence. Currently, there is an incomplete knowledge of the identity of bioluminescent species due to the difficulties associated with optical detection of bioluminescence, arising from inter- and intraspecific variability in bioluminescence properties. In this study, PCR primers were designed to amplify the dinoflagellate luciferase gene (*lcf*) from genetically distant bioluminescent species. One of the primer pairs was “universal” while others amplified longer gene sequences from subsets of taxa. The primers were used to study the distribution of *lcf* and assess potential bioluminescence in dinoflagellate strains representing a wide variety of taxa as well as multiple strains of selected species. Strains of normally bioluminescent species always contained *lcf* even when they were permanently unable to produce light, thus proving this methodology to be a powerful tool in identifying bioluminescent species. Bioluminescence and *lcf* were confined to the Gonyaulacales, Noctilucales and Peridiniales. Considerable variation was observed among genera, or even species within some genera that contained this gene. Partial sequences of *lcf* were obtained for the genera *Ceratocorys, Ceratium*, *Fragilidium* and *Protoperidinium* as well as from previously untested species or *loci* of *Alexandrium* and *Gonyaulax*. The sequences revealed high intracopy variation that obscured the boundaries between species or even genera, some of which could be explained by the presence of two genetic variants within the same species of *Alexandrium*. Highly divergent sequences within *Alexandrium* and *Ceratium* show a more diverse composition of *lcf* than previously known.

2.1 Introduction

Bioluminescence, the production of light by living organisms, is a widespread phenomenon in nature occurring predominantly in the marine environment (Widder 2010). The only protists that are known to produce bioluminescence are marine dinoflagellates and radiolarians (Haddock et al. 2010, Widder 2010). Dinoflagellates are the most ubiquitous protists that can produce light (Tett 1971, Haddock et al. 2010, Widder 2010) and represent the only known photosynthetic organisms with this property (Sweeney 1987). Many of the dinoflagellate species that are bioluminescent are ecologically important either because they form blooms, including harmful blooms, or simply because of their cosmopolitan distribution. Their bright bioluminescence in surface oceanic and coastal waters during the night, induced by mechanical stimulation, that has been measured in all oceans of the world (Raymond and DeVries 1976, Lapota and Losee 1984, Lapota et al. 1992, Swift et al. 1995) often reported as 'glowing water' (Lynch 1978), highlights the global occurrence of these organisms.

Dinoflagellate bioluminescence is thought to function as a defence against predators. Laboratory experiments have demonstrated that bioluminescence, induced by contact with a grazer, could potentially act to increase dinoflagellate survival as the grazer is either startled (Buskey et al. 1983) or, like a 'burglar alarm', its position is revealed to a higher level predator which in turn consumes it (Burkenroad 1943, Abrahams and Townsend 1993, Fleisher and Case 1995). Therefore, bioluminescence could play a significant role in bloom formation and could influence ecosystem dynamics. However, these observations need to be tested *in situ* or with predator-prey systems that co-occur in nature.

In dinoflagellate cells light production is localised in specialised organelles termed scintillons (DeSa and Hastings 1968) which contain the substrate luciferin and the enzyme luciferase (Johnson et al. 1985, Nicolas et al. 1987) and, in at least three genera, a luciferin binding protein (Schmitter et al. 1976, Erdner and Anderson 2006, Liu and Hastings 2007, Uribe et al. 2008). Mechanical agitation of the cell triggers a cascade of events that ultimately leads to a decrease in pH within the scintillons that activates the luciferase and allows it to react with the luciferin, resulting in the emission of a short flash of light (~475 nm wavelength) (reviewed by Hastings 1996, Haddock et al. 2010).

Great insight has been gained into the evolution of dinoflagellate bioluminescence systems through the study of the dinoflagellate luciferase gene (*lcf*) which has been fully sequenced in the heterotrophic dinoflagellate species *Noctiluca scintillans* (Liu and Hastings 2007) and in seven closely related photosynthetic dinoflagellates of the Gonyaulacales genera *Alexandrium*, *Lingulodinium*, *Protoceratium* and *Pyrocystis* (Li and Hastings 1998, Liu et al. 2004). In photosynthetic species *lcf* is composed of three tandemly repeated domains (D1, D2 and D3) each consisting of a highly conserved central region that encodes a catalytically active site (Li et al. 1997), which is flanked by more variable N- and C-terminal regions with roles in the pH response and activity of the enzyme, respectively (Li et al. 2001, Suzuki-Ogoh et al. 2008); an N-terminal gene region similar to that of the dinoflagellate luciferin binding protein gene (*lbp*) precedes the three catalytic domains in all studied species (Li and Hastings 1998, Liu et al. 2004). The bioluminescence gene of *N. scintillans* is unique as the *lcf* consists of only one domain which is shorter than that of the photosynthetic species and which is attached to *lbp* as a single gene (Liu and Hastings 2007). Furthermore, it does not contain the N-terminal gene region found in the photosynthetic species. Despite these differences, the functionally relevant central part of the *lcf* domain retains high sequence similarity to each of the three domains of the photosynthetic species, particularly at the amino acid level. The triplication of the catalytic domains in the seven studied photosynthetic dinoflagellates is thought to have occurred before their speciation, as corresponding domains of different species are more similar to each other, than are the three domains within each organism (Liu et al. 2004). Whether or not the single domain *lcf*/*lbp* hybrid gene of *N. scintillans* is ancestral is currently unknown. The unstable position of *N. scintillans* in dinoflagellate phylogenetic trees (e.g. Hoppenrath and Leander 2010, Ki 2010) means that they cannot be used to infer if there has been fission leading to, or fusion resulting from, the separate *lcf* and *lbp* genes of photosynthetic species (Liu and Hastings 2007). Therefore, the compositions of *lcf* from a diverse array of dinoflagellate taxa need to be examined in order to fully understand the evolution of dinoflagellate bioluminescence.

The identity of bioluminescent species, their biogeographical distribution and the conditions selecting for the production of light remain largely unknown. Central to the understanding of the ecology, physiology and evolution of bioluminescence is to determine which organisms possess the *lcf* gene. Bioluminescence has been reported in 18 dinoflagellate genera (Poupin et al. 1999) through optical observations in cultures, single cells or field communities. However,

the magnitude of light produced, or indeed even the capability to produce it, varies both inter- and intra-specifically (Biggley et al. 1969, Swift et al. 1973, Schmidt et al. 1978, Baker et al. 2008), diurnally through control by circadian rhythms (Hardeland and Nord 1984, Colepicolo et al. 1993, Sullivan and Swift 1994, Li et al. 1996), and it is also affected by the organisms' physiological state (Swift and Meunier 1976, Latz and Jeong 1996). Additionally, identification of the bioluminescent source species in diverse natural planktonic communities is difficult as they may be far less abundant than other co-occurring organisms. Consequently, the characterisation of bioluminescent species based on optical assessment alone is a complex task which can be subject to significant errors.

In this study, PCR primers that target the most conserved and functionally relevant regions of the dinoflagellate *lcf* gene are described. Using these primers and concomitant bioluminescence measurements, we evaluated the genetic potential for bioluminescence in 34 dinoflagellate species of 17 genera, spanning 5 taxonomic orders and including intraspecific comparisons. Additionally, novel partial *lcf* sequences were obtained from distantly related organisms. These results provide significant insight into the lineages that contain bioluminescent members, the intraspecific differences in the ability to produce bioluminescence as well as new information on the genetic diversity of the dinoflagellate *lcf* gene.

2.2 Materials and methods

2.2.1 Dinoflagellate strains

Dinoflagellate cultures were obtained from culture collections or were donated by colleagues from other research institutes (see Table 2.2). Photosynthetic species were cultured in a 12 h: 12 h light: dark cycle at 70 µmol m^{-2} s^{-1} light irradiance. Cultures were grown in the seawater media f/2 (Guillard and Ryther 1962), L1 (Guillard and Hargraves 1993) or K/5 (Keller and Guillard 1985), all without silicate, at 15 ºC, 19 ºC or 22 ºC, depending on the requirements of each strain. *N. scintillans* (Macartney) Kofoid et Swezy was maintained in seawater supplemented regularly with *Dunaliella tertiolecta* CCMP1320 as a food source.

Protoperidinium spp. were not maintained in culture, but instead DNA extracts used in previous studies were provided (Yamaguchi and Horiguchi 2008, Hoppenrath and Leander 2010) (Table 2.2). The taxonomic identity of strains that were difficult to assess by light microscopy were verified by their corresponding SSU rDNA sequences that were available on the GenBank database.

2.2.2 Bioluminescence tests

Dinoflagellate cultures were tested for bioluminescence visually by agitating the culture 4 hours after the onset of the dark phase. If light was not visible, dense cultures at late exponential to early stationary phase were then tested using a FLUOstar Optima luminometer (BMG Labtech, Aylesbury, UK). A 200 µL aliquot of culture was placed in a well of a Costar 96 microwell plate (Corning Life Sciences, Amsterdam, NL), inserted into the instrument and left to recover from handling for 5 minutes. Bioluminescence was stimulated chemically using 2% v/v acetic acid (Sullivan and Swift 1995, von Dassow and Latz 2002).

2.2.3 DNA extraction

The DNA extraction method was modified from Doyle and Doyle (1990). Dinoflagellate cultures were harvested by centrifugation at 4000g for 15 minutes or by filtration onto 5 µm pore size Nuclepore polycarbonate membrane filters (Whatman, Maidstone, UK) for *N. scintillans* and stored at -80 ºC. Cells were disrupted by grinding with a micropestle after freezing in liquid nitrogen and subsequently lysed by incubation at 65 ºC for 1 hour in prewarmed cetyl- trimethylammoniumbromide (CTAB) buffer [2% CTAB, 2% polyvinylpyrrolidone (PVP), 0.5% 2-mercaptoethanol, 1.4 M NaCl, 100 mM Tris HCl and 20 mM EDTA]. Proteins were removed by two extractions in an equal volume of chloroform: isoamylalcohol (24:1). The DNA was precipitated in 1x volume of isopropanol with 0.1x volume of 7.5 M ammonium acetate at -20 ºC, collected by centrifugation at 16000g for 30 minutes and washed with 1 mL 75% ethanol containing 10 mM ammonium acetate. The resulting DNA pellet was air-dried and then resuspended in 30 µL TE buffer [10 mM Tris and 1 mM EDTA]. The DNA purity and quantity were determined using a Nanodrop ND-3000 spectrophotometer (Nanodrop, Wilmington, USA) and PCR quality was assessed by

amplification of the SSU ribosomal DNA using primer pairs 18ScomF1/Dino18SR2 (Zhang et al. 2005, Lin et al. 2006) or Euk1A/Euk516r-GC (Sogin and Gunderson 1987, Amann et al. 1990, Diez et al. 2001).

2.2.4 Primer design

The Consensus Degenerate Hybrid Oligonucleotide Primer (CODEHOP) program (icodehop v1.0: https://icodehop.cphi.washington.edu/i-codehop-context) (Rose et al. 1998) was used to design the dinoflagellate *lcf* (DinoLcf) primers. Eight complete luciferase amino acid sequences were retrieved from GenBank. Sequences of photosynthetic species were split into the three catalytically active repeat domains and the N-terminal region was excluded to facilitate comparison with the single *lcf* domain of *N. scintillans*. Sequences were aligned using ClustalW2 (www.ebi.ac.uk/ClustalW) and following visual examination, the most conserved *loci* in the alignment of the D3 of *lcf* of photosynthetic species and the domain of *N. scintillans* were selected for primer design. The icodehop software predicted primers in each of these conserved amino acid blocks with the consensus clamp being based on a codon usage table for *Lingulodinium polyedrum*. Several primers that corresponded to previously identified suitable conserved regions of the gene were selected on the basis of least core degeneracy and highest score of the consensus clamp (Rose et al. 1998); all primers had similar melting temperatures. Primers were checked for sequence similarity to other genes within dinoflagellates or other organisms using the Basic Local Alignment Search Tool (BLAST) of the National Centre for Biotechnology Information (NCBI) database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Self complementarity, hairpins and primer pair compatibility were assessed using the program PrimerList (http://primerdigital.com/tools/PrimerList.html). The best four forward and two reverse primers (Table 2.1) corresponding to several conserved *loci* of *lcf* (Figure 2.1) were selected and tested accordingly, along with primers for the N-terminal region (Baker et al. 2008).

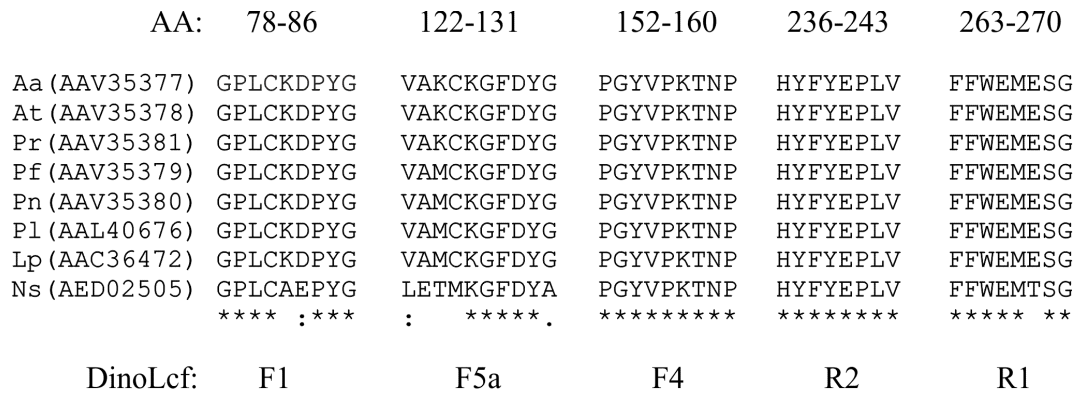

Figure 2.1 Amino acid (AA) alignment of *lcf* sequences from the D3 of photosynthetic species and single domain of *Noctiluca scintillans* at the target sites of each of the primers designed in this study. Species abbreviations are: Aa = *Alexandrium affine*, At = *Alexandrium tamarense*, Pr = *Protoceratium reticulatum*, Pf = *Pyrocystis fusiformis*, Pn = *Pyrocystis noctiluca*, Pl = *Pyrocystis lunula*, Lp = *Lingulodinium polyedrum*, Ns = *N. scintillans.* Accession numbers of the sequences are given in brackets. Only the suffix of the DinoLcf (D-Lcf) primers is shown. The positions shown correspond to the AA in each domain of *L. polyedrum*. The central region corresponding to the catalytic core is from AA 126-275.

Table 2.1 Luciferase gene PCR primers used in this study. The consensus and degenerate regions of the primers are indicated by capital and lower case letters, respectively.

Primer name	Sequence (5'-3')	Source
LcfUniCHF3	TC CAG GTT GCA CGG CTT CGA gcn gcn tgg c	Baker et al. (2008)
LcfUniCHR4	G GGT CTT GTC GCC GTA GTC AAA ncc ytt rca	Baker et al. (2008)
DinoLcfF1	C CCC CTG TGC GAG gan ccn tay gg	Present study
DinoLcfF5a	G GAG CTG TGC AAG GGC tty gay tay g	Present study
DinoLcfF4	C GGC TAC GTG CCC aar acn aay cc	Present study
DinoLcfR1	T GCC GGA CTC CAT Ctc cca raa raa	Present study
DinoLcfR2	CAC CAG GGG CTC Gta raa rta rtg	Present study

2.2.5 PCR reactions

All PCR protocols were initially optimized for *L. polyedrum* (F.Stein) J.D. Dodge and subsequently modified during further testing of other organisms. The final annealing temperature allowed for primers to be combined yet still minimising background amplification. PCR reactions were carried out in 25 μL volumes containing: 0.12 μM each primer, 250 μM each dNTP, 1x standard PCR buffer and 0.5U GoTaq DNA polymerase (Promega, Southampton, UK). The amount of DNA template added to each reaction never exceeded 1 μg. PCR reactions commenced with 5 minutes at 95 ºC for initial denaturation, followed by 35 cycles of 95 ºC for 45 seconds, 55 ºC for 30 seconds, 68 ºC for 1 minute and a final extension at 68 ºC for 5 minutes. PCR reactions for N-terminal regions were conducted with the primers described by Baker et al. (2008). Their PCR conditions were followed only when both their primers were used, but when their forward primer was combined with our own reverse primers then the protocol described herein was followed.

2.2.6 Cloning and sequencing

The PCR products of *lcf* were excised from agarose gels, extracted using the QIAquick gel extraction kit (Qiagen, Crawley, UK) and cloned using the pGEM-T Easy kit (Promega). Sequencing with M13 primers was done by Geneservice (Source Bioscience, Nottingham, UK) and Macrogen (Seoul, South Korea). Two or three clones were sequenced from PCR products of the N-terminal regions or individual domains, respectively. The *lcf* PCR product from the genomic DNA of *N. scintillans* was not sequenced in this study because a PCR product from this strain generated by the same primers using cDNA as the template had been previously sequenced during another study (Valiadi et al. *in prep*; Chapter 5); this sequence has been included in the present analysis. A PCR product from the SSU rDNA amplified from *Alexandrium monilatum* during the DNA PCR quality tests was partially sequenced directly for verification of the strain identity.

2.2.7 Sequence analyses

Sequences trimmed of vector and primers were analysed using the BLASTn tool of the NCBI database, to identify that the correct gene had been amplified and to determine similarities of

lcf to other published sequences. All subsequent analysis was done in MEGA v.5 (Tamura et al. 2011). Nucleotide sequence alignments were carried out using ClustalW (Thompson et al. 1994) and were subsequently manually improved. The similarities between sequences amplified from multiple catalytic domains were investigated based on genetic distance (p-distance). Sequences of variable lengths could be compared by eliminating gaps only in pairwise comparisons. A cluster analysis on the resulting distance matrix was visualised in the form of a dendrogram constructed by the unweighted pair group method with arithmetic mean (UPGMA) and was statistically assessed by 1000 bootstrap replications. In order to further analyse the new sequences from the variable N-terminal to beginning of D1 regions, phylogenetic analysis was undertaken using the maximum likelihood (ML) method. The model that best fit the data was the Tamura-Nei model (Tamura and Nei 1993) with gamma distributed rates among sites (TN93+G) and was used to generate the tree. The reliability of the tree was assessed by 100 bootstrap replications using ML and maximum parsimony (MP).

2.3 Results

2.3.1 Amplification of the luciferase gene

An approximately 270 bp fragment of *lcf* was amplified from 23 dinoflagellate strains belonging to 10 genera, including all 20 bioluminescent strains (Table 2.2), by primers DinoLcfF4 and DinoLcfR2 (Table 2.1) that target the conserved central region of the *lcf* catalytic domains (AA 126- 275; Figure 2.1). Additionally, *lcf* was amplified from the non bioluminescent strain of *Ceratium longipes* (Bailey) Gran by substituting the reverse primer for DinoLcfR1. Primer pairs targeting *loci* outside this central region amplified longer fragments of the gene but from fewer organisms. Generally, with decreased proximity of primer target *loci* to the central domain region, amplification was successful from increasingly fewer organisms and became increasingly non-specific. In protocols amplifying *lcf* from the catalytic domains non-specific products were rare and easy to distinguish from *lcf* by their differing length but significant background amplification occurred in primer combinations involving the forward primer LcfCHF3 (Baker et al. 2008) due to the large difference in the optimal annealing temperatures of the primers. These protocols could therefore not be used for

the purpose of detecting *lcf* but only to amplify longer fragments once the presence of the gene was confirmed with the primer pair DinoLcfF4/R2.

2.3.2 Bioluminescence and luciferase in dinoflagellates

Bioluminescence measurements underestimated the presence of *lcf* in some strains (Table 2.2). Light production was not detected in some strains of typically bioluminescent species, such as *Alexandrium tamarense* (Lebour) Balech PLY 173 and *C. horrida* Stein CCMP 157 which did, however, contain *lcf*. Likewise *lcf* was detected in the non-bioluminescent strains of *C. longipes*, *Fragilidium subglobosum* (von Stosch) Loeblich III and *Fragilidium* sp. Balech ex Loeblich III. It is noteworthy that bioluminescence could not be detected in either strain of *Fragilidium* despite indications from the lab of origin that the cultures were originally bioluminescent.

Bioluminescence and *lcf* were restricted to the orders Gonyaulacales, Noctilucales and Peridiniales (Table 2.2). In the Gonyaulacales, all eight genera tested contained at least two species possessing *lcf*. In the families Gonyaulacaceae, Goniodomataceae and Pyrocystaceae (Fensome et al. 1993) all species contained *lcf* with the exception of *Alexandrium minutum* Halim. The distribution of *lcf* within species of *Ceratium* was more heterogeneous, with *lcf* present only in *Ceratium digitatum* Schutt and *C. longipes* out of the five species tested. Similarly, in the genus *Protoperidinium lcf* was only found in *Protoperidinium crassipes* (Kofoid) Balech but not in *Protoperidinium steidingerae* Balech.

2.3.3 Luciferase gene sequences

The longest *lcf* PCR products of 927 bp were obtained from the N-terminal to D1 region of *Alexandrium* spp. and *C. horrida* (primers F3/R1). *Alexandrium monilatum* (J.F.Howell) Balech *lcf* amplified only with primers that were successful on domains of *Gonyaulax spinifera* (Clap. & Lachm.) Diesing and *Pyrocystis fusiformis* (C.W.Thomson ex Haeckel) Blackman (primers F1/R1-R2) yielding products of 493-572 bp. Additionally, *A. monilatum* was the only species of this genus that did not amplify *lcf* from the N-terminal region. The longest products obtained from *Ceratium*, *Fragilidium* and *Protoperidinium* were 361-440 bp

Table 2.2 Details of dinoflagellate strains tested in this study with results of bioluminescence (BL) and *lcf* PCR tests. Species are subdivided into taxonomic order and family in the order Gonyaulacales. Taxonomy is according to Fensome et al. (1993).

Order / Family / Species	Source	Strain	Isolation location	BL	*lcf*
Order Gonyaulacales					
Family Ceratiaceae					
Ceratium digitatum	MDP	P47B6	Villefranche-sur-Mer, France	+	+
Ceratium horridum	CCAP	1110/6	Unknown	-	-
Ceratium horridum	MH	-	Unknown	-	-
Ceratium horridum	MDP	P38B2	Villefranche-sur-Mer, France	-	-
Ceratium longipes	CCMP	1770	Bigelow lab dock, ME, USA	-	+
Ceratium massiliense	MDP	P36C1	Villefranche-sur-Mer, France	-	-
Ceratium ranipes	MDP	P36A2	Villefranche-sur-Mer, France	-	-
Family Goniodomataceae					
Alexandrium affine	CCMP	112	Vigo, Spain	+	+
Alexandrium fundyense	CCMP	1978	Bay of Fundy, USA	+	+
Alexandrium minutum	PLY	669	Fleet Lagoon, Dorset, UK	-	-
Alexandrium monilatum	MIL	-	Mississippi Sound, USA	+	+
Alexandrium tamarense	CCMP	1598	West Hong Kong Island, China	+	+
Alexandrium tamarense	CCMP	115	Tamar Estuary, Plymouth, UK	+	+
Alexandrium tamarense	PLY	173A	Tamar Estuary, Plymouth, UK	-	+
Family Gonyaulacaceae					
Ceratocorys horrida	CCMP	157	Banda Sea, SE Asia	-	+
Ceratocorys horrida	MIL	-	Sargasso Sea	+	+
Fragilidium cf. *subglobosum*	ALISU	I097-01	Lagoa de Obidos, Portugal	+[b]	+
Fragilidium sp.	ALISU	I091-02	Sines, Portugal	+[b]	+
Gonyaulax spinifera	CCMP	409	Bigelow lab dock, ME, USA	+	+
Lingulodinium polyedrum	CCMP	1738	Texas Coast, USA	+	+
Lingulodinium polyedrum	NOCS	M22	La Jolla Pier, CA, USA	+	+
Lingulodinium polyedrum	NOCS	2810	La Jolla Pier, CA, USA	+	+
Protoceratium reticulatum	CCMP	1889	Friday Harbour, WA, USA	+	+
Family Pyrocystaceae					
Pyrocystis fusiformis	MIL	-	Halmehara Sea, SE Asia	+	+
Pyrocystis lunula	CCMP	731	North Atlantic Ocean	+	+
Pyrocystis lunula	CCAP	1131	Unknown	+	+
Pyrocystis noctiluca	CCMP	732	Santa Barbara, CA, USA	+	+
Order Gymnodiniales					
Akasiwo sanguinea	ALISU	I018-01	Cascais, Portugal	-	-
Amphidinium longum	SPMC	100	Anacortes, WA, USA	-	-
Gymnodinium catenatum	ALISU	I013-04	Espinho, Portugal	-	-
Gymnodinium nagasakiense	PLY	561	North West Pacific	-	-

Table 2.2 continued

Order / Family / Species	Source	Strain	Isolation location	BL	*lcf*
Gymnodinium simplex	PLY	368	Plymouth Sound, UK	-	-
Karlodinium veneficum	PLY	517	Oslofjord, Norway	-	-
Order Noctilucales					
Noctiluca scintillans	MIL	-	Gulf of Mexico	+	+
Order Peridiniales					
Glenodinium foliaceum	CCAP	1116/3	Chesapeake Bay, USA	-	-
Glenodinium hallii	CCMP	2770	Florida, USA	-	-
Glenodinium sp.	SPMC	102	Anacortes, WA, USA	-	-
Lessardia elongata	SPMC	104	Anacortes, WA, USA	-	-
Protoperidinium crassipes	AY	-	Otaru Bay, Hokkaido, Japan	+[b]	+
Protoperidinium steidingerae	MH	-	Vineyard Sound, MA, USA	N/A	-
Order Prorocentrales					
Prorocentrum micans	CCMP	693	Bigelow lab dock, ME, USA	-	-
Prorocentrum micans	CCMP	691	Monhegan Island, ME, USA	-	-
Prorocentrum minimum	CCMP	699	Bigelow lab dock, ME, USA	-	-
Prorocentrum minimum	CCMP	1529	Ecuador	-	-

N/A –Not applicable

[a] Source abbreviations: ALISU-Universidade de Lisboa culture collection, Portugal; CCMP- Culture collection of marine phytoplankton, USA; CCAP-Culture collection of algae and protozoa, UK; PLY-Plymouth culture collection, UK; NOCS- National Oceanography Centre Southampton, UK; SPMC-Shannon point marine center, USA (Kelley Bright/Susanne Strom); MDP-M.D. Pizay, Laboratoire d' Oceanographie de Villefranche/Mer, France; MH- M. Hoppenrath, Senckenberg, Germany; MIL- M.I. Latz, Scripps Institution of Oceanography, USA; AY- Aika Yamaguchi, University of British Columbia, Canada.

[b] Bioluminescence reported by lab of origin not confirmed/tested in this study .

(primers F5a/R1-R2) while *N. scintillans* could only be amplified with the "universal" (F4/R2) primer pair. It should also be noted that the apparent *lcf* pseudogene of *C. longipes* contained unique modifications at several nucleotide positions within the binding site of primer R2 (Figure 2.1) and thus only amplified when primer R1 was used. Details of the primer pairs successful in amplifying *lcf* from each strain, the PCR product lengths and the fragments that were sequenced are shown in Table 2.3.

Selective sequencing of amplified PCR products encompassed representatives of all genera with previously uncharacterised *lcf*, undescribed *loci* of some previously studied species, as well as intraspecific comparisons. As a result, we report the first partial *lcf* sequences from the genera *Ceratium*, *Ceratocorys*, *Fragilidium* and *Protoperidinium* and extend the *lcf* sequence information in the genera *Alexandrium* and *Gonyaulax*. Sequence alignments of D1, D2 and D3 domains of previously known and new sequences, indicated a large number of potential pseudogenes as sequences consisted of deletions of up to 60 bp in some sequences of *P. crassipes*, the majority of which caused shifts in the reading frame. These were common in both non bioluminescent (e.g. *F. subglobosum* and *C. longipes*) and bioluminescent species (e.g. *C. horrida* and *P. fusiformis*) (not shown). Additionally high intracopy variation was observed, with identities as low as 91.1% among sequences of *Alexandrium fundyense* Balech CCMP 1978 originating from the region of the N-terminal to the beginning of first domain.

2.3.4 Cluster and phylogenetic analyses

Several novel sequences were amplified from the catalytic domains of *lcf*. However, due to the highly conserved nature of the *loci* amplified, the clusters in the sequence dendrogram (Figure 2.2) did not receive significant statistical support and the results were only interpreted qualitatively. The dendrogram of *lcf* sequences from the catalytic domains (Figure 2.2) showed that gonyaulacoid dinoflagellates, except *L. polyedrum*, clustered according to analogous domains and each cluster incorporated novel sequences from closely related organisms such as *Alexandrium* spp., *C. horrida* and *G. spinifera*. *Alexandrium monilatum* was more similar to *L. polyedrum* D2 and D3 than to other members of its genus; the identity of the *A. monilatum* strain was confirmed by its SSU rDNA sequence that was identical to that on GenBank from another strain of this species (Rogers et al. 2006). *Pyrocystis* spp. always clustered together within the wider Gonyaulacales and incorporated *P. fusiformis* sequences in D2. Sequences of the Gonyaulacales members *Fragilidium* and *Ceratium* also showed similarities to, but remained distinct from, the tight clusters formed by *Alexandrium*, *Gonyaulax*, *Protoceratium* and *Ceratocorys*. In general, *Ceratium* sequences were most similar to *Alexandrium* spp. with identities ranging from 73.8 - 81.6% in *loci* F5a-R2 or F4-R1. However, four sequences of *C. digitatum* that were in the lower part of this range (<78%)

Table 2.3. Details of PCR primer pairs that amplified *lcf* in dinoflagellate strains containing this gene. D-Lcf is short for primers DinoLcf. The expected PCR product size is indicated below each primer pair. (+) indicates that PCR product was generated, while (–) did not. Asterisks indicate the products that were cloned and sequenced. N/A is not applicable.

Species name	Source	Strain	LcfCHF3 D-LcfR1 927bp	LcfCHF3 D-LcfR1 848bp	LcfCH F3/R4 ~525bp	D-Lcf F1/R1 572bp	D-Lcf F1/R2 493bp	D-Lcf F5a/R1 440bp	D-Lcf F5a/R2 361bp	D-Lcf F4/R1 350bp	D-Lcf F4/R2 271bp
Alexandrium affine	CCMP	112	+	+	+	+	+	+	+	+	+
Alexandrium fundyense	CCMP	1978	+*	+	+	+	+	+	+	+	+
Alexandrium monilatum	MIL		-	-	-	+	+	+	+	+	+
Alexandrium tamarense	CCMP	115	+*	+	+	+	+	+	+	+	+
Alexandrium tamarense	CCMP	1598	+*	+	+	+	+	+	+	+	+
Alexandrium tamarense	PLY	173A	-	-	+	+	+	+	+	+	+
Ceratium digitatum	MDP	P47B6	-	-	-	-	-	-	+*	+*	+
Ceratium longipes	CCMP	1770	-	-	-	-	-	-	-	+*	-
Ceratocorys horrida	CCMP	157	+*	+	+	+	+	+	+	+	+
Ceratocorys horrida	MIL		+*	+	+	+	+	+	+	+	+
Fragilidium subglobosum	ALISU	I097-01	-	-	-	-	-	+*	+	+	+
Fragilidium sp.	ALISU	I091-02	-	-	-	-	-	+	+	+	+
Gonyaulax spinifera	CCMP	409	-	-	+	+*	+	+	+	+	+
Lingulodinium polyedrum	CCMP	1738	+	+	+	+	+	+	+	+	+
Lingulodinium polyedrum	NOCS	M22	+	+	+	+	+	+	+	+	+
Lingulodinium polyedrum	NOCS	2810	+	+	+	+	+	+	+	+	+
Noctiluca scintillans	MIL		-	-	-	-	-	-	-	-	+*
Protoceratium reticulatum	CCMP	1889	+	+	+	+	+	+	+	+	+
Protoperidinium crassipes	AY		N/A	N/A	-	-	-	+*	N/A	+	+*
Pyrocystis fusiformis	MIL		-	-	+	-	+*	+	+	+	+
Pyrocystis lunula	CCMP	731	+	+	+	+	+	+	+	+	+
Pyrocystis lunula	CCAP	1131	+	+	+	+	+	+	+	+	+
Pyrocystis noctiluca	CCMP	732	+	+	+	+	+	+	+	+	+

were divergent and appeared as a separate cluster. The *lcf* sequences of *P. crassipes*, representing the first sequences from the order Peridiniales, were most similar to *A. tamarense* (73.3 - 79.5% identity in *locus* F5a-R1); these sequences formed a cluster that was embedded among the sequence clusters of the Gonyaulacales. A *N. scintillans lcf* sequence was most similar to the previously reported sequence of this organism which was the most distinctive relative to all other dinoflagellates included in this analysis.

The phylogenetic tree of the variable N-terminal to beginning of D1 region (Figure 2.3), which only included some members of the Gonyaulacales, showed 4 main clades all with significant statistical support: an *Alexandrium* clade (A), a *Lingulodinium* clade (L), a *Pyrocystis* clade (P) and a clade consisting *C. horrida* (Ch), *Protoceratium reticulatum* (Clap. & Lachm.) Butschli (Pr) and *G. spinifera* (Gs). *Ceratocorys horrida* grouped closely with *P. reticulatum* as in the catalytic domains (Figure 2.2) and in both cases the sequences of the latter were embedded among dissimilar sequences of the former. Both species were similar to *G. spinifera*. Two further clades within *Alexandrium* spp. that were statistically well supported, separated dissimilar *lcf* sequences of the same organisms that were amplified using different primer pairs. Species with only one representative sequence clustered within either one of these clades.

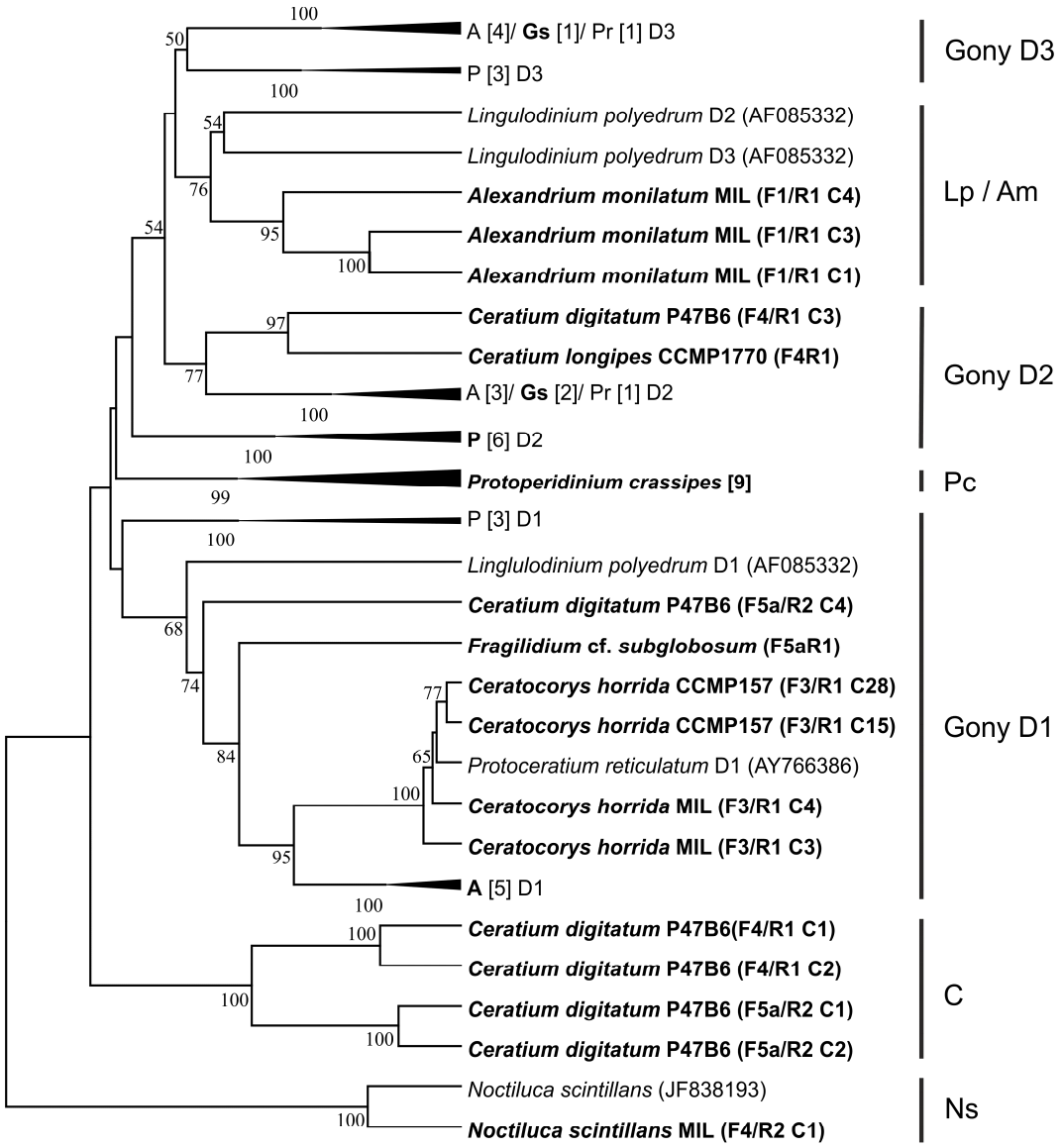

Figure 2.2 UPGMA dendrogram of *lcf* sequences from the catalytic domains based on p-distance. In the interest of clarity, clusters are collapsed when there are more than three highly similar sequences. In collapsed clusters, genus or species names are abbreviated and the number of sequences of each within that cluster is given in square brackets. Bootstrap values are derived from 1000 replications; values less than 50 % are not shown. Accession numbers are shown in brackets following the sequences obtained from GenBank. Sequences obtained in this study are in bold font and are additionally labelled with the primer pair used in the amplification and a clone identifier. A clone identifier is not shown when all clones of a PCR product produced identical sequences. The scale has been excluded as the sequences compared are not of equal length and absolute p-distance values are therefore misleading. A = *Alexandrium*, Am = *Alexandrium monilatum*, C = *Ceratium*, Gony = Gonyaulacales, Gs = *Gonyaulax spinifera*, Lp = *Lingulodinium polyedrum*, Ns = *Noctiluca scintillans*, Pr = *Protoceratium reticulatum*, Pc=*Protoperidinium crassipes*, P = *Pyrocystis*.

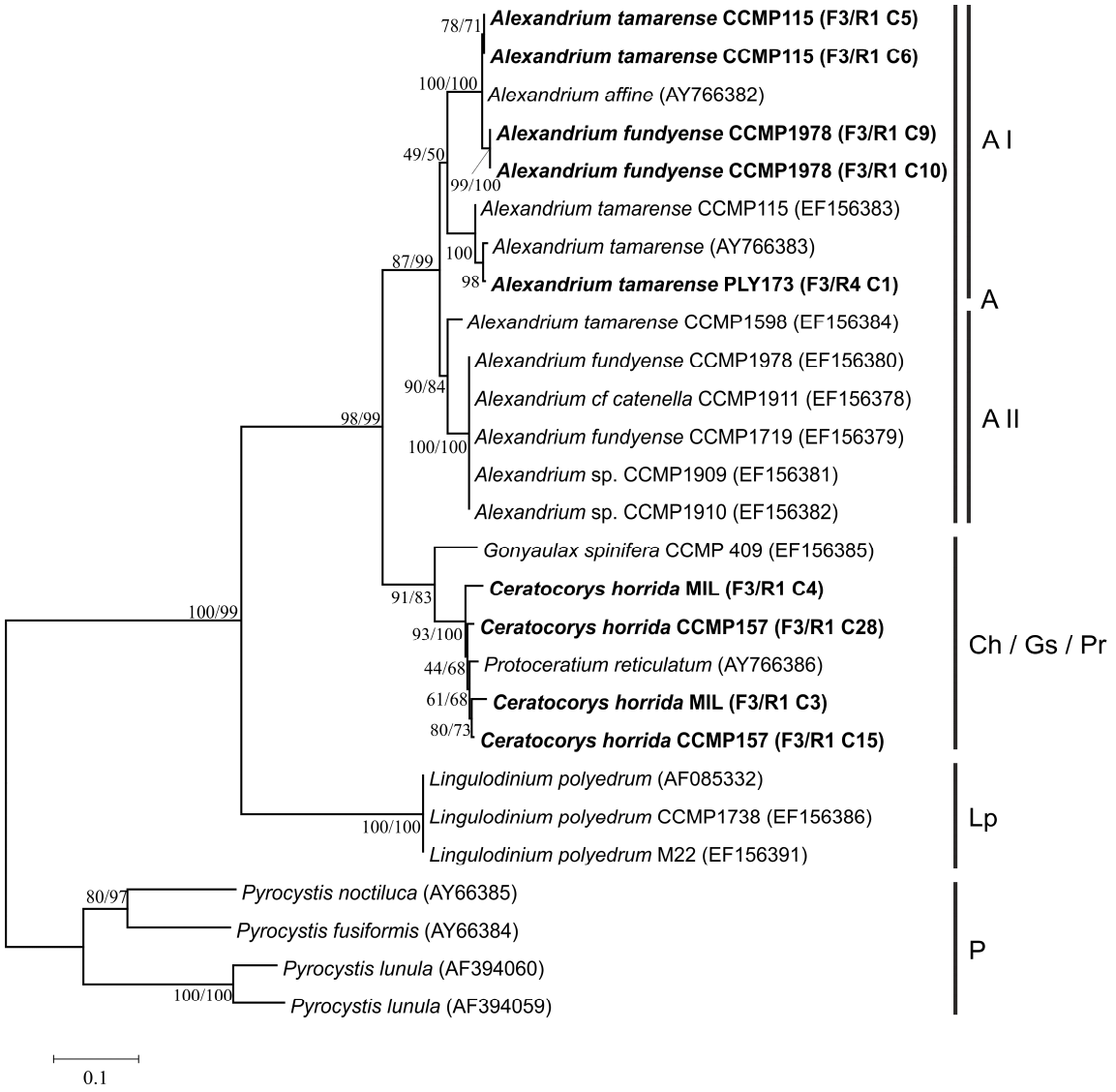

Figure 2.3 Maximum likelihood phylogenetic tree based on a nucleotide alignment of *lcf* sequences amplified from the N-terminal region to the beginning of the first domain. Bootstrap values shown at the nodes are based on 100 replications (maximum likelihood / maximum parsimony). Note that the topology of trees generated in both ML and MP analyses were identical. The scale of branch lengths shows 1 substitution per 10 sites. Accession numbers are shown in brackets following the sequences obtained from GenBank. Sequences obtained in this study are in bold font and are additionally labelled with the primer pair used in the amplification and a clone identifier. A= *Alexandrium*, Ch = *Ceratocorys horrida*, Gs = *Gonyaulax spinifera*, Lp = *Lingulodinium polyedrum*, Pr = *Protoceratium reticulatum*, P = *Pyrocystis.*

2.4 Discussion

2.4.1 Amplification of the luciferase gene

This study presents an analysis of the distribution of the genetic potential for bioluminescence within dinoflagellates and provides novel sequence information to assess the diversity of *lcf* in all major bioluminescent dinoflagellate taxonomic groups. This was achieved through the development of primers that amplify *lcf* from *loci* that encode functionally relevant regions of the gene and are thus likely to be highly conserved across distant dinoflagellate species. Primers targeting more variable *loci* flanking the functional central domain regions (Liu and Hastings, 2007, Liu et al., 2004) were biased in amplifying *lcf* from genera (and close relatives) of the Gonyaulacales that were highly represented in the sequences used to design the primers. Indeed, a diverse taxonomic representation is important when developing protocols to determine the presence of distantly related protein coding genes. Accordingly, primers for the amplification of *lcf* N-terminal gene region (Baker et al. 2008) designed prior to the publication of the *N. scintillans* gene, failed to amplify *lcf* in several genera including one species of *Alexandrium*. This also suggests that, even among photosynthetic species, where this region is assumed to be conserved (Liu et al. 2004, Baker et al. 2008), dinoflagellate *lcf* may have a more diverse composition than previously thought.

2.4.2 Distribution of the luciferase gene within dinoflagellates

The ability to produce light was found to vary among strains of the same species even when isolated from the same location (e.g. *A. tamarense*), in agreement with previous findings in strains of *A. tamarense* (synonym *G. excavata*) (Schmidt et al. 1978) and *Pyrocystis lunula* (Swift et al. 1973). The finding that the genetic basis for bioluminescence is conserved and still detectable among these strains suggests that *lcf* remains largely unmutated, akin to similar findings in the bacterium species *Vibrio cholerae* which also shows intraspecific variation in bioluminescence but not in the presence of the luciferase genes (Palmer and Colwell 1991). It is likely that this may be similar in other dinoflagellate processes which vary intraspecifically, such as the production of toxins and allelochemicals (Tillmann et al. 2009). Our results show that laboratory measurements of phenotypic traits, such as bioluminescence, in monoclonal

cultures may misrepresent the functional capabilities of the species. These can be assessed at the genetic level just as the approach to detect *lcf* is the basis for identifying bioluminescent species.

Bioluminescence was found to be restricted to the orders Gonyaulacales, Noctilucales and Peridiniales. Bioluminescence has been reported for a few *Gymnodinium* and *Prorocentrum* species (compiled by Poupin et al. 1999) but these observations were based on unfounded assumptions. For example, Kofoid and Swezy (1921) reported *G. flavum* as bioluminescent only because it was the most abundant organism in a light producing bloom, even though cells of *Gonyaulax* spp. were also present. We tested some of the species mentioned by Poupin et al. (1999) as well as other members of the Gymnodiniales and Prorocentrales and none of them were found to emit light or possess *lcf*. As a result of our data and the few isolated reports of bioluminescence in the Gymnodiniales and Prorocentrales, it is highly unlikely (although impossible to exclude) that these orders contain bioluminescent members.

Within bioluminescent groups, the presence of *lcf* was remarkably conserved in the families Gonyaulacaceae and Pyrocystaceae (Fensome et al. 1993) but in the genera *Alexandrium*, *Ceratium* and *Protoperidinium*, the distribution of *lcf* was not homogeneous among species. This was particularly pronounced in *Ceratium* and our findings did not agree with previously reported bioluminescent species of this genus (Poupin et al. 1999). For example, *Ceratium horridum* (Cleve) Gran has been reported to be bioluminescent from field testing of single cells (Lapota and Losee 1984, Batchelder et al. 1992), but all three strains tested in the present study lacked *lcf*. In contrast, we found *lcf* in *C. longipes* which is morphologically almost indistinguishable from *C. horridum* (Kraberg et al. 2010), which suggests it may instead have been the origin of the detected bioluminescence in these studies. Consequently, several reports of bioluminescent species that have not been confirmed in culture should be re-evaluated and complemented by detailed morphological analysis or molecular taxonomy.

In the predominantly bioluminescent genus *Alexandrium*, only *Alexandrium minutum* Halim lacks *lcf*, in agreement with the absence of this gene in expressed sequence tag (EST) libraries of two strains of this species (Yang et al. 2010). Additionally, bioluminescence has not been reported in this species. Phylogenetic analysis of *Alexandrium* spp. using SSU rDNA (John et al. 2003) has indicated that *A. minutum* does not belong to the same clade as the bioluminescent *Alexandrium* spp. included in our study and mentioned in the literature (e.g.

A. ostenfeldii; Kremp et al. 2009). Also, *C. digitatum*, found here to be bioluminescent, is the closest phylogenetic relative to *Ceratium fusus* (Gomez et al. 2010b), another well known bioluminescent dinoflagellate species (Sullivan and Swift 1994). This could indicate that *lcf* is present within specific clades of species within certain genera while it is absent, or only undetectable gene remnants remain, in others. It would be interesting to investigate this further in the context of evolutionary events and potential environmental pressures that have selected for the production of light.

2.4.3. Diversity and phylogeny of the luciferase gene in dinoflagellates

Novel partial *lcf* sequences from genera with previously uncharacterised *lcf* genes, particularly *Ceratium* and *Protoperidinium*, meant that similarities of particular *loci* of this gene could be compared across all bioluminescent dinoflagellate genera. This was complemented by the phylogenetic analysis of N-terminal regions of some organisms which together revealed several novel features of dinoflagellate *lcf*. Large sequence variation of up to ~9 % in *A. fundyense* among sequences originating from the non-repeated N-terminal gene region, revealed that there are multiple non-identical copies of the gene within some strains. Additionally, some of these copies are potentially pseudogenes and occur in several organisms regardless of whether or not they are bioluminescent. Large variations among gene copies are well known in dinoflagellate gene families (Zhang and Lin 2003, Kim et al. 2011) and the presence of pseudogenes has also been reported (Lin et al. 2009, Kim et al. 2011). The variation among clones of *lcf* amplified in this study is comparable in magnitude to *L. polyedrum lbp* gene variants whose sequences vary by more than 10% (Lee et al. 1993, Tanikawa et al. 2004). Two distinct clades of *lcf* in *Alexandrium* spp. (Figure 2.3) suggest some of the intracopy variation could indeed be explained by the presence of two distinct variants of *lcf* , as found in the transcripts *P. lunula lcf* (Okamoto et al. 2001) and *L. polyedrum lbp* (Lee et al. 1993, Machabée et al. 1994, Tanikawa et al. 2004). This could also explain the divergence among sequences of the *C. horrida* strains that are intermingled with *P. reticulatum* in both regions of the gene examined (Figures 2.2 and 2.3). Additionally, this meant that *G. spinifera* could not be designated its own clade as suggested by Baker et al. (2008) because the dissimilarity of its sequence to those of *C. horrida* and *P. reticulatum* was still within the range exhibited among sequences of gene copies obtained from single strains. Kim et al (2011) recently demonstrated that sequences from multiple gene copies of the actin

gene varied so much that they overlapped between two species of *Dinophysis*. Our results show that the sequence variability among gene copies is so large that this overlap can even extend into closely related genera. Therefore, comparisons of these organisms based on genomic sequences of multicopy protein coding genes such as *lcf* could be distorted by insufficient sampling of gene variants/copies and lead to misguided interpretations of their relationships.

Important insights into the similarities of *lcf* between closely and distantly related taxa resulted from the sequences obtained from the catalytic domains. However, as the primers had to target conserved *loci* in order to detect *lcf* in all species, this inevitably meant that the resulting *lcf* sequences contained very few phylogenetically informative sites, which are, in contrast, predominantly found in the regions flanking the central parts of the domains (Liu et al. 2004, Liu and Hastings 2007). This was particularly true for the shorter sequences originating from *loci* nearer to the highly conserved central region of each domain. Therefore, the dendrogram presented in Figure 2.2 was not intended to draw any conclusions on the relatedness of species but it rather illustrates the sequence distance matrix in order to identify features of the sequences that could be interesting to investigate further.

Sequences amplified from multiple catalytic domains grouped with corresponding domains of similar organisms (Figure 2.2). All D1 sequences of the Gonyaulacales grouped together, in agreement with previous studies on these genes (Liu et al. 2004, Liu and Hastings 2007), but this was not the case with D2 and D3 due to the more conserved nature of the shorter sequences amplified from these domains. Gonyaulacoid dinoflagellates tended to group together in individual clusters of D1, D2 and D3 and showed similar affiliations to those observed in the N-terminal tree (Figure 2.3). *Ceratocorys horrida* was closely related to *P. reticulatum* in both regions of the gene examined (N-terminal and catalytic domains) and *F. subglobosum* was similar to other members of the Gonyaulacales, consistent with their positions in rDNA phylogenies (Gomez et al. 2010b) and taxonomic placement (Fensome et al. 1993), respectively. The *lcf* sequences of most *Alexandrium* spp. are highly similar which agrees with them being members of, or closely related to, the *Alexandrium tamarense* species complex (John et al. 2003). However, the dissimilarity of *A. monilatum* to other *Alexandrium* spp. that led it to preferentially cluster with *L. polyedrum* is intriguing, because this species diverged early within *Alexandrium* (Rodger et al., 2006) and it has a unique toxin profile relative to other species of this genus (Hsia et al. 2006). Thus, further sequence information

from *A. monilatum* could potentially provide important insight into the evolution of *lcf* within *Alexandrium* and the phylogenetic relation to *lcf* of other gonyaulacoid dinoflagellates.

Sequences from the remaining genera, *Ceratium* and *Protoperidinium*, were small in length and from the most conserved region of the *lcf* gene, but still showed variable and interesting similarity features. The similarity of all sequenced clones of PCR products from both species of *Ceratium* to *Alexandrium* spp. is consistent with their taxonomic placement in the Gonyaulacales (Fensome et al. 1993) and SSU rDNA phylogenies (Gomez et al. 2010b). However, the divergent sequence cluster formed by the majority of *C. digitatum* clones may represent distinct *lcf* variants or domains and would thus be interesting to investigate further. The same holds true for *P. crassipes* whose sequences could potentially represent a new group in dinoflagellate *lcf* genes. The phylogenetic position of the *Protoperidinium* genus relative to the Gonyaulacales is not well resolved by either rDNA (Yamaguchi and Horiguchi 2005, Yamaguchi et al. 2006) or heat shock protein 90 (Hoppenrath and Leander 2010) and possibly the more variable *lcf* genes could be valuable in investigating the phylogenetic relations between these organisms.

2.3.4 Conclusions

This study has significantly increased our knowledge on the distribution and diversity of bioluminescence and *lcf* in dinoflagellates. Novel insights have been gained into the identity of bioluminescent species and the genetic conservation of bioluminescence within species, a capability underestimated by optical testing. The first partial sequences of *lcf* from several dinoflagellate genera have revealed new aspects of the diversity, phylogeny and similarities of this gene among distant dinoflagellate taxa and have led us to identify *A. monilatum*, *Ceratium* spp. and *Protoperidinium* spp. as key organisms for further studies on the evolution of this gene. The “universal” *lcf* primers presented in this study have been used to field test the detection of bioluminescent dinoflagellates in their natural environment (Valiadi et al. in prep.) and we also anticipate that they will have useful applications in facilitating full *lcf* gene sequencing of key species by combination with rapid amplification of cDNA ends (RACE), therefore providing a tool that will increase our understanding of the ecology and evolution of dinoflagellate bioluminescence.

Acknowledgements

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Chapter 3

DETECTION AND MOLECULAR PHYLOGENY OF THE LUCIFERIN BINDING PROTEIN GENE IN GONYAULACOID DINOFLAGELLATES

Abstract

Dinoflagellate bioluminescence systems can operate with or without a luciferin binding protein, representing two distinct modes for the production of light. However, which mode is more common is currently unknown. This study used PCR primers to detect and partially sequence a large part the luciferin binding protein gene (*lbp*) from a group of ecologically important bioluminescent gonyaulacoid dinoflagellates. The presence of *lbp* is reported for the first time in the genera *Ceratocorys*, *Gonyaulax* and *Protoceratium*. Additionally, *lbp* is shown to be widespread in bioluminescent species of *Alexandrium*. The sequences of *lbp* showed the presence of pseudogenes and high intracopy variation within organisms that, in some cases, was consistent with the presence of more than one gene variant. Phylogenetic analysis of novel and previously described *lbp* sequences revealed four clades with highly divergent sequences. *Alexandrium monilatum lbp* was found to be divergent from other *Alexandrium* species and grouped with *Gonyaulax spinifera*, *Ceratocorys horrida* and *Protoceratium reticulatum*, in the only clade composed by more than one genus. Overall, this study shows that *lbp* is very common in dinoflagellate bioluminescence systems and the results are discussed in the context of the validity of the current biochemical model of dinoflagellate bioluminescence

3.1 Introduction

Bioluminescence in dinoflagellates is brought about by the reaction of a luciferin substrate and a luciferase (LCF) enzyme within the scintillons, which are the organelles that contain the components for the bioluminescence reaction (DeSa and Hastings 1968, Johnson et al. 1985, Nicolas et al. 1987). The dinoflagellate bioluminescence system can operate with or without a luciferin binding protein (LBP) (Schmitter et al. 1976, Knaust et al. 1998), thus representing two distinct mechanisms for the production of light. The function of LBP is thought to be to protect luciferin from autoxidation at physiological pH because it is naturally highly reactive with oxygen (Morse et al. 1989b, Nakamura et al. 1989). A reduction in pH within the scintillons activates LCF and also triggers the release of luciferin from LBP making it available for oxidation by LCF (reviewed by Haddock et al. 2010). It is currently unknown how many of the bioluminescent dinoflagellate species utilise LBP in their bioluminescence system.

The dinoflagellate *Lingulodinium polyedrum* has been the main model organism for studies of LBP, representing the first organism where this protein was first isolated and where *lbp* was first sequenced and cloned (Morse et al. 1989b, Lee et al. 1993, Machabée et al. 1994). The *lbp* gene family is composed by two variants, *lbp*a and *lbp*b sharing 86% sequence identity, that are expressed in equal amounts forming native proteins that exist as dimers (Morse et al. 1989b, Machabée et al. 1994). Recent studies on the transcriptomes of several bioluminescent species of *Alexandrium* found *lbp* in *A. catenella (Uribe et al. 2008)*, *A. fundyense* (Erdner and Anderson 2006) and *A. tamarense* (Hackett et al. 2005). Further evidence that bioluminescent systems utilising LBP are common in dinoflagellates came with the sequencing of a gene in *Noctiluca scintillans* where *lbp* is attached to the 3' end of *lcf* forming a single gene (Liu and Hastings 2007). It therefore seems that most of the organisms whose bioluminescence genes have been studied in detail contain *lbp* with the only exception so far being the genus *Pyrocystis* where the absence of LBP has been confirmed in its protein extracts using an antibody raised against *L. polyedrum* LBP (Schmitter et al. 1976, Knaust et al. 1998).

The *lbp* is composed of four tandemly repeated domains, although the sequence similarity among them is low when compared to the high similarities among the three domains of *lcf* in

photosynthetic species (Liu and Hastings 2007). The domains of *lbp* of photosynthetic species are preceded by an N-terminal gene region which is found in highly similar form in *lcf* of the same species (Li et al. 1997). This N-terminal region is not found in the *lbp* domain of *N. scintillans* (Liu and Hastings 2007). Among the bioluminescence genes of *N. scintillans*, the *lbp* is more divergent at the amino acid level (40% identity) than its *lcf* (60% identity) relative to *L. polyedrum* which is the species with the most similar bioluminescence genes to this organism (Liu and Hastings 2007). This suggests that while the composition of *lcf* has been shown to vary significantly among bioluminescent dinoflagellates (Chapter 2), it is likely that *lbp* may be yet even more diverse.

In order to further explore the evolution and physiology of bioluminescence in dinoflagellates, a greater knowledge of the genes composing the bioluminescent systems in different dinoflagellate species is required. In this study, the presence of *lbp* was investigated in several bioluminescent dinoflagellates by PCR. Although it was not possible to create primers that could amplify *lbp* from all species known to contain it, it could be amplified from several ecologically important gonyaulacoid dinoflagellates, including all the toxic bioluminescent genera known to date. Novel *lbp* sequences obtained from these organisms provided insights into the molecular evolution of *lbp* in this key dinoflagellate group.

3.2 Materials and Methods

3.2.1 Dinoflagellate cultures and DNA extraction

The dinoflagellate cultures or DNA extracts used in this analysis as well as the DNA extraction methods are as described in Chapter 2.

3.2.2 Primer design

Primers for *lbp* were designed using the Consensus Degenerate Hybrid Oligonucleotide Primer (CODEHOP) program (Rose et al. 1998). Six complete sequences of *lbp* were obtained from GenBank: four from *L. polyedrum* (not identical), one from *A. catenella* and one from *N.*

scintillans. Sequences were aligned using ClustalW2 (www.ebi.ac.uk/ClustalW) and the CODEHOP program predicted primers in conserved *loci*. The consensus clamp was based on a codon usage table for *L. polyedrum*. Primers that were selected based on least core degeneracy and highest score of the consensus clamp, were checked for sequence similarity to other genes within dinoflagellates or other organisms using the Basic Local Alignment Search Tool (BLAST) of the National Centre for Biotechnology Information (NCBI) database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and their compatibility was assessed using the program PrimerList (http://primerdigital.com/tools/PrimerList.html). The final primer set DinoLbpF2 and DinoLbpR1 amplified ~1180bp of the *lbp*; details of the primer sequences and target positions are given in Table 3.1.

Table 3.1 Primers for the luciferin binding protein gene designed in this study. The corresponding consensus amino acid (AA) sequences are shown and the target amino acid positions correspond to the LBP of *Lingulodinium polyedrum*. F denotes the forward primer and R denotes the reverse primer. Capital letters show the consensus region and lower case letters show the degenerate region of the primers.

Primer	Sequence 5'-3'	Consensus AA	AA position
DinolbpF2	C GAG CGC GTG ATC gcn gtn aar ga	DERVIAVKE	246-254
DinolbpR1	C CAC GAT GCA CAT GGA Gck ytg cat rta	YMQRSMCLLD	631-640

3.2.3 PCR reactions

All PCR protocols were initially optimized on *L. polyedrum* and subsequently modified during further testing of other organisms. PCR reactions were carried out in 25 µL volumes containing: 0.12 µM each primer, 250 µM each dNTP 1x PCR buffer, 0.5 U GoTaq polymerase (Promega, Southampton, UK) and 50-100 ng of DNA. PCR reactions commenced with 5 minutes at 95 ºC for initial template denaturation, followed by 35 cycles of 95 ºC for 45 seconds, 61 ºC for 30 seconds, 68 ºC for 1.5 minutes and a final extension at 68 ºC for 5

minutes. In a second test the annealing temperature was lowered to 58 ºC from 61ºC, with all other parameters remaining the same.

3.2.4 Cloning and sequencing

The PCR products of *lbp* were excised from agarose gels, extracted using the QIAquick gel extraction kit (Qiagen, Crawley, UK) and cloned into the pCR4 vector in the TOPO TA cloning kit (Invitrogen, Paisley, UK). Two clones were sequenced for each PCR product from both directions using primers M13F and M13R. The resulting sequences were reconstructed to the full PCR product using the CAP3 sequence assembly program (http://pbil.univ-lyon1.fr/cap3.php). Sequencing was done by Eurofins MWG Operon (Ebersberg, Germany).

3.2.5 Sequence analyses

Sequences trimmed of vector and primers were analysed using the BLASTn tool of the NCBI database, to ensure that the correct gene had been amplified. All subsequent analysis was done in MEGA v. 5 (Tamura et al. 2011). Nucleotide sequence alignments were carried out using ClustalW (Thompson et al. 1994) and were subsequently improved manually. Phylogenetic analysis was undertaken using the GTR+G model implemented in the maximum likelihood method (ML). The tree was statistically assessed by 100 bootstrap replications using ML and maximum parsimony (MP).

3.3 Results

3.3.1 Detection of the luciferin binding protein gene

The dinoflagellate strains tested in this study along with results of PCR tests are shown in Table 3.2. The *lbp* primers yielded PCR products of approximately 1180 bp from 11 strains out of the 18 tested. Sequencing was done in a selective manner and focused on organisms where *lbp* was found for the first time and organisms whose *lbp* had only been sequenced from

a short locus in previous studies. Novel sequences obtained from four species of *Alexandrium*, *Ceratocorys horrida*, *Gonyaulax spinifera* and *Protoceratium reticulatum* represent the first reports of *lbp* in the latter three genera and the first long partial sequences of *Alexandrium affine*, *Alexandrium fundyense*, *Alexandrium monilatum* and *Alexandrium tamarense*. The only species known to contain *lbp* that was not amplified by the Dinolbp primers was *N. scintillans*. Therefore, the negative PCR results in *Ceratium longipes*, *Fragilidium* cf. *subglobosum*, *Protoperidinium crassipes* and *Pyrocystis* spp. were not conclusively indicative of the absence of the gene as it is possible they were present but not detected. Indeed, the difficulties with detecting *lbp* by PCR were highlighted in the fact that, even where successful, detection was not possible using a single PCR protocol. The reduction of the annealing temperature in a second round of tests resulted in some additional non-specific PCR products but it was only with this protocol that the *lbp* in *A. monilatum*, *C. horrida* and *G. spinifera* could be detected.

3.3.2 Luciferin binding protein gene sequences

The sequences obtained from two clones of every PCR product were never identical, with identities among clones ranging from 91.8% to 99.7% in *P. reticulatum* and *A. monilatum* respectively. The sequence identities between species ranged from 99.7% between *A. affine* and *A. tamarense* CCMP 115 to 50% between *G. spinifera and N. scintillans.* Pseudogenes were identified in five sequences. Insertions of one or two nucleotides in *A. affine* C3 (position 811), *A. monilatum* C4 (positions 858, 883) and *P. reticulatum* C1b (868) caused shifts in the reading frame. A single nucleotide insertion was found in *G. spinifera* C1.2 which shifted the reading frame. However, a deletion found in both clones of this species at position 615 resulted in loss of the reading frame in C2 but reinstated the correct reading frame of C1.2 after 100 amino acids of sequence that did not resemble LBP.

A sequence alignment of the translated amino acid *lbp* sequences (Figure 3.1) showed that the *lbp* of photosynthetic species were highly similar with more than 60% of the residues consistently being identical. By contrast, *N. scintillans* showed the greatest dissimilarities, in agreement with the low nucleotide and amino acid identities exhibited to all other organisms. There was no part of the sequences examined that showed long regions containing a large number of conserved positions, but rather these were spread out throughout the alignment.

Table 3.2 Details of the dinoflagellate cultures tested in this study with the results of *lbp* PCR tests.

Species	Source	Strain	LBP
Alexandrium affine[a]	CCMP	112	+*
Alexandrium fundyense[b]	CCMP	1978	+*
Alexandrium monilatum	MIL		+*
Alexandrium tamarense[b]	CCMP	115	+*
Alexandrium tamarense[b]	CCMP	1598	+*
Alexandrium tamarense[b]	PLY	173	+
Ceratium longipes	CCMP	1770	-
Ceratocorys horrida	CCMP	157	+
Ceratocorys horrida	MIL		+*
Fragilidium cf. *subglobosum*	ALISU	I097-01	-
Gonyaulax spinifera	CCMP	409	+*
Lingulodinium polyedrum[b†]	NOCS	M22	+
Noctiluca scintillans[b†]	MIL		-
Protoceratium reticulatum[a]	CCMP	1889	+
Protoperidinium crassipes	AY		-
Pyrocystis lunula[c]	CCMP	731	-
Pyrocystis lunula[c]	CCAP	1131	-
Pyrocystis noctiluca[c]	CCMP	732	-

[a] Species reported to contain LBP (L. Liu and J.W. Hastings, unpublished data, reported in Liu et al. 2004)

[b] The presence of *lbp* is known in the species

[c] The absence of LBP is known in the species

[†] *Lbp* has been has been fully sequenced

* PCR product sequenced in this study

3.3.3 Phylogenetic analysis

The differences between sequences of several species were difficult to interpret because it is unknown which variant of *lbp* is amplified by the PCR primers in each species. In order to define the clades in the phylogenetic analysis (Figure 3.2) the variation among the two *lbp* variants in *L. polyedrum* and the normal intracopy variation likely within each variant (e.g. 4.2% among two sequences of *C. horrida*) had to be considered. Based on this, sequences sharing 81- 91% identity could reflect two different *lbp* variants within the same organism and consequently the differences between species were significant when sequences were less than 81% identical. The phylogenetic analysis of novel *lbp* sequences (Figure 3.2) revealed four clades in the *lbp* sequences that were highly statistically supported. *N. scintillans* was distinct from all other species forming its own distinct clade. Within gonyaulacoid dinoflagellates three clades were identified, all with high statistical support: a *L. polyedrum* clade, an *Alexandrium* clade and a clade consisting of *A. monilatum*, *C. horrida*, *G. spinifera* and *P. reticulatum* sequences. Within the latter clade sequences of *C. horrida* and *P. reticulatum* were intermingled and they were all similar to *G. spinifera* (80-82.3% identity). This clade also included *A. monilatum* which was most similar to *C. horrida* (82.3 % maximum identity) than to any other *Alexandrium* species (81% maximum identity with *A. affine*).

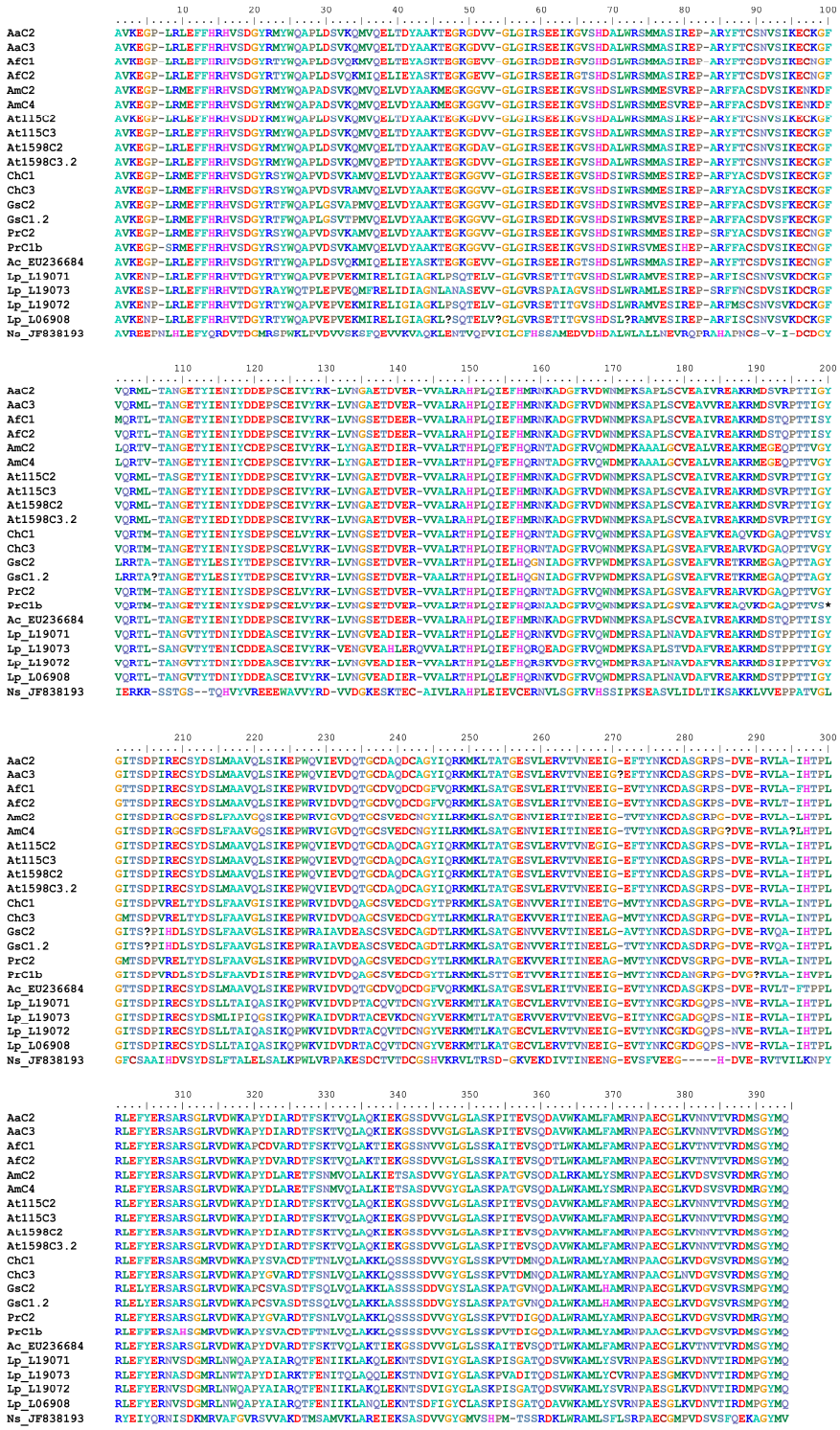

Figure 3.1 Alignment of translated *lbp* sequences. A unique colour is given to every residue in order to highlight similarities among sequences and conserved positions. Accession numbers are given for sequences obtained from GenBank and clone identifier for sequences generated in this study. Aa=*Alexandrium affine*, Ac=*A.catenella*, Af=*A. fundyense*, Am=*A.monilatum*, At=*A. tamarense*, Ch=*Ceratocorys horrida*, Gs=*Gonyaulax spinifera*, Ns=*Noctiluca scintillans*, Lp=*Lingulodinium polyedrum*, Pr=*Protoceratium reticulatum.*

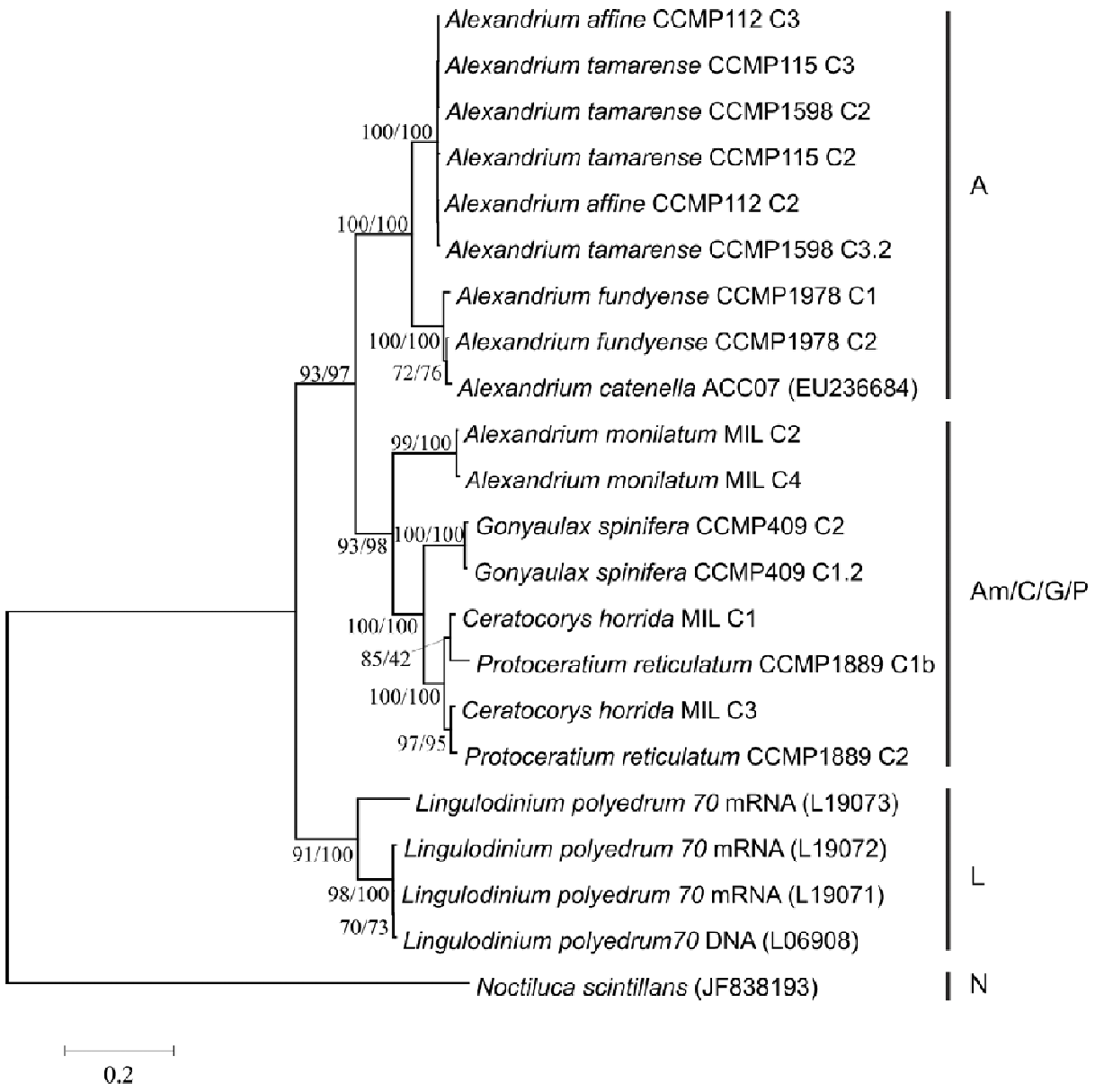

Figure 3.2 Maximum likelihood tree based on a nucleotide alignment of partial *lbp* sequences. Bootstrap values shown at the nodes are based on 100 replications (maximum likelihood/ maximum parsimony). The scale of branch lengths shows 2 substitutions per 10 sites. Accession numbers of sequences obtained from GenBank are given in brackets. Four clades of *lbp* are shown, indicated by vertical lines. A = *Alexandrium*, Am = *Alexandrium monilatum*, C = *Ceratocorys horrida*, G = *Gonyaulax spinifera*, L= *Lingulodinium polyedrum*, N = *Noctiluca scintillans*, P = *Protoceratium reticulatum*.

3.4 Discussion

The primary aim of this study was to determine which dinoflagellates contain *lbp* and indeed it was found in several closely related gonyaulacoid dinoflagellates. However, the lack of detection of *lbp* in *N. scintillans* which is known to contain this gene meant that no negative PCR result could be interpreted as an absence of *lbp*. *Noctiluca scintillans* bioluminescence genes are very distinct to other bioluminescent dinoflagellates, particularly in the *lbp* domain (Liu and Hastings 2007). It is therefore not surprising that a "universal" primer pair cannot be designed for *lbp* as has been previously done with *lcf* (Chapter 2). Within Gonyaulacales though, there is a possibility that *lbp* could be detected in all species that contain it, but this cannot be determined from the present results. The absence of expressed LBP has been previously confirmed in *Pyrocystis* spp. (Schmitter et al. 1976, Knaust et al. 1998) and is thus consistent with the negative PCR results in our analyses. However, *Ceratium* and *Fragilidium* spp. have never been tested for LBP at the proteomic or genomic level. The fact that *lbp* was not been detected with the Dinolbp primers could indicate that these genera represent further species with a bioluminescence system that does not utilize LBP or that *lbp* within Gonyaulacales is highly diverse as is *lcf*. Indeed, the *Fragilidium* spp. and *Ceratium* spp. tested in Chapter 2 did not amplify with the same primers as other gonyaulacoid dinoflagellates which itself suggests that there are as yet unknown differences between their bioluminescence genes. It is, however, also possible that there were deleterious mutations at the target sites of the *lbp* primes in the two strains tested which were both non bioluminescent and contained only *lcf* pseudogenes when tested in Chapter 2. Unfortunately, no DNA was available for testing from bioluminescent species of *Fragilidium* and *Ceratium* at the time of this study; the culture of the bioluminescent *Ceratium digitatum* used in Chapter 2 had collapsed and all the DNA material had been used up in that study.

It is well known that protein coding genes in dinoflagellate genomes occur in large gene families with sequence variation among gene copies that also include numerous pseudogenes (Zhang and Lin 2003, Lin et al. 2009, Kim et al. 2011). This makes it difficult to predict if a genomic sequence reflects an expressed gene, without sequencing numerous copies of the gene in both the genomic and transcript pool. In this study, two clones were sequenced from each PCR product. Indications of pseudogenes with frameshifting mutations were identified in one of two sequences of three species, which were highly bioluminescent at the time of

harvesting for DNA analyses. Thus, the mutations in these sequences did not have a harmful effect on the bioluminescence system. However, *G. spinifera* was the only species that yielded two sequences that were both mutated. While this is possibly a coincidence and it is likely that some functional *lbp* copies remain, it is also possible that this *G. spinifera* strain which emits bioluminescence so dim that it is visually undetectable (Baker et al. 2008), contains mutations in more *lbp* gene copies than in other species. It would be interesting to investigate whether mutations in *lbp* that inhibit the binding of luciferin or result in the expression of only few functional gene copies, are responsible for the underperformance of the bioluminescence system in non bioluminescent strains of normally bioluminescent species (Chapter 2, Swift et al. 1973, Schmidt et al. 1978). The potentially immense importance of *lbp* is highlighted in transcriptional studies on *Alexandrium* species and *Lingulodinium polyedrum* where it was found to be one of the most highly expressed genes in all these organisms, even surpassing that of *lcf* (Tanikawa et al. 2004, Hackett et al. 2005, Erdner and Anderson 2006, Uribe et al. 2008, Toulza et al. 2010). Likewise, LBP can make up 1% of the total protein of *L. polyedrum* (Morse et al. 1989b). The findings of these studies and indications of potentially more pseudogenes in *G. spinifera* sequences generated in this study, suggest that bioluminescence is a highly important process in these organisms and that *lbp* is potentially as important as *lcf* in the functioning of the bioluminescent system.

An amino acid alignment of translated *lbp* sequences showed that the binding site of LBP to the luciferin cannot be readily deduced. The region coding for the luciferin binding site in *lcf* is characterized by a long region of the gene that is highly conserved at both the nucleotide and amino acid level, even in the most genetically distant organisms examined to date (Chapter 2, Liu and Hastings 2007). However, no such features exist in *lbp* and additionally there is no part of the sequence that is similar to *lcf*. It could be that the only two conserved regions that were used in this study to design the primers and are thus not included in the alignment, have a role in binding the luciferin. Considering, that both *lcf* and *lbp* produce proteins that bind the same substrate, it would be interesting to investigate the crystal structure of native LBP, as the binding site could also result from the interaction of the two protein molecules that form the functional dimer.

The phylogenetic analysis of *lbp* sequences showed four distinct clades. Within gonyaulacoid dinoflagellates, the associations of organisms were remarkably similar to a previous phylogenetic tree of N-terminal *lcf* sequences of the same organisms (Chapter 2). *Ceratocorys*

horrida and *P. reticulatum* are virtually undistinguishable in this study and also with respect to their *lcf* (Chapter 2) and ribosomal genes (Gomez et al. 2010b), indicating that they are genetically very closely related despite the large differences in their morphology (e.g. presence of spines in *C. horrida*). This could suggest that these two species have diverged only recently and that their morphological differences quickly evolved in response to their respective environments. For example, the spines of *C. horrida* are thought to aid the floatation of the cells and are rapidly retracted when turbulence increases (Zirbel et al. 2000). Consequently, morphological characters may not describe the evolution of these species correctly and are another typical example where morphology and molecular phylogeny demonstrate the complexities in phytoplankton systematics.

The *lbp* of the dinoflagellate species *G. spinifera* is closely related to both of *C horrida* and *P. reticulatum*. This finding further supports the conclusions of Chapter 2 with much shorter *lcf* sequences from the N-terminal region, where it was considered inappropriate to designate a separate clade for this species as was initially suggested by Baker et al. (2008). In order to robustly investigate the relationships of bioluminescence genes of *Gonyaulax* species, more species need to included and deeper sequencing of gene variants must be conducted.

Alexandrium monilatum lbp is divergent from that of other *Alexandrium* spp. This is in agreement with its SSU rDNA (Rogers et al. 2006), its toxin profile (Hsia et al. 2006) and with previous findings using partial *lcf* sequences (Chapter 2). In the latter study, the short sequences used resulted in this organism clustering with *L. polyedrum* which is not the case using a long fragment of *lbp*. As previously suggested (Chapter 2), the bioluminescence genes of *A. monilatum* could be an excellent model to study the evolution of bioluminescence and possibly the phylogenetic relation between the ecologically important gonyaulacoid dinoflagellate species. Additionally, the inclusion of other representative bioluminescent *Alexandrium* species that are distinct from the *Alexandrium tamarense* 'species complex' such as *A. ostenfeldii* (Kremp et al. 2009) could help to better constrain the relationships among diverse *Alexandrium* species.

The finding that *lbp* is present in several gonyaulacoid dinoflagellate species is indicative of bioluminescence systems utilizing LBP being more common than those that do not. At present, the only bioluminescence system that has been shown to lack LBP is that of *Pyrocystis* spp. (Schmitter et al. 1976, Knaust et al. 1998). *Pyrocystis lunula* has been a model

organism to study the chemistry of dinoflagellate bioluminescence and it is the only species whose luciferin has been structurally characterised; it was found to be an open chain tetrapyrrole that was suggested to be a breakdown product of chlorophyll *a* (Nakamura et al. 1989). This luciferin is thought to be universal in dinoflagellates because LCF from any dinoflagellate can use it as a substrate to produce light (Nakamura et al. 1989). It has been hypothesized that heterotrophic dinoflagellates either acquire luciferin nutritionally or that it is synthesized from the conversion of chlorophyll in ingested prey (Liu and Hastings 2007). However, a recent study showed that the heterotrophic dinoflagellate *Protoperidinium crassipes* maintained its bioluminescence intensity for one year in the absence of chlorophyll or luciferin containing food, indicating a different origin of luciferin in this organism (Yamaguchi and Horiguchi 2008). The presence of *lbp* in several photosynthetic species indicates that perhaps even within these species there is an alternative luciferin molecule that requires LBP for stabilization in contrast to *P. lunula* luciferin. There are indeed a large number of highly similar tetrapyrrole molecules in cells of both photosynthetic and heterotrophic organisms with diverse functional roles (Kadish et al. 2003) that could act as precursors for luciferin. Therefore, more studies on the structure of dinoflagellate luciferin, particularly of LBP containing species, are necessary so that the biochemistry of bioluminescence and its link with other physiological pathways in the cell can be fully understood. The phylogenetic analysis of novel *lbp* sequences suggests that the phylogenetically distinct and ecologically important dinoflagellate species *L. polyedrum* and *A. tamarense* would be good model organisms to further investigate the chemistry of dinoflagellate bioluminescence.

Chapter 4

BIOLUMINESCENT DINOFLAGELLATES OF THE PATAGONIAN SHELF DURING EARLY AUSTRAL SUMMER 2008

Abstract

The distribution of bioluminescent dinoflagellates in the Patagonian Shelf region was investigated using "universal" PCR primers for the dinoflagellate luciferase gene (*lcf*). Coincidental discrete measurements of bioluminescence were undertaken and sequences of *lcf* as well as single cell PCR tests allowed for the identification and quantification of bioluminescent dinoflagellates by microscopy. Molecular detection of *lcf* showed that bioluminescent dinoflagellates were widespread in the majority of the study region. Their presence was comparatively underestimated by optical bioluminescence measurements which were additionally affected by interspecific differences in bioluminescence intensity and by other bioluminescent organisms. Molecular and microscopy data showed that the complex hydrography of the area played an important role in determining the distribution and composition of dinoflagellate populations. Dinoflagellates were absent south of the Falkland Islands where nutrient rich, well mixed and cold Falklands Current Waters harboured diatoms instead. Diverse populations of dinoflagellates appeared when the Shelf and Falklands Current Waters warmed and stratified as they flowed northwards. The shelf break front, supported the highest concentrations of a bioluminescent dinoflagellate population (~4000 cells L^{-1}), which was dominated by *Gonyaulax* sp., as well as a marked bloom (~2 million cells L^{-1}) of the non bioluminescent *Prorocentrum* cf. *minimum*, and was therefore catalytic for dinoflagellate growth, possibly due to upwelling of nutrient rich water. By contrast, low salinity and nutrient waters that were influenced by the Rio de la Plata contained bioluminescent *Noctiluca scintillans* along with non bioluminescent *Ceratium tripos*. This study represents the first application of a molecular approach to detect bioluminescent dinoflagellates in natural water samples and is shown to be a promising tool for ecological studies of these organisms.

4.1 Introduction

Dinoflagellates are the most ubiquitous protists in the marine environment that produce light (Kelly 1968, Tett 1971, Haddock et al. 2010, Widder 2010). Their bright bioluminescence in surface oceanic and coastal waters has been reported, often as 'glowing water' (Lynch 1978), in all oceans of the world (Raymond and DeVries 1976, Lapota and Losee 1984, Lapota et al. 1992, Swift et al. 1995). Light is produced by dinoflagellates intracellularly in organelles called scintillons (DeSa and Hastings 1968) which contain the enzyme luciferase and a luciferin substrate, which in some species is stabilised by a luciferin binding protein (Liu et al. 2004, Liu and Hastings 2007, Uribe et al. 2008). When cells are mechanically agitated, the luminescent chemistry is activated producing blue light in the form of brief and bright flashes (Hastings 1996). Bioluminescence in dinoflagellates is thought to have a defensive role protecting them from their predators by either startling them (Buskey et al. 1983) or by attracting higher level predators which in turn consume them (Abrahams and Townsend 1993, Fleisher and Case 1995). Bioluminescence may therefore be an important survival strategy playing a key role in bloom formation and may have significant consequences in terms of its potential to restructure oceanic food webs. However, little is known about the distribution and ecological characteristics of bioluminescent dinoflagellates.

Ecological studies on bioluminescent dinoflagellates are made difficult by the lack of suitable methods to detect these organisms in mixed planktonic communities. Instruments which measure *in situ* stimulated light emission, termed bathyphotometers, have thus far been the only tool to assess the presence and magnitude of bioluminescence in the water column. They typically consist of a grid or impeller that stimulates bioluminescence in both dinoflagellates and zooplankton, followed by a photodiode or photomultiplier that measures the light emitted by the passing bioluminescent organisms (reviewed in Herren et al. 2005). Detailed *in situ* investigations on light producing organisms have shown that both dinoflagellates and zooplankton can be significant contributors to the stimulated bioluminescence depending on the location and the season, and that in most cases they occur simultaneously (Lapota and Losee 1984, Swift et al. 1985, Lapota et al. 1992, Swift et al. 1995). However, the contribution of each of these groups to a given bioluminescence measurement cannot be readily discerned.

Dinoflagellate bioluminescence varies inter- and intraspecifically, with some species producing light so dim that it is only detectable at very high cell densities by sensitive instruments (Baker et al. 2008). Bioluminescence is 'switched off' during the day by photoinhibition and by diurnal rhythms that are controlled by a circadian clock, which also modulate its intensity at night (Hardeland 1982, Fritz et al. 1990, Buskey et al. 1992). Additionally, environmental and physiological factors can impact the intensity of bioluminescence produced by an organism (Buskey et al. 1992, Sullivan and Swift 1994, Sullivan and Swift 1995, Latz and Jeong 1996). Accordingly, optical bioluminescence measurements in dinoflagellate cultures have been found to underestimate the potential for bioluminescence in these organisms (Chapter 2; Baker et al. 2008). It is therefore unlikely that complex bioluminescent signatures can be used to reveal the distribution of bioluminescent dinoflagellates in diverse oceanic plankton communities.

The use of gene specific primers has been widely reported in a variety of environments to detect the presence of genes of bacteria (McDonald et al. 1995, Voytek and Ward 1995, Allen et al. 2001), diatoms (Allen et al. 2005) and dinoflagellates (Godhe et al. 2001, Godhe et al. 2002b) associated with particular biogeochemical or ecological processes of interest. In this context, primers designed for the amplification of bacterial luciferase genes resulted in the detection of diverse assemblages of uncultivated marine bioluminescent bacteria in bathypelagic waters of the Tyrrhenian Sea (Gentile et al. 2009). Similarly, a study by Baker et al. (2008) showed that bioluminescent dinoflagellates could be detected in natural samples using PCR primers targeting the dinoflagellate luciferase gene (*lcf*). While the primers described by Baker et al. (2008) were later found to amplify *lcf* only from a limited number of species, a new primer set described in Chapter 2 that amplifies *lcf* from all the main bioluminescent dinoflagellate genera, could be used to detect diverse bioluminescent dinoflagellate populations in oceanic waters.

A research cruise to the Patagonian Shelf region provided an ideal opportunity to test the molecular approach for the detection of bioluminescent dinoflagellates in the environment. The Patagonian Shelf is located in the South West Atlantic along the eastern seaboard of Argentina (Figure 4.1). It is a broad continental shelf up to 850 km wide and hosts the South East South American Large Marine Ecosystem (Bisbal 1995). The Patagonian Shelf is one of the most productive regions in the world's oceans and a globally important CO_2 sink (Schloss et al. 2007, Bianchi et al. 2009). The region is well known for its yearly recurring

coccolithophore blooms along the shelf break front which are evident as highly reflective ‘white’ waters in satellite images due to the calcite liths covering these organisms (Brown and Podestá 1997, Iglesias-Rodriguez et al. 2002). However, high chlorophyll concentrations that are not related to coccolithophores (i.e. high calcite) have been attributed to diverse assemblages of diatoms and dinoflagellates (Negri et al. 1992, Gayoso and Podestá 1996, Carreto et al. 2003, Olguín et al. 2006, Carreto et al. 2008, Garcia et al. 2008) including blooms of the bioluminescent *Alexandrium tamarense* during spring and early summer along the coast of Argentina (Carreto et al. 1986, Gayoso 2001, Gayoso and Fulco 2006).

The hydrography of the South West Atlantic is highly dynamic and complex due to the interaction of subpolar, subtropical and riverine waters derived from the Falklands (Malvinas) and Brazil Currents and the Rio de la Plata outflow, respectively (Figure 4.1). The Falklands Current is a branch of the Antarctic Circumpolar Current, carrying cold water northwards over the continental shelf slope, until it meets the southward flow of the warm and saline Brazil Current. The region of mixing and offshore movement of both currents between 36 and 38 ºS forms the Brazil Falklands confluence zone (BFCZ) (Olson et al. 1988, Gordon 1989, Piola and Gordon 1989, Provost et al. 1996, Brandini et al. 2000). On the Patagonian Shelf, northward flowing shelf waters which are characterised by warmer temperature and lower salinity due to water inputs from the Pacific through the Magellan Strait, flow parallel to the Falklands Current (Carreto et al. 1995, Sabatini et al. 2004). The interaction of shelf waters and the Falklands Current results in the formation of the Shelf Break front (SBF). This permanent hydrographic feature is characterised by a pronounced thermal gradient and strong biological productivity of both calcifying and non calcifying phytoplankton (Romero et al. 2006, Franco et al. 2008, Painter et al. 2010) whose growth is thought to be supported by upwelling of nutrient rich water along the SBF (Romero et al. 2006, Garcia et al. 2008).

In this study, the distribution of bioluminescent dinoflagellate populations across hydrographically contrasting surface waters and frontal regions of the Patagonian Shelf was evaluated using PCR primers for dinoflagellate *lcf* in conjunction with bioluminescence measurements. Analyses of *lcf* sequences retrieved from mixed plankton samples as well as single cell PCR tests allowed the identification and quantification of bioluminescent dinoflagellates by microscopy. The molecular and microscopy data led to the first investigation of these populations relative to their physical and chemical environment as well

as to other protists. The molecular detection of *lcf* is demonstrated to be a promising tool for ecological studies of bioluminescent dinoflagellates.

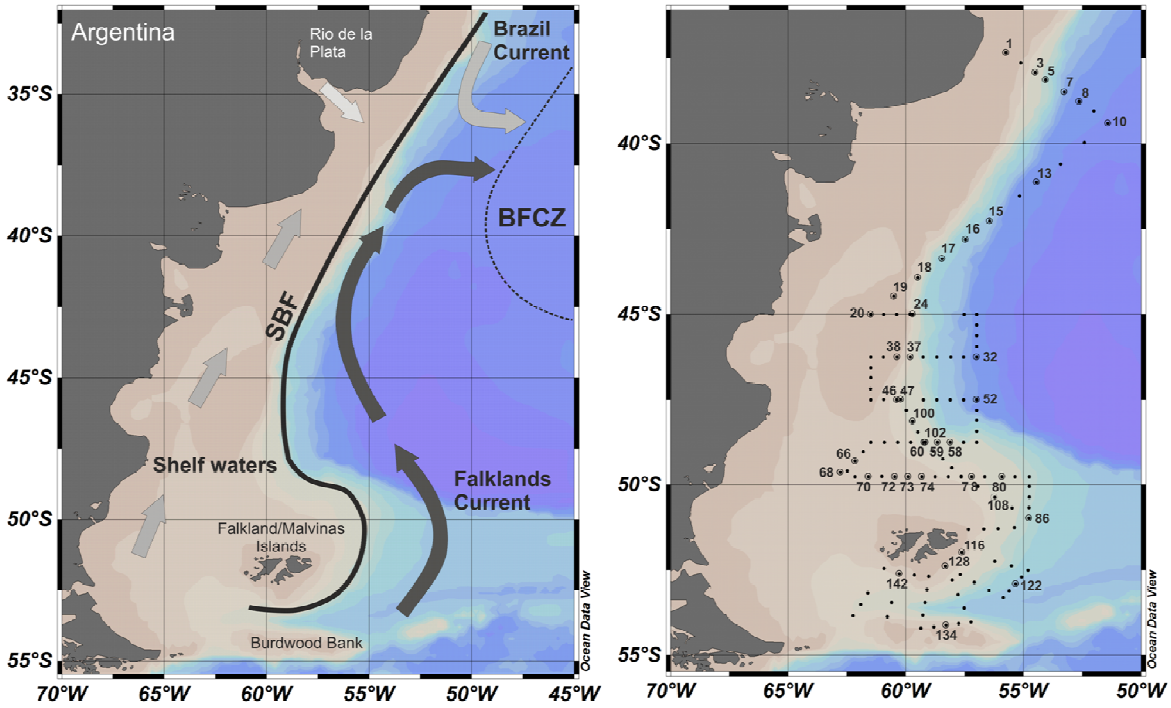

Figure 4.1 Maps of the Patagonian Shelf with the general bathymetry; gradient from darkest brown to darkest blue signifying the increasing depth from approximately 100 m to more than 2000 m. Left panel shows the routes of major currents and the areas where their interactions cause well known features such as the shelf break front (SBF) and the Brazil Falklands Currents confluence zone (BFCZ). The SBF becomes sharper moving northward, coinciding with steepening of the shelf break (sharp transition from brown to blue in the bathymetry), and so south of approximately 47 ºS it covers a less well defined and wider area than depicted by the black line. Right panel is a close up of the cruise area; black dots show all CTD stations of the cruise and enlarged and labelled dots show the stations sampled in this study.

4.2 Materials and Methods

4.2.1Sample collection

Samples were collected during the COPAS ’08 (Coccolithophores of the Patagonian Shelf ‘08) cruise on board the R/V Roger Revelle. The cruise took place from the 4th of December 2008 to the 2nd of January 2009 during which several transects were conducted across the Patagonian Shelf break (Figure 4.1). Forty stations were sampled, mainly during the night, by a CTD rosette at surface (1-5 m) and subsurface chlorophyll maximum (SCM) depths. For the purpose of this study, the SCM is defined as the peak in chlorophyll fluorescence occurring below the surface, largely corresponding to the base of the mixed layer, regardless of whether or not another peak was present at the surface. Twenty litres of seawater were pre-filtered through 1mm nylon mesh and collected in carboys that had been made light impermeable using black tape to prevent the photoinhibition of bioluminescence. Two litre subsamples were taken for bioluminescence measurements and the rest of the water was filtered onto 12 µm pore size Nuclepore polycarbonate membrane filters (Whatman, UK). For mixed community DNA extractions, the filters were immediately frozen at -80 ºC. For the subsequent isolation of single cells for PCR analysis, cells were rinsed off the filters using 2 mL >99.9% ethanol (molecular biology grade, Sigma, UK) and the resulting cell suspension was frozen at -20 ºC. Subsamples of 100 mL were collected and fixed for microscopy analysis with a Lugols’ iodine solution acidified with 10% acetic acid; these samples were initially collected only when a bioluminescence signal was seen but increased in frequency after station 16 of the cruise.

4.2.2 Bioluminescence measurements

Bioluminescence was measured using a Glowtracka bathyphotometer (Chelsea Technologies, UK) that had been converted for benchtop use and was able to process 2 L discrete samples. The setup consisted of a 2 L reservoir attached to a pipe leading into the measuring chamber. The water was kept in the chamber and reservoir by a closed tap at the outlet of the instrument. Excitation of bioluminescence was achieved using a 1mm nylon mesh at the entrance of the detection chamber. This setup had been tested in the lab prior to its use at sea

using cultures of *Lingulodinium polyedrum* and *Pyrocystis lunula* diluted to a large volume. A later improved version of this setup has been described by Marcinko et al. (2011).

Bioluminescence measurements commenced after allowing the samples to recover from handling for 15 minutes. For each measurement, 2 L of seawater were accurately measured using a measuring cylinder, poured into the reservoir of the instrument and left to recover from handling for another 5 minutes. The tap that held the water in the reservoir was then released allowing the water to flow through the instrument. Bioluminescence was measured every millisecond for approximately 15 seconds in the form of a voltage signal recorded by a data logger attached to a computer operating the program LabView (National Instruments, UK). Two litres of fresh water were run through the instrument between every sample measurement in order to clean the instrument of residual cells and to obtain a baseline measurement.

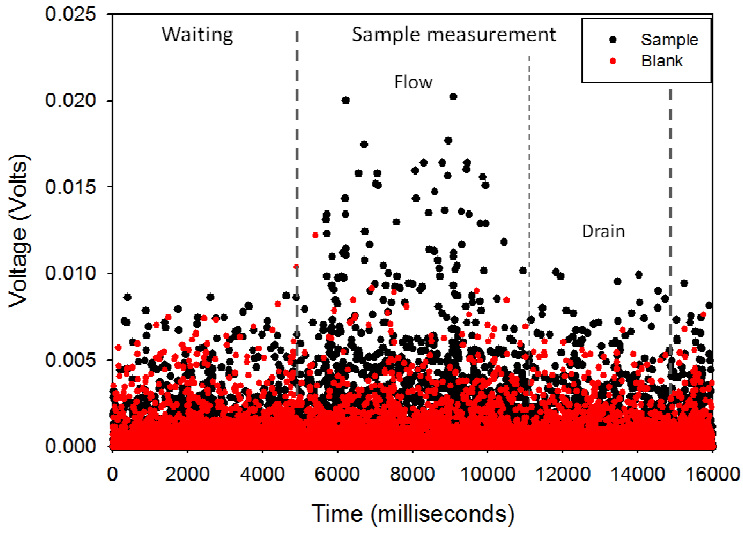

Figure 4.2 Example of a bioluminescence measurement registering voltage at 1 KHz resolution. This sample was taken at Station 1 at a depth of 4m and the corresponding blank measurement is shown. The sample was released after approximately 5 seconds (i.e. 5000 milliseconds). When the sample flowed through the detection chamber, high voltage corresponding to the bioluminescence was recorded relative to the blank. After approximately 11 seconds most of the sample had completed its passage through the detection chamber while small amounts were still draining. The measurement was complete after 15 seconds.

Raw bioluminescence data were processed by summing the voltages recorded in each sample over approximately 15 seconds (Figure 4.2). This voltage value was then converted to a value of photons cm^{-2} s^{-1} using a calibration equation (Voltage * 1.157 $x10^{11}$) provided by the instrument manufacturer, as derived during the calibration process. Blank samples were treated in the same way, making sure that the measurement time of the sample and its corresponding blank were trimmed to exactly the same time interval. The final bioluminescence value was calculated by subtracting the blank value from every corresponding sample value. In order to calculate the detection limit of the method only samples that had been measured for precisely the same length of time could be used. Twenty nine blank samples that were measured for exactly 15 seconds were selected and the final detection limit of 2.5 x 10^{11} photons cm^{-2} s^{-1} corresponds to three times the standard deviation of the average blank value.

4.2.3 DNA extraction

The frozen samples of filtered plankton were processed directly on the filters. Cells were ruptured by immersing the tubes in liquid nitrogen and grinding the frozen cells on the filter using a micropestle until they began to thaw; this procedure was repeated three times. Further rupture of cells was achieved by the addition of 300 µl boiling buffer (1.4 M NaCl, 100 mM Tris HCl, 20 mM EDTA) and incubation at 90 ºC for 10 minutes. This step was important for disrupting 'tough' organisms such as *Ceratium* spp. and was found to increase the DNA yield by up to 5 times. Cells were lysed by incubation at 65 ºC for 1 hour in prewarmed cetyl-trimethylammoniumbromide (CTAB) buffer (2% CTAB, 2% polyvinylpyrrolidone (PVP), 0.5% 2-mercaptoethanol, 1.4 M NaCl, 20 mM EDTA and 100 mM Tris HCl) achieved by adding an equal volume (300 µl) of this lysis buffer with double concnetrations of CTAB, PVP and 2-mercaptoethanol to the boiling buffer containing the ruptured cells. The rest of the extraction followed the modified version of Doyle and Doyle (1990) that was described in Chapter 2. The DNA was dissolved in 30 µl of TE buffer (10 mM Tris and 1 mM EDTA), its purity and quantity were measured using a Nanodrop spectrophotometer (model) and its PCR quality was assessed by amplification of eukaryotic small subunit ribosomal DNA using primers Euk1A/Euk516r-GC (Sogin and Gunderson 1987, Amann et al. 1990, Diez et al. 2001).

4.2.4 Preparation of cells of single cell PCR

Single cells of all *Ceratium* species found were subjected to PCR. This analysis was focused on this genus because several of its species were present in significant numbers at some locations but there was great uncertainty over which of these were bioluminescent (Chapter 2). Also, they could be more easily taxonomically identified after ethanol fixation due to their size and shape, than species of other genera such as *Protoperidinium*. The suspensions of cells that had been stored in ethanol were concentrated by gentle centrifugation at 4000 x g, the supernatant discarded and replaced with TE buffer. This was repeated three times in order to thoroughly remove the ethanol. Individuals identified under an inverted microscope were isolated in 1 µl using a micropipette and then transferred to a PCR tube. Cells that were not used immediately were stored frozen at -80 ºC.

4.2.5 PCR amplification of the luciferase gene

Dinoflagellate *lcf* was amplified using primers DinoLCF_F4 and DinoLCF_R2 and the protocol as described in Chapter 2. The templates for the PCR were either 1µl of extracted DNA (maximum 1 µg), or a single cell in 1 µl TE buffer that had been disrupted by boiling at 90 ºC for 10 minutes followed by rapid cooling on ice, immediately prior to the addition of the PCR components.

4.2.6 Cloning and sequence analyses

The PCR bands from 10 of the mixed community DNA samples that produced a PCR product with the “universal” dinoflagellate *lcf* primers were excised from agarose gels, extracted using the QIAquick gel extraction kit (Qiagen, UK) and cloned into the pCR 4 vector using the TOPO TA cloning kit (Invitrogen, USA). Colonies were screened for inserts using M13 primers and four clones from each sample were sequenced using the M13 forward primer. Sequencing was done by Geneservice (UK). The sequences obtained from the clones of the *lcf* PCR products were trimmed of vector and primers and analysed using the BLASTn tool of the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi), to confirm that the correct gene had been amplified and to determine the similarities of these sequences to those published from other dinoflagellates. All further sequence analysis was conducted in the MEGA v. 5 software

(Tamura et al. 2011). Nucleotide sequence alignments were carried out using ClustalW (Thompson et al. 1994) and were subsequently improved manually. A genetic distance (p-distance) matrix was generated to compare the similarities of environmental *lcf* sequences to those of cultured isolates.

4.2.7 Microscopy

Samples fixed in Lugol's iodine were examined under an inverted light microscope (X200; Brunel microscopes) using the Utermöhl method (Utermöhl 1958). A 50 mL aliquot was left to settle overnight and cells were enumerated at 100x or 200x magnification depending on their size and density in the sample. Cells larger than approximately 8 µm were identified according to Hasle and Syvertsen (1997) and Steidinger and Tangen (1997).

4.2.8 Nutrient and chlorophyll measurements

The macronutrients nitrate, phosphate, silicic acid and ammonium were measured using an autoanalyser and standard protocols (Grasshoff et al. 1983); data was provided by Dan Shuller (Scripps Institution of Oceanography, California, USA). Chlorophyll analyses were conducted by the group of William M. Balch (Bigelow Laboratory for Ocean Sciences, Maine USA) who provided this data for the stations sampled in this study where available.

4.3 Results

4.3.1 Environmental setting

The study area consists of a range of hydrographic provinces which have been described in detail for this cruise by Painter et al. (2010). The characteristics of six key water masses relevant to this study are summarised in Table 4.1. Using these characteristics and surface salinity data, the positions of these waters masses at the surface relative to the stations sampled during this study, are shown in Figure 4.3. During the beginning of the cruise at the northern part of the study region until the latitude of 40 ºS, the surface waters were influenced by the

Rio de la Plata Water (stations 1 and 5) followed by Brazil Current Water (stations 8-11). The remainder of the cruise repeatedly crossed the northward flowing Falklands Current Waters found mostly off or near the shelf break as well as Shelf Waters west of the Falklands Current. The distinction between Shelf and Falklands Current Waters signified the position of the SBF. The Shelf Waters were further subdivided from east to west into Subantartic Shelf Water, High Salinity Shelf Water and Low Salinity Shelf Water, which extended in parallel meridionally.

Table 4.1 Characteristics of surface water masses present in the study area (modified from Painter et al. 2010).

Water mass	Salinity	Temperature
Rio de la Plata outflow	<33	>18
Brazil Current Waters	>34	>16
Falklands (Malvinas) Current Water	>33.9	-
Shelf Water: Subantarctic	33.78-33.9	-
Shelf water: High salinity	33.58-33.78	-
Shelf water: Low salinity	<33.58	-

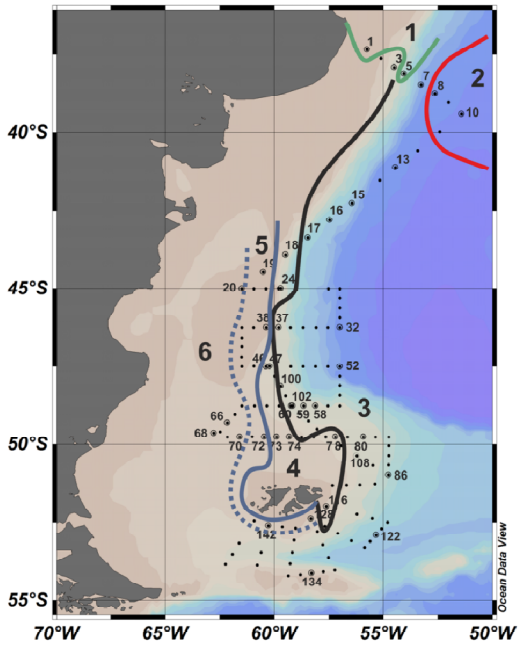

Figure 4.3 Map of the Patagonian Shelf showing the positions of surface water masses relative to the stations sampled during this cruise. 1 = Rio de la Plata Water; 2 = Brazil Current Water; 3 = Falklands Current Water; 4 = Subantarctic Shelf Water; 5 = High Salinity Shelf Water; and 6 = Low Salinity Shelf Water.

The surface distributions of some key physical and chemical variables as well as mixed layer depth which, for consistency with Painter et al (2010), was defined as the depth where temperature decreased by 0.5 ºC relative to the surface values (Levitus 1982), are shown in Figure 4.4. The area south of the Falkland Islands (i.e. south of approximately 52 ºS) was characterised by colder waters (<10 ºC) and a deeper mixed layer (>50m). As these waters flowed towards the north, surface temperature increased to approximately 13 ºC complemented by a shoaling of the mixed layer depth to 20-30 m. East-west gradients in temperature were not pronounced even though the salinity data showed the distinction between the Shelf and Falkland Current Waters, with salinity below or above 33.9, respectively.

Nitrate and phosphate followed a similar latitudinal gradient to temperature with high surface nitrate and phosphate concentrations (>10 µM and >0.8 µM respectively) found south of the Falkland Islands, which declined rapidly northwards in both the Shelf and Falkland Current Waters. Despite this decline, concentrations generally remained high (approximately 2 µM nitrate and 0.2 µM phosphate) across most of the study area until 40 ºS. Ammonium concentrations, although patchy, similarly decreased northwards. Silicate did not follow the distribution patterns of other macronutrients and was generally low (<2 µM) throughout the study region with a few exceptions such as the area north of 40 ºS. East-west gradients were evident in most macronutrients, particularly nitrate and phosphate, with lower nutrient concentrations on the west side of the SBF within the Shelf Waters, particularly in the Low Salinity Shelf Water where nitrate was depleted. Additionally, nitrate, phosphate and ammonium were higher near the SBF between the latitudes 47-50 ºS, compared to adjacent areas.

The northern area of the study region, north of 40 ºS, was characterised by waters with temperatures exceeding 15 ºC which corresponded to low salinity (<30) water from the Rio de la Plata (stations 1 and 5) and high salinity (>34.9) water from the Brazil Current (stations 8-11). Both water masses were poor in surface macronutrients (<0.01 µM nitrate and <0.3 µM phosphate) but had a high silicate load (3-14 µM), particularly near the Rio de la Plata outflow.

Figure 4.5 shows a comparison of nitrate phosphate and discrete chlorophyll concentrations at the surface and the depth of the subsurface chlorophyll maximum at the stations sampled

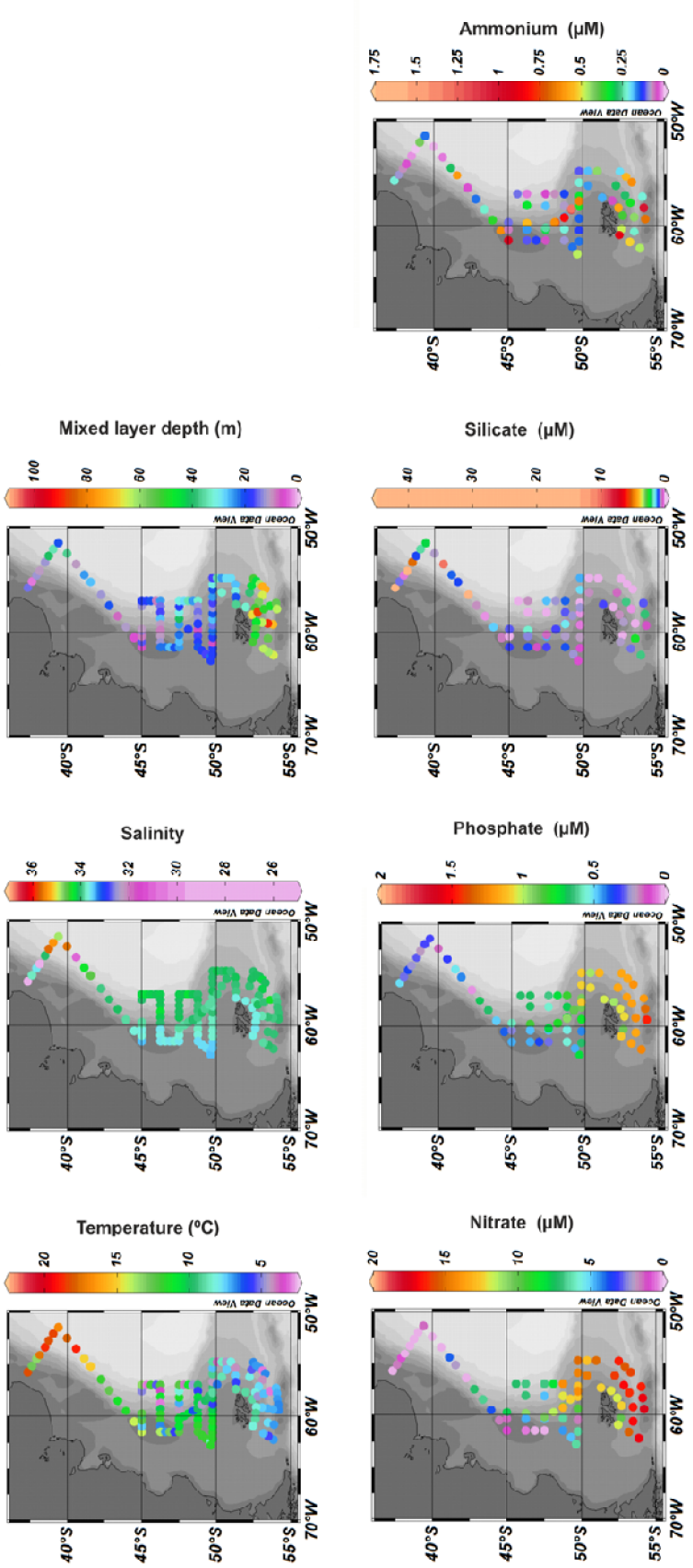

Figure 4.4 Maps of the Patagonian Shelf showing the large scale surface distributions of physical and chemical variables from the whole cruise dataset. The colour scales are adjusted for resolution at the most common concentrations.

during this study. South of 50 ºS where the mixed layer depth was deeper than 30 m, the high surface and subsurface chlorophyll maximum nutrient concentrations (>10 μM nitrate and >0.8 μM phosphate) were nearly homogeneous. Further north the major latitudinal northward decline in concentrations of nitrate and phosphate in surface waters (Figure 4.4) was not evident at the subsurface chlorophyll maximum depths where nutrient concentrations were more variable. For example, high concentrations of nitrate and phosphate (>10μM and >1 μM respectively) at the subsurface chlorophyll maximum depth at stations 18 and 24, situated on the SBF, were not reflected in the corresponding surface concentrations of these nutrients, where surface concentrations of both nutrients at both stations were equal to or less than half of those at depth. Chlorophyll concentrations generally ranged from 0.1-3.9 μg L^{-1} (Figure 4.5) and showed a patchy distribution not corresponding to trends in nutrient concentrations at either the surface or at depth. Vertical differences were examined at 30 stations where data was available at both depths. Out of these 30 stations 11 showed similar chlorophyll concentrations with less than 0.2 μg L^{-1} difference between the surface and the subsurface chlorophyll maximum (e.g. station 142). Higher chlorophyll concentrations were found at the subsurface chlorophyll maximum at 13 stations while only 6 stations had higher chlorophyll concentrations at the surface. Some of the highest chlorophyll concentrations were associated with stations near the SBF (e.g. stations 5, 18, 46, 60 and 74).

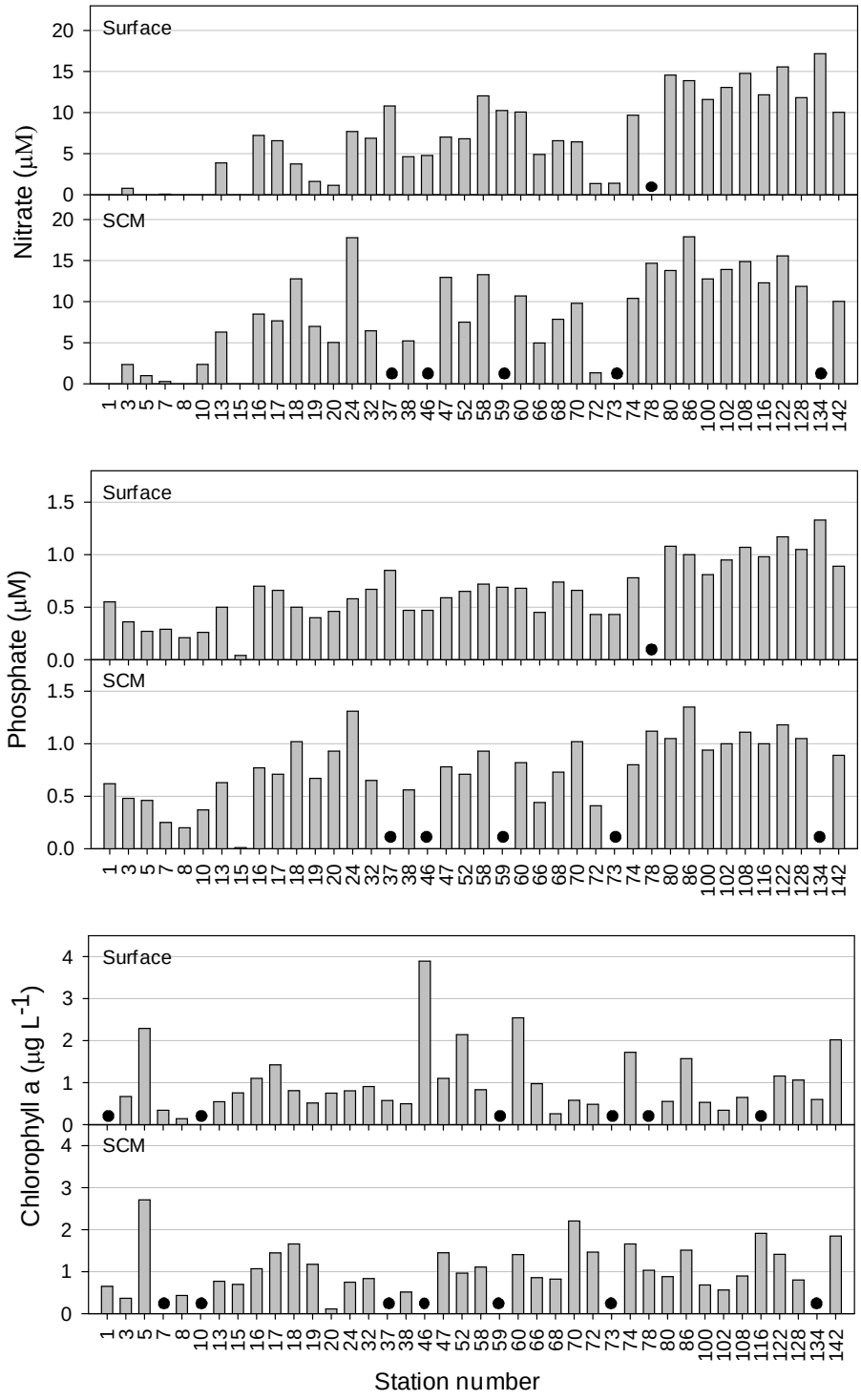

Figure 4.5 Comparison of nitrate, phosphate and chlorophyll concentrations at the surface (upper panel) and at the depth of the subsurface chlorophyll maximum (SCM; lower panel), at the stations sampled in this study. A black circle indicates no data at that point rather than a zero value.

4.3.2 Luciferase gene detection in mixed communities and single cells

The amplification of dinoflagellate *lcf* from natural samples was highly specific producing well defined PCR bands without non specific background amplification (Figure 4.6). Of the 72 samples analysed (after three being omitted due to poor purity and PCR failure), 48 produced a PCR product corresponding to the expected 280 bp band for *lcf*. The specificity of the primers to the correct gene was confirmed by sequencing 40 clones from 10 samples, representing approximately 20% of the samples that produced PCR product. The PCR assay was also applied to single cells in order to distinguish bioluminescent from non bioluminescent species in the genus *Ceratium* (Figure 4.7). Three cells of *C. fusus* produced a PCR band in this assay and thus were the only ones found to contain *lcf* in the single cell PCR tests; *lcf* was not found in *C. furca*, *C. lineatum*-like, *C. tripos* and a *C. teres*-like species.

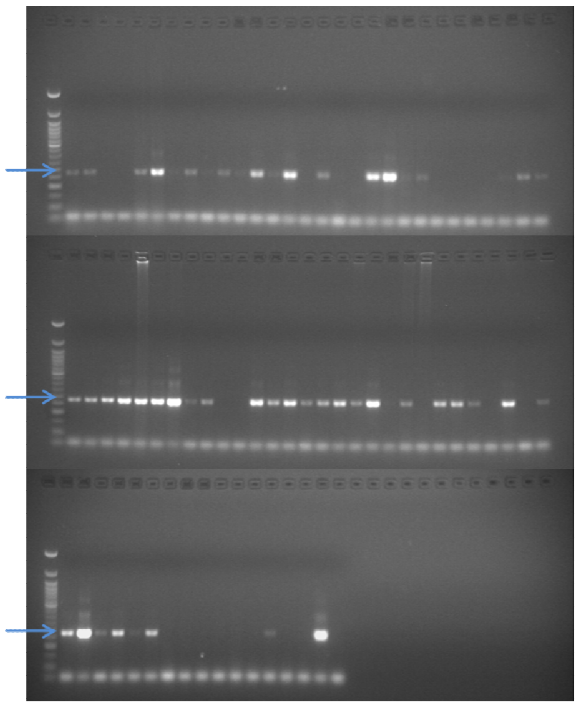

Figure 4.6 Gel photograph of the luciferase gene PCR on samples collected during the COPAS cruise, showing the very specific and efficient detection of the gene from mixed plankton community DNA samples. The first lane in each row is a 50 bp DNA marker and last two lanes are positive and negative control respectively. The 270 bp band marked by a blue arrow corresponds to the luciferase gene PCR product. Samples are in order of collection i.e. consecutive stations with chlorophyll maximum depth sample first followed by the surface sample, but individual numbering is omitted as the results are shown on a map in Figure 4.8.

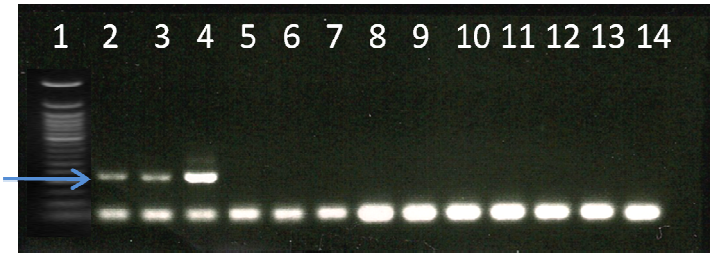

Figure 4.7 Gel photograph of representative PCR tests for the detection of the luciferase gene in single cells of various *Ceratium* species. Lane contents: 1) 50 bp DNA marker; 2-4) *Ceratium fusus*; 5-7) *Ceratium furca*; 8-10) *Ceratium tripos*; 11-12) *Ceratium lineatum*; 13-14) *Ceratium* cf. *teres*.

4.3.3 Luciferase gene sequences

Four *lcf* sequences were obtained from each of the 10 samples selected for sequencing, resulting in a total of 40 sequences from the study area (Table 4.2). Sequences with 92-97% identity to *N. scintillans* were only detected in samples from stations 1 and 5 and made up all the sequences obtained from station 1. The rest of the samples were dominated by sequences of an organism that was most similar to *L. polyedrum* (83-85% identity), accounting for 26 out of the 32 remaining sequences. Samples from stations located near the SBF (17, 24, 47 and 74) along with station 134 situated near the southern coast of the Falkland Islands, were composed exclusively by sequences of this *L. polyedrum*-like organism. Samples from stations located away from the SBF, yielded *lcf* sequences with similarities to a more diverse array of organisms: *Alexandrium tamarense*, *Ceratocorys horrida*, *Gonyaulax spinifera* and *Protoceratium reticulatum*.

Table 4.2 Similarities of *lcf* sequences amplified from selected locations to *lcf* of other organisms measured as genetic distance (p-distance). Surface and deep samples are indicated by S and D, respectively. When a sequence was equally similar to that of two organisms this is indicated by 'and' between the species names.

Station (S/D)	Most similar organism	p-distance	No. of sequences
1 (D)	*Noctiluca scintillans*	0.03-0.08	4
5 (D)	*Noctiluca scintillans*	0.06	1
	Alexandrium tamarense and *Gonyaulax spinifera*	0.17-0.18	2
	Ceratocorys horrida	0.03	1
17 (D)	*Lingulodinium polyedrum*	0.15-0.18	4
24 (D)	*Lingulodinium polyedrum*	0.15-0.17	4
46 (S)	*Lingulodinium polyedrum*	0.17	2
	Gonyaulax spinifera and *Protoceratium reticulatum*	0.1	1
	Protoceratium reticulatum	0.1	1
47 (S)	*Lingulodinium polyedrum*	0.16-0.17	4
68 (S)	*Lingulodinium polyedrum*	0.15-0.17	2
	Ceratocorys horrida	0.12-0.13	2
74 (S)	*Lingulodinium polyedrum*	0.16-0.18	4
80 (S)	*Lingulodinium polyedrum*	0.17-0.18	2
	Alexandrium tamarense	0.19	1
	Protoceratium reticulatum	0.17	1
134 (S)	*Lingulodinium polyedrum*	0.17	4

4.3.4 Distribution of luciferase and bioluminescence

The presence of *lcf*, shown in Figure 4.8, was widespread in most of the study region with 48 of the 72 (67%) of the samples analysed containing this gene. A marked absence of *lcf* was evident south of the Falkland Islands with only one surface sample at station 134 being positive for this gene. The absence of *lcf* was also evident in several samples in the vicinity of station 20. In addition, *lcf* was more frequent in surface waters than at the subsurface chlorophyll maximum with 27 of 37 (73%) and 18 of 33 (54%) of samples containing *lcf* in the former and the latter, respectively.

The distribution of bioluminescence, shown in Figure 4.9, showed that bioluminescence above the detection limit was only measured in 13 samples. Detectable bioluminescence was found in the northernmost stations, some stations near the SBF and in surface waters of the southernmost stations. The highest values, above 5 x 10^{11} photons cm^{-2} s^{-1}, were recorded in the deep sample of the first station and the surface samples of stations 46, 47 and 142. Bioluminescence was not more frequent at a particular depth, as it could be absent in the surface but present in the deep sample, or vice versa, depending on the location.

4.3.5 Prediction of bioluminescent dinoflagellate groups

In order to conduct a quantitative analysis of bioluminescent dinoflagellates in relation to the sensitivity of the *lcf* PCR detection, the bioluminescence intensity and to environmental factors, it was necessary to estimate the abundance and composition of the bioluminescent dinoflagellate population. For this purpose, dinoflagellate cells that were enumerated in 43 samples were separated into two groups according to their bioluminescence based on literature reports (Chapter 2; Poupin et al. 1999) and the PCR results of this study (Figure 4.7; Table 4.2).

The bioluminescent group consisted of *Gonyaulax*-like dinoflagellates (including *Alexandrium, Lingulodinium* and *Protoceratium* species), *C. fusus, Noctiluca scintillans, Protoperidinium* spp., *Pyrocystis* spp. and *Pyrophacus* sp. Other dinoflagellates that were present were mainly of the genus *Prorocentrum* and the order Gymnodiniales along with some *Dinophysis* spp.; all these dinoflagellates were classified as non bioluminescent.

4.3.6 Sensitivity of the luciferase gene detection

Comparison of cell counts extrapolated to the 3 L volume of DNA samples and detection of *lcf* revealed a high sensitivity of the PCR protocol. *Lcf* was even weakly detected in one sample where bioluminescent cells were not identified. However, inconsistent detection at low cell concentrations was highlighted by 5 samples where bioluminescent cells were present but *lcf* was not detected. Detection became consistent above an estimated 900 bioluminescent cells.

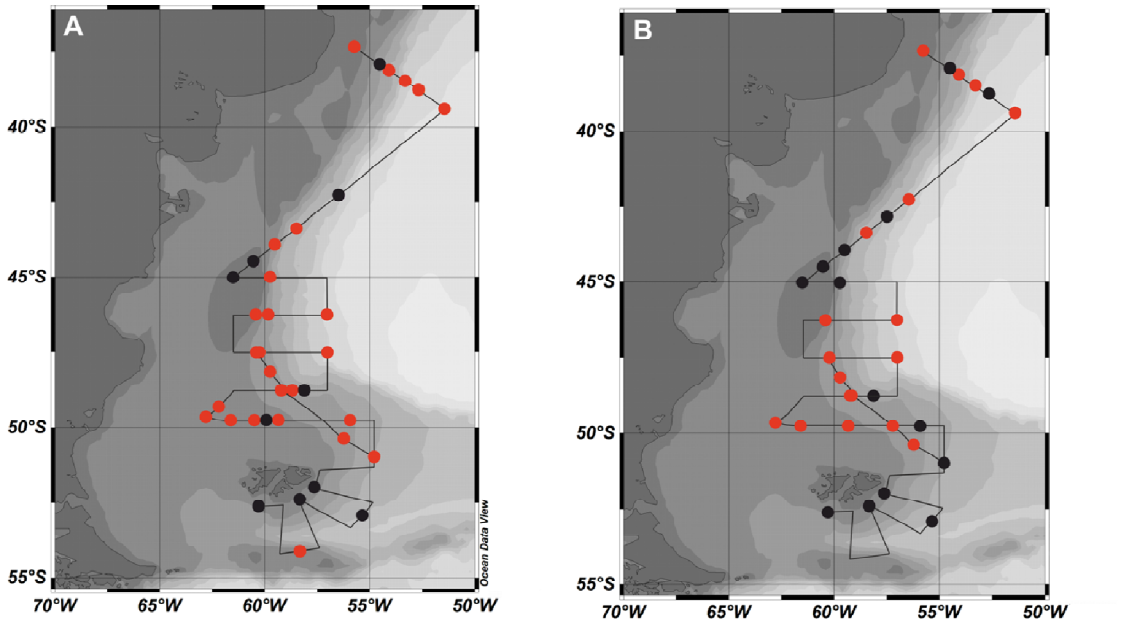

Figure 4.8 Maps of the Patagonian Shelf showing the distribution of the luciferase gene at surface (A) and chlorophyll maximum depths (B).Red circles indicate that luciferase was detected while black dots indicate that luciferase was not detected. Note that some stations overlap.

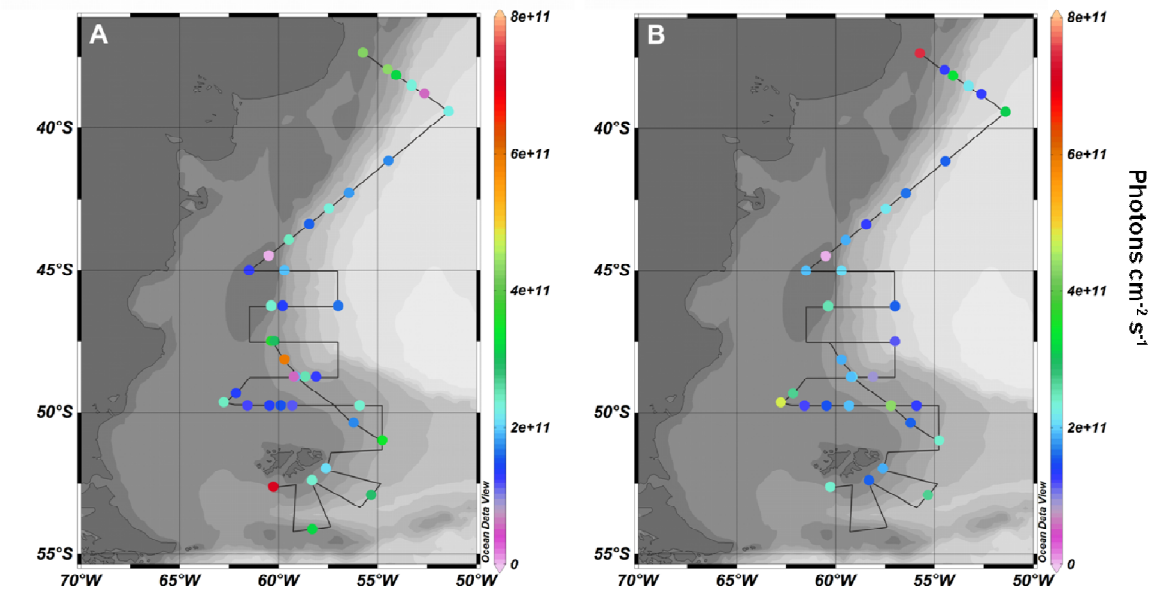

Figure 4.9 Maps of the Patagonian Shelf showing the distribution of bioluminescence at surface (A) and chlorophyll maximum depths (B).Note that values below 2.5 x 10^{11} photons cm^{-2} s^{-1} (i.e. blue shades) are below the detection limit. Note that some station overlap.

4.3.7 Comparison of bioluminescence to luciferase gene presence and bioluminescent dinoflagellates

The number of samples containing bioluminescent dinoflagellates was comparatively underestimated more than 3-fold by bioluminescence measurements (Figure 4.9) relative to the detection of *lcf* (Figure 4.8). Additionally, two samples where bioluminescence was detected, did not correspond to a positive detection of *lcf* (stations 3 and 142 surface) and another four samples were associated with very low concentrations (<0.2 $x10^3$ cells L^{-1}) of bioluminescent dinoflagellates (Figure 4.10; stations 134 surface and 5, 60 and 78 subsurface chlorophyll maximum). In samples where measured bioluminescence coincided with the presence of bioluminescent dinoflagellates, the magnitude of bioluminescence did not correspond to the number of bioluminescent cells present. In one example, a similar amount of light (3-3.5 $x10^{11}$ photons cm^{-2} s^{-1}) was measured in the surface of stations 5 and 46 while bioluminescent cell densities differed by an order of magnitude (120 cells L^{-1} and ~ 1000 cells L^{-1}, respectively). It was, however, noted that the bioluminescent dinoflagellate population was dominated by different organisms at these stations, with *N. scintillans* at station 5 and *Gonyaulax* spp. at station 46. In another example, surface bioluminescence was less intense at station 47 than station 46, even though the former contained a 4-fold higher number of similar bioluminescent *Gonyaulax*-like dinoflagellates. However, the time of the night that bioluminescence was measured in these samples differed, with samples at station 46 and 47 having been collected at 22.30 and 03.00, respectively.

4.3.8 Distribution and composition of dinoflagellate populations identified by microscopy relative to the environment

Microscopy data showed that all dinoflagellates, not just bioluminescent ones, were barely detectable or absent in the southernmost stations and were less abundant in the subsurface chlorophyll maximum throughout the study area (Figure 4.10). Waters south of the Falkland Islands supported some diatom populations. Between these two latitudinally contrasting environments, station 86 located off the Patagonian Shelf contained both a diatom bloom as well as significant numbers of bioluminescent dinoflagellates (Figure 4.10).

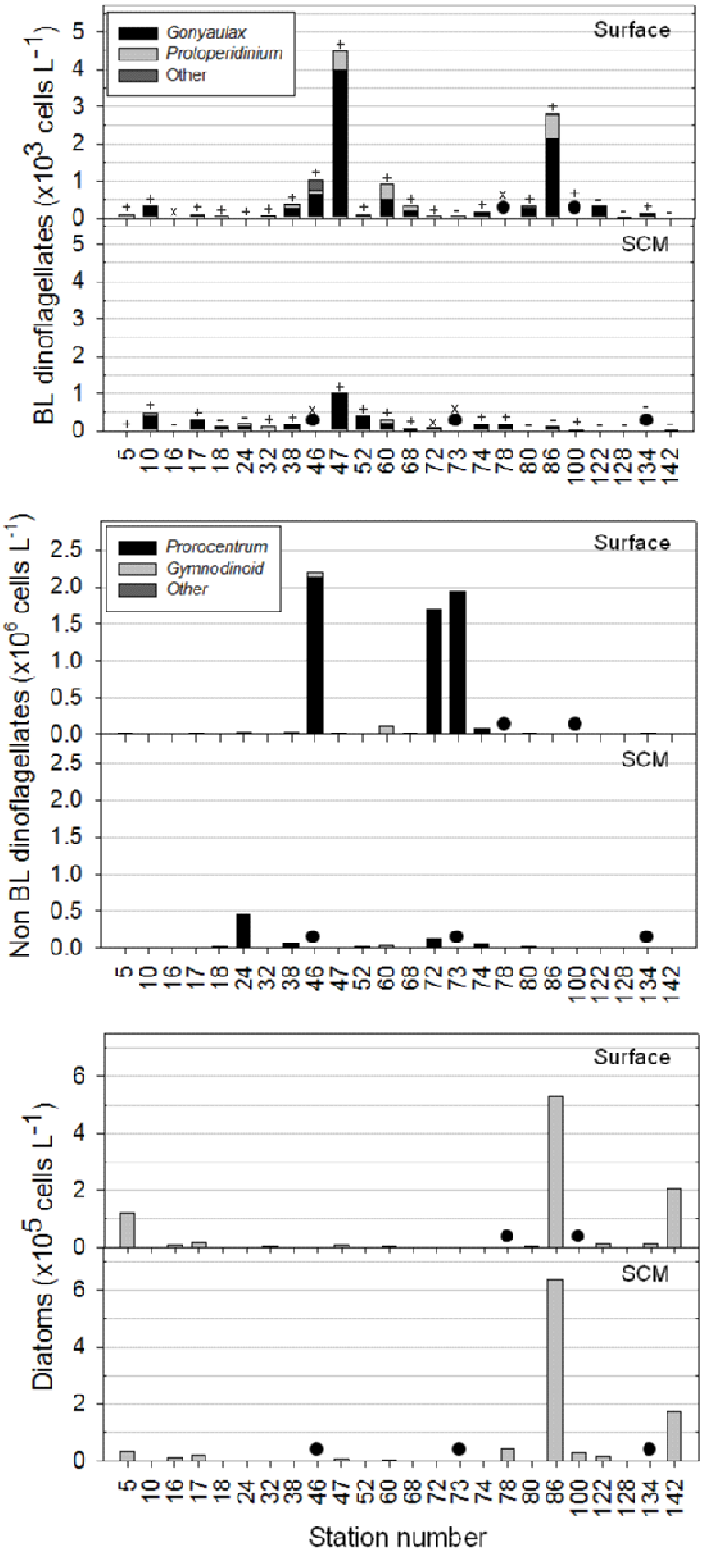

Figure 4.10 Abundances of key protists groups at surface (upper panel) and at the depth of the subsurface chlorophyll maximum (SCM; lower panel), at the stations sampled in this study. A black circle indicates no data at that point rather than a zero value. *Lcf* detection results are superimposed on the plot of bioluminescent dinoflagellates showing positive (+), negative (-), or missing data (x) for each sample.

Diverse populations of dinoflagellates were present in the area north of the Falkland Islands. The highest concentrations of dinoflagellates (>2 million cells L^{-1}) were found near the SBF at station 46, coinciding with the sample showing the highest chlorophyll concentration of nearly 4 μg L^{-1} (Figure 4.5). However, there were also stations near the SBF and elsewhere that had high chlorophyll concentrations (> 1 μg L^{-1}) that did not correspond to high dinoflagellate or diatom abundance, such as, for example, the surface samples of station 60 and 74 and most subsurface chlorophyll maximum samples.

Bioluminescent cells, which were well below bloom densities (<100,000 cells L^{-1}) throughout the study region, were dominated by gonyaulacoid dinoflagellates (Figure 4.10). Microscopic observations revealed that most of these cells belonged to the genus *Gonyaulax* with members of other morphologically similar genera being rare, in agreement with only a few sequences showing similarities to *Alexandrium* and *Protoceratium* species (Table 4.2); these latter species were therefore included in the *Gonyaulax* group. The dominant *Gonyaulax* spp. were responsible for all the samples where the highest abundances of up to ~ 4000 bioluminescent cells L^{-1} were recorded at station 47 (Figure 4.10), coincidental with high bioluminescence (Figure 4.9) at that location. Non bioluminescent dinoflagellates were present in much higher abundance than the bioluminescent ones (Figure 4.10). The numerically most important organism was *Prorocentrum* cf. *minumum*, which formed a sharp bloom along the SBF in surface waters at stations 46, 72 and 73 reaching concentrations of more than 1.5 million cells L^{-1} at these stations which resulted in visible discoloration of the water.

The locations where the *P*. cf. *minimum* bloom was found were spatially distinct to the station of maximum abundance of *Gonyaulax* spp. (station 46 versus 47; Figure 4.10), even though the salinity at stations where both taxa were found remained between 33.8-33.9 corresponding to the Subantarctic Shelf Waters (Figure 4.3). Additionally, there were no clear relationships of either *Gonyaulax* spp. or *Prorocentrum* cf. *minumum* to nutrients. For example, when *Prorocentrum* cf. *minumum* were found in very high abundance at station 73, the waters displayed a near complete drawdown of nitrate (Figure 4.5), but this was not observed at the adjacent station 72 or further north at station 46 where cell concentrations were even higher.

The warmer waters in the region north of 40 ºS (Figure 4.4) supported different dinoflagellate assemblages relative to the rest of the surveyed area. Both microscopy data (Figure 4.10) and *lcf* sequences (Table 2) indicated that low nutrient and salinity waters influenced by the Rio de

la Plata outflow (Figure 4.3) contained *N. scintillans* as the main bioluminescent dinoflagellate present, coinciding with stations of high bioluminescence (Figure 4.9), along with the highest recorded abundance of non bioluminescent *Ceratium* (~12000 cells L^{-1}; Figure 4.10) which was mainly due to *C. tripos*. Waters associated with the oligotrophic saline Brazil Current at station 10 (Figure 4.3) were dominated by *Alexandrium* spp., representing the only station where this genus was a significant component of the protist community.

4.4 Discussion

4.4.1 Application of the molecular detection of the luciferase gene in natural water samples

The application of primers specific to dinoflagellate *lcf* to natural water samples collected from the Patagonian Shelf region provided a unique and novel view of the distribution of bioluminescent dinoflagellates in shelf and open ocean environments. Comparison with bioluminescence measurements readily showed that bioluminescence detection did not always correspond to the presence of bioluminescent dinoflagellates. The molecular detection of *lcf* in mixed communities and in single cells also enabled realistic predictions of which dinoflagellates were likely to produce bioluminescence. The synergistic use of molecular, microscopic and optical data made it possible to conduct a quantitative analysis of abundance and composition of bioluminescent dinoflagellate populations in relation to the sensitivity of *lcf* detection, bioluminescence measurements and environmental conditions.

Identifying which dinoflagellates produce bioluminescence in the water column is not straightforward as there are several ambiguous reports of bioluminescence in some species, which are exacerbated by taxonomic misidentifications (see discussion in Chapter 2). In this study, *lcf* sequences obtained from mixed community samples as well as the identification of *lcf* in single cells of *Ceratium* spp. were employed to obtain a robust classification of the bioluminescent and non bioluminescent dinoflagellate groups. The single cell PCR on species of *Ceratium* was particularly important because there were many *Ceratium* species in the study area, but this genus was known to contain only a few bioluminescent species which are largely un- or mischaracterised (Chapter 2). For example, *C. furca* has been reported

previously to be bioluminescent (Poupin et al. 1999) but was found here not to contain *lcf*. *Ceratium fusus* was the only species found to contain *lcf*, in agreement with studies in the north Atlantic (Swift et al. 1995) and California (Sweeney 1963). In fact, the only organisms that could be directly labelled as bioluminescent with confidence, without the single cell PCR analysis, were *Gonyaulax* and morphologically similar species. This is because bioluminescence and *lcf* had previously been reported in nearly all members of the *Gonyaulacaceae* and *Goniodomataceae* studied previously (Chapter 2; Poupin et al. 1999) and sequences from these taxonomic groups represented the majority of *lcf* sequences retrieved from mixed samples.

Bioluminescence in the genus *Protoperidinium* remains largely elusive. It is generally considered that the majority of *Protoperidinium* species are bioluminescent because many of them have been found to produce light (Swift et al. 1995, Poupin et al. 1999), however, several species have also been found to lack bioluminescence or *lcf* (Chapter 2; Buskey et al. 1992). In the present study it was not possible to test the *Protoperidinium* species (or the *Gonyaulax* species) found in the area by single cell PCR because the long term (over 2 years) ethanol fixation resulted in morphological distortion that made species identification impossible. Moreover, the absence of *Protoperidinium*-like *lcf* sequences in the sequenced samples could be attributed to them being masked by those of gonyaulacoid dinoflagellates that were much more abundant in all the samples (Figure 4.10); considering that only four clones were sequenced from each sample it is unlikely that *Protoperidinium lcf* would be detected. All species of *Protoperidinium* were therefore placed in the bioluminescent group under the assumption that their low contribution to the bioluminescent group (<15% overall) (Figure 4.10) did not significantly affect the overall patterns in the distribution of bioluminescent dinoflagellates. The non bioluminescent dinoflagellates were easier to categorise due to strong evidence for the lack of bioluminescence and *lcf* in the dominant genus *Prorocentrum* as well as members of the Gymnodiniales (Chapter 2).

The discovery that bioluminescent dinoflagellates were widespread (although not highly abundant) in the Patagonian Shelf region was due to the high sensitivity of the molecular detection of *lcf*, that could not have been revealed by microscopy alone. The variation in estimated sensitivity of the PCR protocol (0-900 cells) could have many explanations. First, the extrapolation of cell concentrations counted in 50 mL samples to a respective value in a 3 L DNA sample, could amplify the error of the cell counts. Second, the dinoflagellates that are

capable of bioluminescence may be overestimated. Third, the sensitivity of the PCR is expected to vary according to the number of domains contained (or amplified) in the *lcf* of different organisms (Chapter 2; Liu et al. 2004, Liu and Hastings 2007). Nevertheless, even the largest number of 900 cells detected which corresponds to a cell density of 0.3 cells mL^{-1} is comparable to the detection limits reported for other dinoflagellate targeted PCR protocols (Godhe et al. 2001, Guillou et al. 2002, Zhang and Lin 2002). This therefore further supports the usefulness of the *lcf* detection in natural water samples in assessing the distribution of bioluminescent dinoflagellates.

4.4.2 Drawbacks of using optical bioluminescence measurements in ecological studies of bioluminescent dinoflagellates

The data generated in this study provided the first opportunity to compare bioluminescence measurements with corresponding molecular and microscopic data, in order to assess the usefulness of optical bioluminescence measurements in ecological studies of bioluminescent dinoflagellates. Bioluminescence measurements underestimated the locations where bioluminescent dinoflagellates were present by more than 3-fold relative to the detection of *lcf*. Even when bioluminescence was high, the magnitude observed was affected by other organisms and variability in the bioluminescence characteristics of the dinoflagellates.

In samples where bioluminescent dinoflagellates were completely or nearly undetectable by PCR or microscopy, bioluminescence was attributed to zooplankton. It is not possible to assess how many bioluminescence measurements resulted from the cumulative effects of zooplankton and dinoflagellates. Moline et al. (2009) used a rough correlation between size and flash intensity of various bioluminescent organisms to distinguish the flashes of dinoflagellates from the larger zooplankton but their data show a considerable overlap in flash intensity of these two groups in the small (<1 mm) size range that was targeted by this study.

When bioluminescence was assumed to originate only from dinoflagellates, the measurements appeared to be affected by both interspecific differences in flash intensity and diurnal rhythms. For example, *N. scintillans* which is known to produce a high level of light (Buskey et al. 1992) was measurable at 120 cells L^{-1} at station 5, while the detection of dimmer *Gonyaulax* spp. (Swift et al. 1995, Baker et al. 2008) required an order of magnitude higher cell

concentration. A diurnal rhythm in bioluminescence has been reported in natural populations of *Gonyaulax* spp. (Kelly 1968, Marcinko et al. 2011) and in cultures of several closely related species (Hastings and Sweeney 1958, Sweeney 1987). This could explain the inverse trend of bioluminescence intensity to cell numbers observed in surface waters of stations 46 and 47, where the latter sample with more abundant cells corresponded to a later 'dimmer' stage of the diel cycle.

The presence of a diurnal rhythm in a north Atlantic dinoflagellate population reported by Marcinko et al. (2011) led the authors to suggest that only bioluminescence measured at the same time of night can be used to monitor changes in bioluminescent cell abundance. The results of the present study additionally show that the interspecific differences in the magnitude of bioluminescence mean that bioluminescence measurements are only comparable when the species composition is constant, a situation that is likely to be encountered only within monospecific dinoflagellate blooms. Therefore, in order to use bioluminescence as a tool to monitor bioluminescent dinoflagellate populations, or indeed even for basic bioluminescence datasets to be comparable, three conditions must be met: 1) no zooplankton must be present, 2) only measurements collected at the same time of night can be compared, 3) the composition of the population must be constant.

4.4.3 Environmental control of the distribution and composition of dinoflagellate populations

Molecular and microscopic analyses showed that environmental conditions were important in driving the distribution and composition of both bioluminescent and non bioluminescent dinoflagellate populations. Large scale features such as the absence of these organisms from the well mixed and cold waters found in the southern extent of the study area were readily apparent from both the PCR of *lcf* and from the cell counts. The area north of the Falkland Islands where dinoflagellates were found was characterised by temperatures typical of temperate latitudes (13-22 ºC), relatively shallow mixed layer depth (<30 m), low but adequate macronutrient concentrations (approximately 2 µM nitrate and 0.2 µM phosphate) in most locations and mostly low (<2 µM) silicate concentrations all over the region with the exception of the stations influenced by the Rio de Plata and Brazil Current Waters. Therefore, this area exhibited features that are known to be key for the growth of certain dinoflagellates

such as stratification, adequate macronutrients and potentially limiting silicate concentrations resulting in the outcompetition of diatoms (Egge and Aksnes 1992, Smayda 1997). Indeed, diatoms were mainly found in the cold, nutrient rich and well mixed waters south of the Falkland Islands, which were more typical for their known environmental preferences. Station 86 was an intermediate of these two environments and supported both diatoms and dinoflagellates. In the region where dinoflagellates were present, the physical and chemical features of the water were critical in determining the composition and abundance of dinoflagellate populations.

Waters north of 40 ºS were characterised by two water masses, the Rio de la Plata Waters and Brazil Current Waters that were physically and chemically distinct between them and to the rest of the area; each harboured distinct dinoflagellate populations. Both cell counts and *lcf* sequences revealed that *N. scintillans* was the main bioluminescent dinoflagellate in the low salinity Rio de la Plata Water. However, station 5 which was also influenced by either Shelf Waters or Falklands Current Waters as indicated by increased salinity relative to station 1, also contained bioluminescent gonyaulacoid species that were more typical of these waters, showing a mixing between distinct populations. *Noctiluca scintillans* and both bioluminescent and non bioluminescent *Ceratium* spp. identified by microscopy in the waters of the Rio de la Plata have also been reported previously in this area at a similar time of year (Carreto et al. 2003, Carreto et al. 2008), suggesting that they represent a typical seasonal community in this location. It has been suggested that the typical association of *N. scintillans* and *Ceratium* spp. is due to excretion of nutrients by *N. scintillans* feeding on diatoms fuelling the growth of *Ceratium* (Baek et al. 2008). This could be the case in the nutrient depleted waters of the Rio de la Plata outflow, which did indeed also contain a bloom of very small (<5 μm) pennate diatoms (Alex Poulton, pers. comm.) that could have been responsible for high chlorophyll concentrations at that location (Figure 4.5). Waters from the Brazil current supported yet another different population dominated by *A. tamarense* although in low numbers, consistent with the oligotrophic conditions of these waters (Bisbal 1995, Willson and Rees 2000, Painter et al. 2010).

The dinoflagellate populations in the area between the Falkland Islands and 40ºS were composed of genetically closely related gonyaulacoid dinoflagellates, mainly of the genus *Gonyaulax*. This area contained four water masses, the Falkland Current Waters and three types of Shelf Waters (Table 4.1; Figure 4.3) that were defined based on very subtle

differences in salinity, but were characterised by similar temperatures (Painter et al. 2010). This suggests that the salinity differences were too small to cause significant shifts in the dinoflagellate population composition. However, the *lcf* sequences also indicated that the population found within the Shelf Waters near the SBF was likely composed of only one species which was identified as a *Gonyaulax* sp. by microscopy and whose *lcf* was approximately 85% identical to that of *L. polyedrum*. By contrast, the bioluminescent populations found away from the SBF, amplified *lcf* sequences that were composed of up to 3 species. The more diverse populations away from the SBF could reflect localised interactions of the different types of Shelf Waters and Falklands Current Waters that may create local environments suited to particular species. For example, the abundance of *A. tamarense* was low even in the most inshore locations, despite several reports of blooms of this species near the Argentinean coast during spring (Carreto et al. 1986, Gayoso 2001, Gayoso and Fulco 2006), indicating its predominant occurrence near the coast and potentially limited advection to the rest of the region due to fronts (Carreto et al. 1986). Alternatively, the mixed populations observed here could be representative of all the Shelf and Falklands Current water masses sampled, while environmental conditions near the SBF favoured only one bioluminescent species of *Gonyaulax*, with the *L. polyedrum*-like *lcf*, leading to its high abundance along this front (station 47) which was indeed the highest abundance of a bioluminescent dinoflagellate recorded in the study region. The differences in the composition of populations associated with distinct water masses and the potential role of the interactions of the water masses in determining their locations and abundance, suggest that the composition of bioluminescent dinoflagellate populations, and possibly other protists, is shaped by the hydrography of the area creating distinct or diffuse environmental zones separated by either sharp of diffuse boundaries depending on the prevalent physical processes occurring at the time. One such zone could be considered the SBF which is of particular importance for dinoflagellate populations.

The SBF favoured not only bioluminescent dinoflagellates but also non bioluminescent species, as both *Gonyaulax* spp. and *Prorocentrum* cf. *minumum* were found in maximum abundances near this front. The bioluminescent *Gonyaulax* spp. and the bloom forming *Prorocentrum* cf. *minumum* found in this study have not been previously reported in this area (Olguín et al. 2006, Schloss et al. 2007, Garcia et al. 2008). It has been suggested that upwelled waters along the SBF supply essential macronutrients and possibly also iron, that are

essential to maintain the persistent phytoplankton blooms along this front throughout the spring and summer (Romero et al. 2006, Garcia et al. 2008). Potential upwelling could explain the higher surface nutrient concentrations along the SBF observed in this study. Indeed, chlorophyll was high at several locations along the SBF, with the highest values coinciding with the exceptionally intense *Prorocentrum* cf. *minutum* bloom at station 46. However, several locations particularly near the SBF (e.g. stations 60 and 74) where high chlorophyll concentrations were not due to dinoflagellates or diatoms, suggest that other phytoplankton not captured in the microscopy data, including a declining coccolithophore bloom (Painter et al. 2010), were also present in high abundance near the SBF. It may therefore be that this area is important for all phytoplankton functional groups at all stages of the population succession.

Both bioluminescent *Gonyaulax* spp. and non bioluminescent *Prorocentrum* cf. *minutum* were found in the Subantarctic Shelf Waters and their maximum abundances were associated with the SBF. However, despite the constant water mass, it was clear that the maxima of bioluminescent and non bioluminescent populations were found at different locations (e.g. stations 46 versus 47). This could reflect different source locations of transported populations or an environmental segregation of these distinct taxa. In either case, one would expect that the two populations would be associated with waters of different chemical properties but such associations could not be identified. This is likely due to intense mixing processes over small spatial scales that are typical near the SBF (Franco et al. 2008, Painter et al. 2010) which cannot be resolved in the present dataset due to the low resolution of biological sampling relative to high spatial heterogeneity of the study area. However, dinoflagellate ecological characteristics are highly complex and several studies have shown that the availability of organic nutrients (Oh et al. 2002, Lee and Kim 2007, Fagerberg et al. 2009, Jauzein et al. 2010), trace metals (Mitrovic et al. 2004, He et al. 2010) as well as suitable prokaryotic or eukaryotic prey (Stoecker 1999, Jeong et al. 2005a, Jeong et al. 2005c, Yoo 2009) are all important for dinoflagellate growth. As the specific requirements differ between dinoflagellate species, any one of these factors could have been responsible for the differences in the distribution of *Prorocentrum* cf. *minutum* and *Gonyaulax* spp.

4.4.5 Conclusions

This study represents the first application of a molecular approach to study natural mixed bioluminescent dinoflagellate populations in non coastal waters and it is the first study focused on non coastal dinoflagellate populations to be conducted in the Patagonian Shelf region. The analysis presented here has resulted in new insight into the distribution of these organisms and has demonstrated the environmental controls that are important in structuring dinoflagellate populations of Patagonian Shelf. Furthermore, the limitations of optical bioluminescence measurements in studies of bioluminescent dinoflagellates emerged from their comparison to molecular and microscopy data. Therefore, the application of PCR primers for dinoflagellate *lcf* to map and identify natural populations of bioluminescent dinoflagellates represents a powerful tool for ecological studies on these organisms.

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Chapter 5

FUNCTIONAL DIVERSITY AMONG GEOGRAPHIC VARIETIES OF *NOCTILUCA SCINTILLANS*: A COMPARATIVE MOLECULAR AND CELLULAR STUDY OF THE BIOLUMINESCENCE SYSTEM

Abstract

Noctiluca scintillans is widely known for its bright bioluminescence worldwide, but one of its varieties that blooms along the west coast of the USA is not bioluminescent. This study used PCR primers for the dinoflagellate luciferase gene (*lcf*) to test two strains of the non bioluminescent variety for the presence and expression of *lcf*. Furthermore, confocal laser scanning microscopy and transmission electron microscopy were used to detect luciferin and to identify scintillons within the cells, respectively. The results show that non bioluminescent *Noctiluca scintillans* contains a bioluminescence system that is now "switched off" as this organism contains *lcf* in its genome but does not expresses it and it also contains scintillons although it does not produce luciferin. Furthermore, a novel single luciferin binding protein gene (*lbp*) was discovered in the course of this investigation showing that *N. scintillans* also has separate genes for *lcf* and *lbp*. Preliminary phylogenetic analysis of one non bioluminescent strain based on SSU rDNA, indicates that the non bioluminescent variety of *N. scintillans* is likely a distinct species and the California Current is proposed to be responsible for the spatial isolation of the bioluminescent and non bioluminescent populations. This study presents the first evidence that the environment is important in maintaining or eliminating bioluminescence, therefore indicating its biological relevance

5.1 Introduction

The heterotrophic noctilucoid dinoflagellate *N. scintillans* has a global distribution and often forms dense blooms (e.g. Elbrachter and Qi 1998). Its blooms are famously brightly bioluminescent, giving it its Latin name which means 'glowing bright by night' and its common name the 'sea sparkle'. Although the genus *Noctiluca* contains only one species, *N. scintillans*, there are regional varieties with distinct characteristics. For instance, the variety occurring in waters of south and east Asia is the only one that contains the photosynthetic green flagellate symbiont *Pedinomonas noctilucae* in its vacuoles (Sweeney 1978) providing it about 70% of its energy requirements (Hansen et al. 2004). Another variety that blooms along the west coasts of the USA differs morphologically by being smaller than the Atlantic and Mediterranean *N. scintillans*, but it is not bioluminescent (Chang 1960, Sweeney 1963). This variety represents the only bioluminescent dinoflagellate known to have a regional variety that does not produce light.

The west coast of the USA stretches from the state of Washington in the north bordering Canada to the state of California in the south bordering Mexico. This region is characterised by an eastern boundary current called the California Current System as part of the North Pacific Gyre (Sverdrup et al. 1942) depicted in Figure 5.1. Surface waters originate from the Subarctic current (or North Pacific current) which flows east along the northern reach of the North Pacific gyre and as these waters reach the Washington coast at approximately 45ºN, part of the flow turn towards the equator until the southern tip of the Baja California peninsula where the current veers west to become the North Equatorial Current (reviewed by Wyrtki 1967). The non bioluminescent variety of *N. scintillans* is therefore found in the waters of the California Current, while, nearby, in the Baja California which is isolated from the California Current (Wyrtki 1967, Baumgartner and Christiansen Jr. 1985), the bioluminescent variety is present (Lapota and Losee 1984). Furthermore, during spring, when *N. scintillans* usually blooms in California, and when the inshore northward countercurrent is negligible (Di Lorenzo 2003), blooms may be retained near the shore by fronts created during upwelling events that may themselves create closed circulation in coastal bays (Barth et al. 2000, Lynn et al. 2003). It is therefore likely that the two functional varieties of *N. scintillans* may be segregated by physical hydrographic barriers.

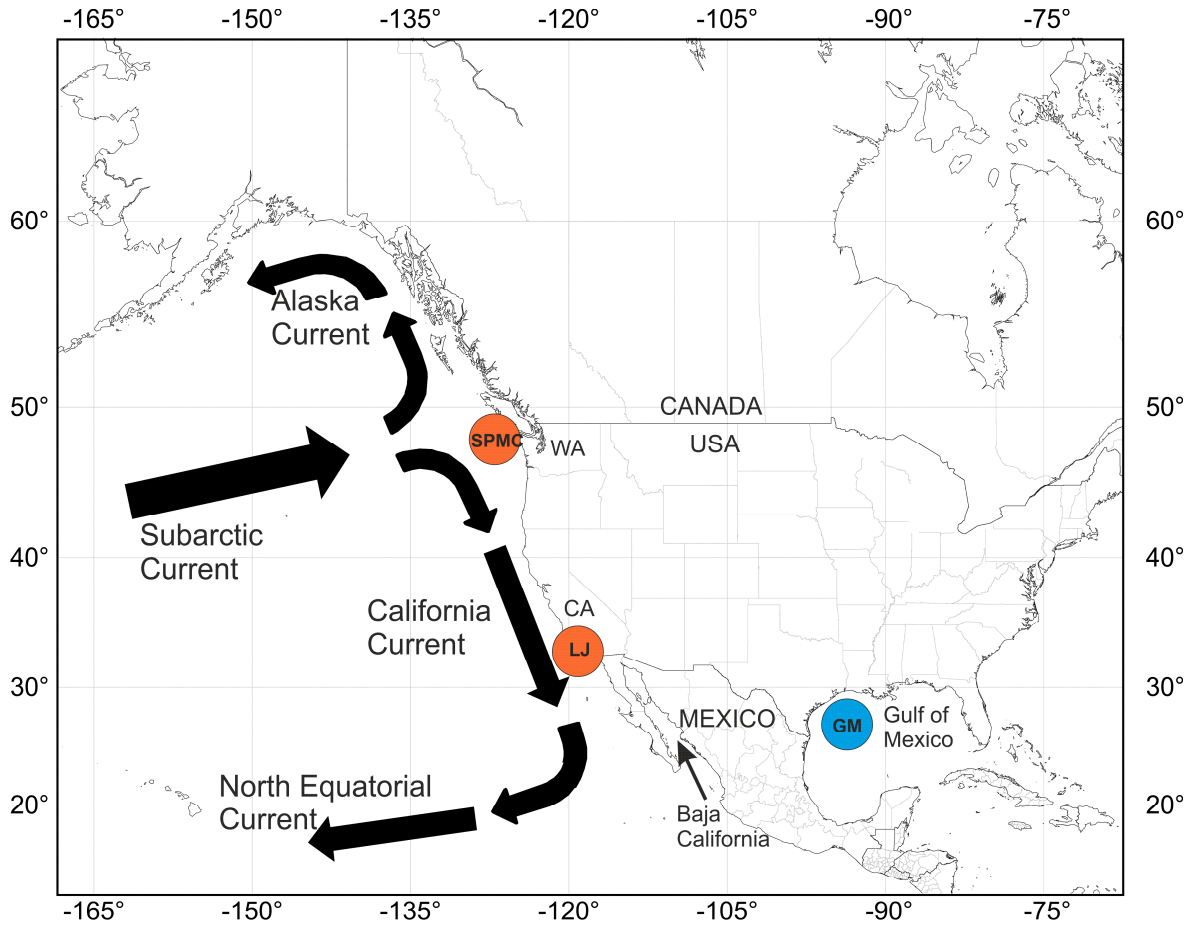

Figure 5.1 Map of North America showing the major current systems that affect the west coast of the USA (drawn after Sverdrup et al. 1942). The geographic origins of three *N. scintillans* strains that formed the basis of this study are shown: Blue circle indicates that the strain is bioluminescent while orange circles indicate that the strains are not bioluminescent. The strain identifiers given in the circles are explained in Table 1.

Noctiluca scintillans is considered to be a primitive taxon among dinoflagellates because noctilucoids have the typical dinokaryotic nucleus only during their gamete life stage (Taylor 2004) and *N. scintillans* lacks mRNA editing in its mitochondria that is typical of species that appeared later in time (Zhang and Lin 2008). Molecular phylogenies have yielded conflicting results on the phylogenetic position of *N. scintillans* depending on the gene considered, showing it to be primitive according to rDNA genes (Gomez et al. 2010a, Ki 2010) and some protein coding genes (Fukuda and Endoh 2008), or more recently derived according to other protein coding genes (Hoppenrath and Leander 2010). This has also caused uncertainties with

interpreting the origin of the hybrid structure of its *lcf/lbp* (Liu and Hastings 2007, Fukuda and Endoh 2008, Hoppenrath and Leander 2010). The case of a non bioluminescent variety is of particular importance because either the common ancestor of *N. scintillans* was bioluminescent and one variety lost the ability to bioluminesce or the gene was transferred horizontally directly and only into the bioluminescent variety. Determining this could be important in understanding how dinoflagellates bioluminescence has evolved through time as well as whether there are particular environments that favour the maintenance or loss of the production of light. Additionally, this information could help understand regional differences in other dinoflagellate functional traits, such as toxicity, where the genes involved are currently unknown.

In aim of this study was to investigate whether there are detectable components of a bioluminescence system in the non bioluminescent variety of *N. scintillans* at the molecular and cellular level. Two strains of the non bioluminescent variety and one strain of the bioluminescent variety (Figure 5.1) were used to comparatively assess the presence of *lcf* in the genome, *lcf* gene expression, production of luciferin and presence of scintillons. This study presents the first combined molecular and cellular investigation on the regional variation of a dinoflagellate functional trait.

5.2 Materials and Methods

5.2.1 *Noctiluca scintillans* cultures

Cultures of *N. scintillans* isolated from three locations (Figure 5.1) were obtained from colleagues (Table 5.1). Cells were maintained in polycarbonate flasks (Nunc, Thermo Scientific, USA) containing 400 mL seawater sterilised by filtration through a 0.22 µm membrane filter (Steritop, Millipore, UK) and supplemented with appropriate amounts of prey culture as required. Subculturing was conducted every 10 days by pouring 100 mL of culture into a new vessel containing 300 mL of seawater with prey. The photosynthetic prey cells *Dunaliella tertiolecta* CCMP 1320 (Chlorophyceae) and *Prorocentrum micans* CCMP 691 (Dinophyceae) were grown in 50 mL volumes of F/2 (Guillard and Ryther 1962) and L1 (Guillard and Hargraves 1993) seawater media, respectively, with silicate omitted from both,

in 100 mL Erlenmeyer flasks. All cultures were maintained at 19 ºC in a 12:12 light:dark cycle. The light irradiance in the incubator was 70 μmol m^{-2} s^{-1} and this was reduced for the *N. scintillans* cultures to prevent overgrowth of the prey, by using neutral density light filters (Lee filters, UK) which reduced the light level to 15 μmol m^{-1} s^{-2}.

Table 5.1 Details of *N. scintillans* cultures used in this study. Bioluminescence is abbreviated as BL. Note: the strains names GM and LJ indicate the geographic origin as these strains do not have another designation. Affiliations: M. I. Latz, Scripps Institution of Oceanography, California, USA; K. Bight and S. Strom, Shannon Point Marine Center (SPMC), Washington, USA.

Strain	**Origin**	**Prey**	**BL**	**Source**
GM	Gulf of Mexico	*Dunaliella tertiolecta*	Yes	M.I. Latz
LJ	Scripps Pier, La Jolla, CA, USA	*Dunaliella tertiolecta*	No	M.I. Latz
SPMC136	Puget Sound, WA, USA	*Dunaliella tertiolecta* and *Prorocentrum micans*	No	K. Bright/ S. Strom

5.2.2 Bioluminescence tests

Bioluminescence tests on dense cultures were conducted as described in Chapter 2 (page 25).

5.2.3 Nucleic acid extraction and reverse transcription

Cells were harvested at stationary phase when 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. Disruption of the cells by liquid nitrogen and micropestle and extraction of DNA was performed as described in Chapter 2. Extraction of RNA was carried out using the Nucleospin RNA II kit (Macherey-Nagel, Germany) which contains an integrated on column DNase digestion step. The quantity and purity of the DNA and RNA was assessed using a Nanodrop spectrophotometer (ND-3000, Nanodrop, USA). The RNA was reverse transcribed to cDNA

using the High Capacity RNA-to-cDNA kit (Applied Biosystems, USA) which utilises random primers to initiate reverse transcription. A control reverse transcription reaction was included for each RNA sample where no reverse transcriptase enzyme was added (-RT reaction). The PCR quality of the DNA and cDNA, as well as the absence of genomic DNA carryover contamination in cDNA samples, was assessed by PCR with primers 18ScomF1/ Dino18SR2 (Lin et al. 2006) (Table 2) on DNA, cDNA and –RT reaction samples.

5.2.4 PCR reactions

The primers used in this study and their corresponding gene targets are detailed in Table 5.2. Detection of *lcf* was achieved using "universal" primers for this gene (see Chapter 2) and the protocol described therein was followed using either DNA or cDNA as a template.

Primers were designed to amplify and sequence nearly the whole open reading frame (ORF) of the *lcf*/*lbp* gene, using the program Primer3 (http://frodo.wi.mit.edu/primer3/) and the published full *lcf*/*lbp* gene of *N. scintillans* as a reference sequence. The sequences of the resulting PCR primers NocORF23F/2418R is shown in Table 5.2. The PCR reactions consisted of 0.1 µM each primer, 250 µM each dNTP, 1x standard PCR buffer, 0.5U GoTaq DNA polymerase (Promega, UK) and 50-100 ng of DNA or cDNA template, in a 25 µL volume. Amplification by PCR was achieved with the following program: 5 minutes at 95 ºC for initial denaturation, followed by 35 cycles of 95 ºC for 45 seconds, 49 ºC for 30 seconds, 68 ºC for 3 minutes and a final extension at 68 ºC for 10 minutes.

The ribosomal genes were amplified using dinoflagellate specific primers (Ki and Han 2005). This was only done on strain GM and LJ because the SPMC136 strain contained another dinoflagellate (*Prorocentrum micans*) as prey. The PCR reactions were conducted using the high fidelity and high yield Advantage 2 PCR kit and Polymerase mix (Clontech, USA). Each 25 µL reaction contained 0.02 µM each primer, 200 µM each dNTP, 1x Advantage 2 PCR buffer and 1x Advantage 2 polymerase mix, as recommended by the manufacturer. The PCR amplification program was as follows: 95 ºC for 3 minutes for initial denaturation followed by 35 cycles of followed by 35 cycles of 95 ºC for 20 seconds, 55 ºC for 30 seconds, 72 ºC for 4 minutes and a final extension at 72 ºC for 10 minutes.

Table 5.2 PCR primers used in this study. The lower case letters indicate degenerate region. Numbers in NocORF and ribosomal gene primer names the indicate 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	Chapter 2
DinoLcfR2	Dino *lcf*	CACCAGGGGCTCGtaraartartg	Chapter 2
NocORF23F	Noc *lcf*/*lbp*	ACCGAAGCTGTTTTGGATTG	Present study
NocORF2418R	Noc *lcf*/*lbp*	TGGCCGAGAGAGAAAAGAAA	Present study
18ScomF1	Dino SSU	GCTTGTCTCAAAGATTAAGCCATGC	Zhang et al. (2005)
Dino18SR2	Dino SSU	TGCTTTCGCAGTAGTYYGTCTTTAAC	Lin et al. (2006)
AT18F01	Dino SSU	ACCTGGTTGATCCTGCCAGTAG	Ki and Han (2005)
PM28-R1318	Dino LSU	TCGGCAGGTGAGTTGTTACACAC	Ki and Han (2005)

5.2.5 Cloning and sequencing

All PCR products were excised from agarose gels and purified using the QIAquick gel extraction kit (Qiagen, UK). The PCR products of *N. scintillans lcf*/*lbp* ORF were cloned using the pGEM-T Easy kit (Promega, UK) and one clone was sequenced using M13 primers and an internal primer designed from the first sequence reads. The ribosomal PCR products were sent for sequencing directly. Initially, the PCR primers used in the amplification were used for sequencing, however, due to the inadequate read lengths obtained, the 18ScomF1 primer (used in the PCR quality tests) was finally used to partially sequence the 18S (SSU) gene only. All sequencing was done by Eurofins (Eurofins MWG Operon, Germany).

5.2.6 Sequence and phylogenetic analyses

Sequences were trimmed of vector and primers and assembled using the CAP3 sequence assembly program (http://pbil.univ-lyon1.fr/cap3.php). The BLASTn tool of the NCBI database was used to identify that the correct gene had been amplified and to determine similarities to other sequences on the database. All subsequent analysis was done in MEGA v.5 (Tamura et al. 2011). Nucleotide sequence alignments were carried out using ClustalW (Thompson et al. 1994) and were subsequently manually improved. Phylogenetic analysis of

the SSU rDNA gene sequences was undertaken by the maximum likelihood method (ML) and the Tamura-Nei model with gamma distribution (TN93+G) as predicted by the MEGA software to be the best model for the substitution pattern. The robustness of the tree was statistically assessed by bootstrapping with 100 replications in ML and maximum parsimony (MP) analyses. The phylogenetic analysis was conducted using partial (800bp) sequences of the SSU rDNA gene that was amplified from strains GM and LJ (Table 5.1) that were obtained with a single read. Seven sequences of *N. scintillans* were used with five of them from previous studies that had been deposited in GenBank. Another two sequences that were on the database were excluded because the cells that had been studied were of unknown geographic origin. Additional dinoflagellate sequences were included in the tree and the ancestral species *Perkinsus* sp. (Ki 2010) was used as an outgroup.

5.2.7 Confocal laser scanning microscopy

Cells that were in the mid dark phase were collected individually using a plastic Pasteur pipette and placed on a glass slide with a raised circular well constructed by double sided tape, that could accommodate the large *N. scintillans* cells. The cells were immobilised by adding a drop of viscous Protoslow quieting solution (Blades Biological Ltd, UK). Cells were imaged using a Leica TCS SP5 laser scanning confocal microscope (Leica Microsystems Inc. USA) at the Institute of Developmental Science of the University of Southampton, which was operated by Dr. David Johnston. Optical slices of 0.5 μm were obtained for part of the cell as long as the focal distance of the microscope did not require pressing against the raised coverslip. The auto-fluorescence of luciferin was induced by a UV-laser with emission at 405 nm. The luciferin fluorescence was then recorded and imaged at 416-520 nm.

5.2.7 Transmission electron microscopy

For transmission electron microscopy (TEM), cultures of *N. scintillans* were fixed with 10% glutaraldehyde, prepared in filter sterilised sea water, to a final concentration of 2%. The cultures and fixative were kept cool in ice before fixation. Cells were concentrated in small baskets using 40 μm plankton net. To avoid further disturbance of the cells all the following steps until embedding in Spurr resin were preformed in these baskets. Glutaraldehyde was

rinsed off in 0.1 M PIPES buffer to which 1% NaCl was added to raise the buffer osmolarity. Cells were post fixed in 1% osmium tetroxide in the same buffer for 1 hour at room temperature. Samples were then rinsed in distilled water and stained in 2% aqueous uranyl acetate for 20 minutes followed by standard dehydration in an ethanol series and a final 10 minute step in acetonitrile before embedding overnight in Spurr resin. Individual specimens were then picked and placed in separate polymerizing moulds. Sectioning was performed on an ultramicrotome (Reichert –Jung Ultracut E.). Sections were mounted on uncoated copper grids stained with lead citrate. Grids were viewed and photographed on a Hitachi H-7000 electron microscope.

5.3 Results

5.3.1 Detection of the luciferase gene

All strains of *N. scintillans* were tested for the presence of *lcf* in their genomic DNA and cDNA using “universal” primers for this gene. All strains amplified the correct sized fragment (~270bp) from the genomic DNA. However, *lcf* was only detected in the cDNA of the bioluminescent strain GM. The representative PCR amplification results in DNA and cDNA of non bioluminescent strain SPMC136 is shown in Figure 5.2. The negative result in the two non bioluminescent strains LJ and SPMC136 was confirmed to not be a result of PCR failure as the cDNA template quality tests by PCR of dinoflagellate SSU rDNA were successful in all strains (Figure 5.3).

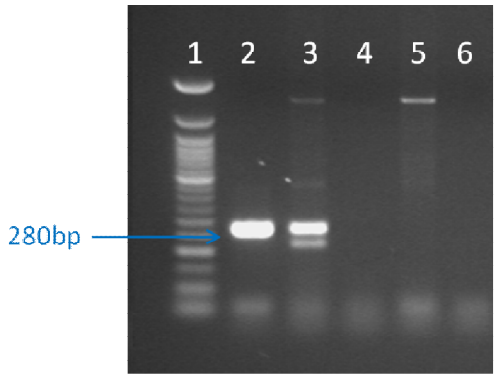

Figure 5.2 Gel photograph of PCR results of the detection of the *lcf* in the non bioluminescent *N. scintillans* strain SMPC136. Lane contents: 1) 50bp DNA marker; 2) Positive control strain GM cDNA; 3) strain SMPC136 DNA; 4) strain SMPC136 cDNA; 5) *Prorocentrum micans* food cells DNA; 6) Negative control without template. The 270bp band of *lcf* amplified by primers DinolcfF4/R2 is labelled with a blue arrow. The identity of the secondary band in strain SPMC136 has not been investigated.

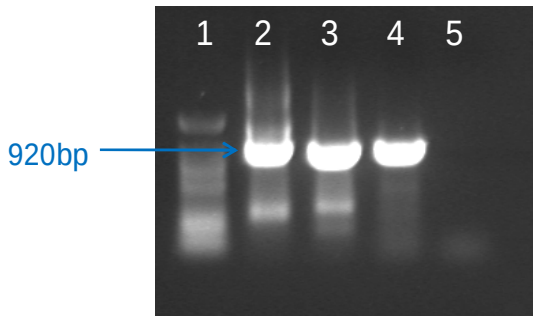

Figure 5.3 Gel photograph of PCR quality tests on cDNA of all three *N. scintillans* strains using primers 18ScomF1/Dino18SR2, which amplify the SSU rDNA gene. Lane contents: 1) 50bp DNA marker; 2) *N. scintillans* GM cDNA; 3) *N. scintillans* SMPC136 cDNA; 4) *N. scintillans* LJ cDNA; 5) No template negative control. The 920bp band of the SSU rDNA gene amplified in all strains is labelled with a blue arrow.

5.3.2 Amplification of the bioluminescence gene and a novel luciferin binding protein gene

Following the detection of *lcf* in the genomic DNA of all *N. scintillans* strains, the sequencing of the full *lcf*/*lbp* ORF was pursued. Primers targeted to the N-terminal region (NocORF23F) and the *lbp* domain (NocORF2418R), were expected to amplify the nearly full length ORF. Extensive optimisation of the PCR conditions was undertaken but the amplification from

genomic DNA was extremely weak and barely detectable. Therefore, the PCR products could not be excised for sequencing. In order to confirm that the PCR conditions were not responsible for this weak amplification, the same protocol was performed using cDNA from strain GM as template. The amplification from cDNA was successful with some surprising results. The primers that were designed to amplify the ORF of *N. scintillans lcf*/*lbp* amplified two distinct cDNA fragments (Figure 5.4). One fragment was 2396 bp long which was the expected size and was excised and cloned. The second fragment which was only 1457 bp long was unexpected and was also seen in the weak amplifications from genomic DNA that could not be excised. As the primers were sequence specific and this product appeared as a clean bright band that was unlikely to be a non specific product, the decision was taken to excise and clone it as well.

Sequencing showed that the ~2.4 kb fragment was indeed the combined *lcf*/*lbp* gene previously reported for this organism (Liu and Hastings 2007). The sequence consisted of part of the N-terminal region, the *lcf* domain and followed by the *lbp* domain and the nucleotide sequence identity between this sequence and that previously reported for *N. scintillans* (Liu and Hastings 2007) was high (94%). The sequence of the ~1.5kb fragment revealed that this was also an *lbp* with high nucleotide sequence identity of 92.2% to the *lbp* domain of the 2.4 kb fragment amplified here. An alignment of the two translated amino acid (AA) sequences revealed that the gene N-terminal region lead directly into the *lbp* domain in the absence of an *lcf* domain. Therefore the sequences that shared the N-terminal region and *lbp* domain but not the *lcf* domain correspond to two different genes: a combined *lcf*/*lbp* and a single separate *lbp*.

The two genes identified are predicted to produce proteins of distinct molecular weight that can be calculated based on the full *lcf*/*lbp* translated AA sequence. Considering the length of full *lbp* domain the expected molecular weight of the resulting protein would be 63.4 kDa. The molecular weight of the N-terminal region is 4.1 kDa. If the full sequence of the second single *lbp* is assumed to be the same length as the corresponding *lbp* domain of the combined *lcf*/*lbp* gene then the estimated molecular weight of the protein resulting from the single *lbp* (i.e. *lbp* domain and N-terminal) can be estimated to be 67.5 kDa.

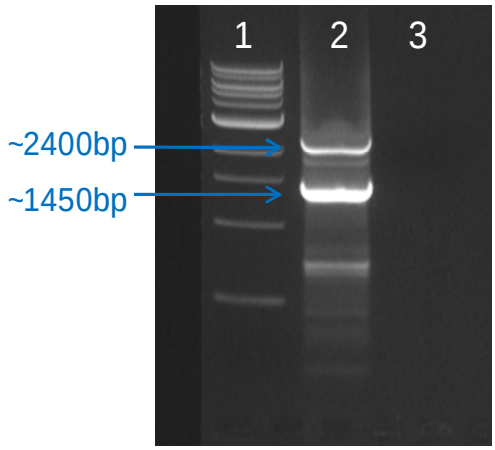

Figure 5.4 Gel photograph of PCR products resulting from amplification of *N. scintillans* GM cDNA with primers NocORF23F/2418R. Lane contents: 1) 1kb DNA marker; 2) *N. scintillans* GM cDNA; 3) Negative control without template. The ~2.4kb band corresponds to the combined *lcf*/*lbp* gene and the ~1.45kb band corresponds to the single *lbp*.

5.3.3 Microscopic identification of scintillons

The presence of scintillons can typically be determined by the fluorescence of luciferin under UV light. All strains were examined by confocal laser scanning microscopy in order to determine the presence and cellular location of luciferin. Only the bioluminescent strain was found to contain luciferin in its scintillons, which appeared as blue dots along the whole cell periphery (Figure 5.5). The number of scintillons in every cell was so high that due to their small size they blended into a nearly continuous fluorescence surrounding the cell. As scintillons were too small in comparison to the large cell their exact number could not be counted in the confocal microscope images.

The presence of scintillons in the cells of the bioluminescent strain and in one of the non bioluminescent strains (SPMC136) was further investigated using TEM (Figure 5.6) The bioluminescent strain contained scintillons appearing as smooth electron dense organelles surrounded by a membrane, around the periphery of the cells near the cell wall, identical to previous reports (Nicolas et al. 1987), occurring in clusters with or near trichocysts. The size of the scintillons ranged from 0.7 to 0.9 μm.

Structures of equivalent morphology and size to the scintillons were present in the non bioluminescent strain SPMC136, that retained their electron density and appeared to be identical to the scintillons of the bioluminescent strain. Additionally, there were several structures of smaller size which also occurred near the cell wall 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.

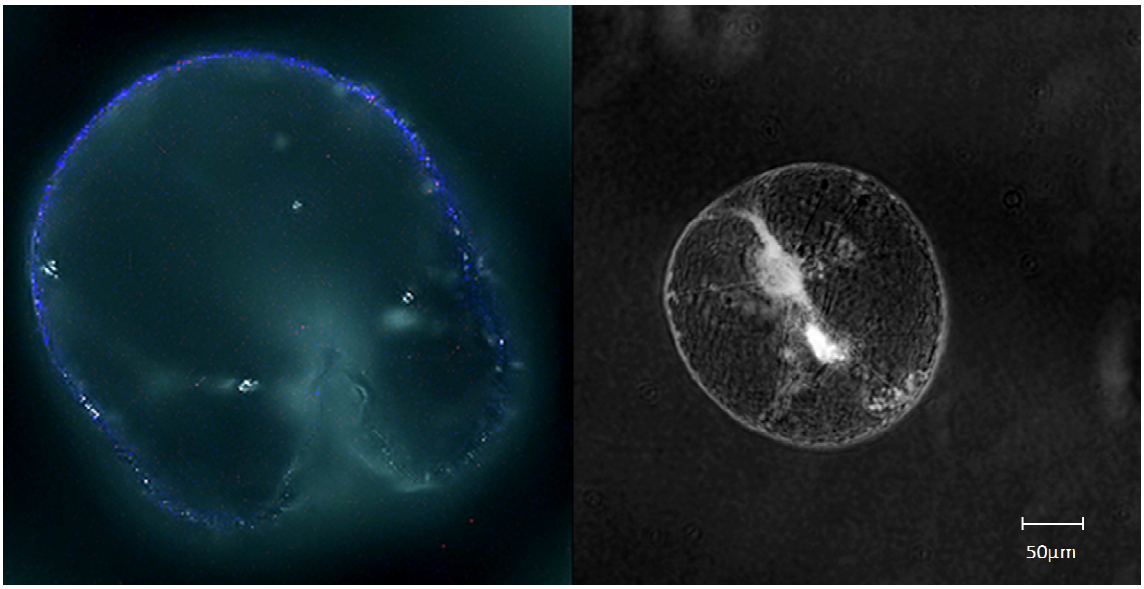

Figure 5.5 Confocal laser scanning microscope images of cells of the bioluminescent (left) and non bioluminescent (right) cells of *N. scintillans*, illuminated with UV light and shown on the same magnification. The blue fluorescence of luciferin shows the distribution of scintillons on the periphery of the bioluminescent cell which is absent in the non bioluminescent cell. The bluish hue in the left image is caused by the intense fluorescence of luciferin beyond the focal plane. The cells are photographed on the same magnification to show the true size difference: maximum widths of the bioluminescent and non bioluminescent cells shown are 440µm and 200 µm, respectively.

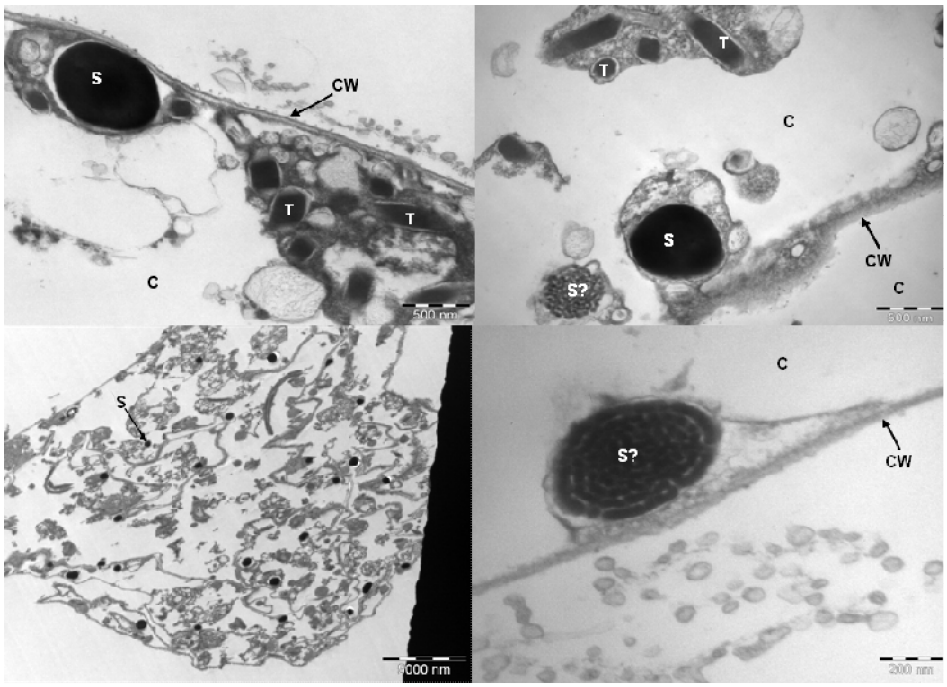

Figure 5.6 Transmission electron micrographs of *N. scintillans* cell sections. Upper left shows detail of the cell periphery of bioluminescent *N. scintillans* GM with a scintillon surrounded by membrane and trichocysts along the cell wall. Lower left shows a slice of the cell surface of *N. scintillans* GM with a large number of scintillons. Upper right shows detail of the cell periphery of the non bioluminescent *N. scintillans* strain SPMC136 with a scintillon surrounded by a membrane near the cell wall and an additional smaller scintillons like structure with a granular content. Lower right shows this scintillons like structure in higher magnification with detail of the internal granular structure. S = scintillons, S? = scintillons like structure with lower density of content, T = trichocyst, CW = cell wall, C = cytoplasm (Images provided by Ana Amorim).

5.3.4 Phylogenetic analysis based on SSU rDNA

During this study, both the SSU and LSU regions of the rDNA of two *N. scintillans* strains (GM and LJ) were amplified by PCR. However, recurrent sequencing failures with sequencing primers designed in this study or published in the literature (Ki 2010) meant that only an 800bp section of the highly conserved SSU rDNA could be obtained in the time frame of this PhD project. Therefore, the phylogenetic analysis presented here is considered to be a preliminary result that will be investigated further.

The ML phylogenetic tree (Figure 5.7) shows that all *N. scintillans* strains are very similar which is consistent with the high identities (>98%) exhibited among these strains at the

nucleotide level. However, the non bioluminescent strain LJ appeared to be divergent from other strains, branching off at the base of the *N. scintillans* group. This divergent branch was the only one within *N. scintillans* that was supported by a very high bootstrap value of more than 95% in both ML and MP analyses. A divergence was noted in one of the Asian strains (AB297469) which was though not as statistically robust and generally the Asian strains were not statistically resolved from the strains originating from the eastern USA.

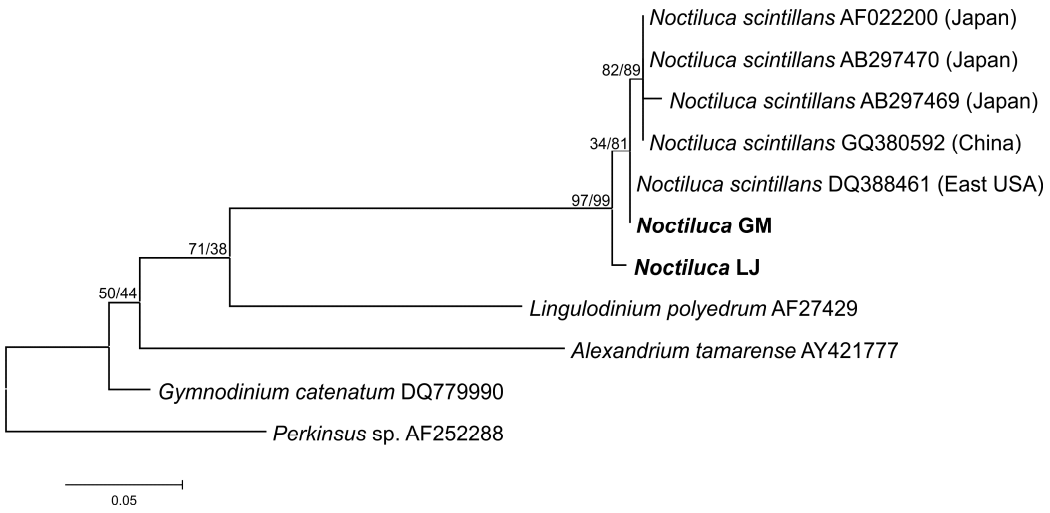

Figure 5.7 Maximum likelihood phylogenetic tree based on a nucleotide alignment of partial SSU rDNA sequences. Bootstrap values shown at the nodes are based on a maximum likelihood analysis followed by a maximum parsimony analysis. The scale of the branch lengths shows 0.5 substitutions per site. Accession numbers are shown after the species name where the sequences were obtained from GenBank and the geographic origin of the corresponding strains are in brackets. Sequences obtained in this study are in bold font.

5.4 Discussion

5.4.1 Discovery of a novel luciferin binding protein gene in *Noctiluca scintillans*

The finding of a second *lbp* in *N. scintillans* that occurs as a separate gene to the combined *lcf*/*lbp* is a new addition to our knowledge of dinoflagellate bioluminescent systems. A second LBP was hypothesised by Liu and Hastings (2007) to be the cause of a ~65 kDa protein being labelled in *N. scintillans* protein extracts by an anti-LBP antibody, although a corresponding

gene was not found in their study. The molecular weight of that protein corresponds well to the estimated 67.5 kDa protein that would result from the single *lbp* gene, thus indicating the likelihood that the single *lbp* does produce a distinct protein.

The presence of a single *lbp* gene shows that *lbp* and *lcf* occur as combined but also as separate genes in *N. scintillans*. The presence of two *lbp* genes in this organism is consistent with the expression of two *lbp* variants with 86% sequence identity that have been reported in *Lingulodinium polyedrum* (Morse et al. 1989b, Machabée et al. 1994) and have been further indicated by divergent *lbp* sequences within the organisms studied in chapter 4. If the *N. scintillans* bioluminescence system were to be ancestral, then the single *lbp* and combined *lcf/lbp* that underwent fission, could have resulted in the two *lbp* variants in photosynthetic species. Conversely, if *N. scintillans* obtained its bioluminescence genes by horizontal gene transfer, potentially only one of the two *lbp* variants fused with *lcf*. Horizontal gene transfer is increasingly thought of as a common occurrence in eukaryotes (Keeling and Palmer 2008) and a particularly well documented example is the transfer of proteorhodopsin genes from bacteria to dinoflagellates (Slamovits et al. 2011). While the likelihood of either scenario cannot be assessed at present due to the unresolved position of *N. scintillans* in dinoflagellate phylogenetic trees (see discussion by Hoppenrath and Leander 2010), further sequences of *lcf* and *lbp* genes from diverse taxa could be key in unravelling the pathways of evolution of dinoflagellate bioluminescence.

The functional role of a second LBP is unknown. Liu and Hastings (2007) speculated that if there were to be a second single *lbp* in *N. scintillans*, it could have a role in binding luciferin in the scintillons or it could act to store luciferin in the cytoplasm. This could indeed also be the case for the two *lbp* variants in photosynthetic species. However, since a physiological role for bioluminescence is unknown, it is difficult to predict the roles of unexpected components of the bioluminescence system. In order to address these questions, molecular physiological studies are needed and in this context *lbp* variants will have to be monitored separately.

5.2.4 Molecular control of the geographic variation in the bioluminescence of *Noctiluca scintillans*

The variety of *N. scintillans* that blooms along the west coast of the USA is morphologically distinguishable only by its smaller size. However, it is functionally distinct as it is not bioluminescent. The presence of *lcf* in the genome of these cells as well as the presence of scintillons suggests that this variety may have been bioluminescent in the past. The finding of scintillons of identical form in the non bioluminescent variety was surprising, because the smooth electron dense contents show that this feature is not related to the components required for bioluminescence, even though as yet only bioluminescence related protein have been found in the scintillons (Desjardins and Morse 1993). The presence of scintillons in cells with dysfunctional bioluminescence systems suggests that scintillons may have an additional function to that of light production. Electron dense dark bodies that appear similar to scintillons under some conditions have been observed in ultrastructural analyses of the freshwater cyanobacteria *Tolypothrix tenuis* (Gantt and Conti 1969) and in rodent aortic muscle cells and retinal Müller cells where they have been identified as acid phosphatase containing lysosomes (James-Kracke et al. 1979, Hori et al. 1980). Thus, there are several organelles that are similar to scintillons and further investigation into the contents of scintillons as well as the newly described scintillon-like granular organelles needs to be undertaken in order to establish their origin.

The findings of this study show that the common ancestor of the *N. scintillans* varieties studied here must have been bioluminescent or at least contained *lcf* in its genome. However, at some point in time, the bioluminescence system of the non bioluminescent variety "switched off" at both the molecular (gene expression) and cellular (lack of luciferin) level. This is consistent with findings in Chapter 2, as well as previous findings in bioluminescent bacteria (Palmer and Colwell 1991). These findings collectively suggest that the loss of gene function is a common cause for variations in bioluminescence. Furthermore, this could be a prevalent phenomenon in dinoflagellates that could, for example, be similar in the regional variations observed in the toxicity of morphological undistinguishable phylogenetic species of *Alexandrium* (Lilly et al. 2007) and in *Pyrodinium bahamense* regional varieties (Steidinger et al. 1980, Azanza 1997). The processes that result in the permanent "switching off" of bioluminescence or other dinoflagellate genes are unknown. The mechanisms that are

involved in pre-transcriptional gene silencing in any eukaryotic organisms are numerous, including structural modifications of transcription initiation sites and regulatory RNAs (reviewed by Wolf et al. 2010), DNA methylation (Curradi et al. 2002), repressor proteins or even heritable enzymatic modifications in histone proteins that encapsulate the particular region of the genome where the silenced gene is found (Ayyanathan et al. 2003). In *N. scintillans*, only the structural differences in gene sequence would be easy to determine. Additionally, the genes involved in the biosynthetic pathway of luciferin that are currently unknown could potentially play a role in acting as a controlling feedback on *lcf* expression. While clearly there needs to be a greater understanding of the bioluminescence system in order to answer questions about its regulation, this system could be a useful model system to investigate the genetic mechanisms that result in functional and ecological diversity within dinoflagellate species.

5.4.3 The role of hydrographic barriers in population segregation and potential speciation

The distinct non bioluminescent variety of *N. scintillans* occurs over a large area along the whole west coast of the USA. Its northern limits are unknown but it definitely does extend until Washington (approximately 50ºN) and possibly beyond, although it does not cross to the North West Pacific where the green *N. scintillans* is found instead. In the south, however, its limits are better defined, as a bioluminescent variety has been reported in the Baja California (Lapota and Losee 1984) and off the Galapagos and Cocos Islands (Staples 1966). This indicates that there is a hydrographic barrier posed by the California Current and associated upwelling fronts (Barth et al. 2000, Lynn et al. 2003) that is important in keeping the two populations separate. Mature cells of *N. scintillans* are positively buoyant non swimmers and therefore depend on surface currents to transport them. Therefore, it is unlikely that coastal blooms of non bioluminescent *N. scintillans* would travel south and into the Gulf of California or vice versa in the case of the bioluminescent *N. scintillans*. However, there has been one report of low number bioluminescent *N. scintillans* cells in Californian waters (Herren et al. 2004), which suggests that if these cells do arrive in the area or if they are present in the background community, the environmental conditions prevent their growth but rather favour the growth of the non bioluminescent variety allowing it to form dense blooms. Thus, these

functionally distinct varieties of *N. scintillans* may have distinct environmental niches that are mutually exclusive.

Geographic isolation results in allopatric populations that can evolve independently to their respective environments and ultimately lead to reproductive isolation and therefore speciation (Muller 1942, Wolf et al. 2010). The genetic mechanisms underlying speciation of this kind is the subject of considerable research and gene expression is hypothesised to play a larger role than structural differences in genes (Wray 2007), as functional traits are differentially selected in distinct environments (Wolf et al. 2010). The phylogenetic analysis of SSU rDNA genes provides preliminary indications that the non bioluminescent *N. scintillans* variety (strain LJ) is distinct from other bioluminescent *N. scintillans* strains. Considering the morphological and functional differences in size and bioluminescence, respectively, of the two varieties examined in this study, this indicates that perhaps these two varieties are not the same species and that potentially, the hydrographic barriers to population dispersal have resulted in reproductive and therefore isolation speciation. When *Noctiluca scintillans* reproduces sexually, nearly 1000 homothallic gametes are produced from each mature cell but they do not form cysts after fusion and additionally, gametogenesis may be seasonally induced (Fukuda and Endoh 2006). Thus in addition to hydrographic barriers separating the two varieties, the reproductive features of *N. scintillans* could limit the opportunities for transport to another area in the form of a cyst, or mating could be prevented by reproductive timing differing in populations adapted to different environments. The ribosomal sequence data included in the present analysis are too few and of a highly conserved region of the rDNA gene, to make a conclusive statement to the species status of *N. scintillans* varieties. Efforts are therefore being focused on obtaining and phylogenetically analysing the more variable LSU rDNA region, while also taking into account sequence differences within each variety. This approach has proved to be powerful as a robust species marker in other until recently unresolved dinoflagellate species complexes such as that of *Alexandrium tamarense* (Lilly, 2007) that are reproductively incompatible (Brosnahan et al. 2010). Indeed, at present, it would be more suitable to regard *N. scintillans* as a species complex with functionally distinct members and it is highly necessary to examine the ribosomal genes of these varieties in order to conclusively determine if they can be considered as the same or different species. For example, in addition to the varieties studied here, the green variety of *N. scintillans* found in the tropical Indian and west Pacific oceans is thought to be in the early stages of endosymbiosis, tending to become

photosynthetic (Hansen et al. 2004) and it is likely that it has or it will ecologically evolve into a different species. Therefore biogeographical and physiological studies on *N. scintillans* varieties could be an excellent model for speciation genetics and determining the control over their bioluminescence could provide valuable insights into the genetic processes that result in functional differentiation.

5.4.4 Wider implications of these findings for the function of bioluminescence

Bioluminescence is not a core function of the cell that it cannot survive without, as it is not present in all dinoflagellates or even in individuals of the same species. However, bioluminescence appears to have been maintained since at least the Cretaceous when *Alexandrium*, the latest bioluminescent genus according to *lcf* phylogenies (Liu et al. 2004, Liu and Hastings 2007), is estimated to have appeared (John et al. 2003). It is unlikely that bioluminescence, a process likely to be energetically expensive, would be maintained if it did not confer a significant advantage. It is possible that bioluminescence could serve more than one function simultaneously, e.g. defence against predators and potentially an as yet unknown physiological role, as discussed by Abrahams and Townsend (1993) which could differ among species. The geographical functional differentiation of *N. scintillans* shows that bioluminescence is likely to be "switched off" when it is not required. This could mean that the environmental conditions in the west coast of the USA do not require *N. scintillans* to produce light. However, as California regularly sees some of the most iconic bioluminescent blooms of *Lingulodinium polyedrum* (Holmes et al. 1967), the conditions that regulate light production in *N. scintillans*, and therefore its physiological or ecological roles, must be different. Establishing these roles would require comparisons of environmental conditions that are prevalent during blooms of the geographically distinct bioluminescent and non bioluminescent varieties. However, there has not yet been a single study on the environmental conditions during which the non bioluminescent *N. scintillans* blooms along the west coast of the USA. Considering the results of the present study, more physiological and ecological studies are warranted in order to understand the environmental conditions that select for the production of light. Combining results of phylogenetic analyses on ribosomal genes, DNA sequence and expression data of bioluminescence genes and environmental data could be catalytic in obtaining a holistic understanding on the function of bioluminescence,

environmental selection pressures acting on it and finally whether the loss of bioluminescence is important in causing ecological speciation or whether it is simply a result of it.

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Chapter 6

GENERAL DISCUSSION

The aim of this thesis was to gain a fundamental understanding of several aspects of bioluminescence in dinoflagellates: the identity of bioluminescent species, the genetic composition of their bioluminescence systems, the diversity and evolution of bioluminescence related genes, the level of gene regulation that results in intraspecific differences of light production at local and regional scales, and the distribution of these organisms in the sea. Applying molecular tools to study these topics resulted in a wealth of new knowledge on dinoflagellate bioluminescence and re-addressed past knowledge which led to raising new questions that need to be investigated. This section summarises and synthesises the findings of the thesis, addresses its limitations and suggests the most immediate research questions that should take priority in the next investigations.

6.1 Detection and genetic diversity of the luciferase gene within marine dinoflagellates

The key objective that made this thesis possible was the development of “universal” primers for dinoflagellate *lcf*. For this purpose, it was important to design several primers and test them in a collection of dinoflagellates that was as broad as possible and inclusive of species that are widely represented in both coastal and open ocean environments. Sourcing cultures or DNA of bioluminescent *Ceratium* spp., *N. scintillans* and *Protoperidinium* spp. proved to be a difficult task as these organisms are not easy to culture. However, it was essential to include these organisms, as highlighted by the only one primer pair that successfully amplified *lcf* from all bioluminescent species as a result the large variation of nucleotide sequences of the gene between organisms. This task was realised after nearly three years of persistent effort and constant redevelopment of the PCR protocol with addition of new organisms and the *lcf* sequence of *Noctiluca scintillans* (Liu and Hastings 2007) was catalytic for the success of this

effort. The *lcf* primers were the main tool for most of the subsequent work and are expected to have even more applications than those reported in this thesis.

The comparison of intraspecific optical and genetic characteristics provided the first view of bioluminescence being "switched off" at the post genomic level. The data indicated that this can be due to normal intraspecific variation but also due to culture effects as both *Fragilidium* spp. permanently lost their bioluminescence after air transport to the UK. The genetic level conservation of *lcf* allows one to assess a species for a particular functional trait even though it may not be expressing it at that time and can therefore allow studies to focus on discovering potential bioluminescence in as many species as possible, rather than having to test many strains of the same species. Furthermore, genetic and phenotypic comparisons of bioluminescence could hold the potential for fundamental explorations of the molecular processes that terminate the expression of protein coding genes in dinoflagellates. It will though be difficult to disentangle culture effects from intraspecific variation and therefore fresh cultures should be used for this purpose.

The application of the *lcf* PCR primers to a large collection of dinoflagellates revealed that in the genera *Alexandrium* and *Ceratium*, the presence of *lcf* in certain species and its absence in others may be explained by their phylogenetic distance. It also showed that there have been several errors in previous reports of bioluminescent species that have to be readdressed. The application of the *lcf* PCR in other culture collections, coupled with molecular phylogenetic information, will provide more information about bioluminescent species, their relatedness, and therefore the evolutionary path of bioluminescence within the dinoflagellates.

The phylogenetic analysis of new *lcf* sequences showed that dinoflagellate phylogenies based on protein coding genes may give invalid results because the sequence variation among gene copies within one organism can be as large as the sequence variation between organisms of different genera. It is not known to what taxonomic level this variability may extend and certainly this will vary for every individual gene depending on its degree of conservation. This may, at least partially, explain why protein coding gene sequences produce conflicting phylogenetic trees. Robust phylogenies are highly necessary to interpret the evolution of a range of morphological and functional features, including bioluminescence. Therefore, when a protein coding gene is to be used for phylogenetic inference, its variability within the

organism, between closely genetically related organisms and between more distant organisms has to be assessed and the resulting phylogeny must be interpreted in that context.

The cluster analysis of *lcf* sequences of *Ceratium* and *Protoperidinium* showed interesting novelties, a highly distinct gene variant or domain is present in the former and potentially a new group of the gene is associated with the latter. The functional and evolutionary significance of these sequence features could not be assessed but they showed that the true diversity of *lcf* is only beginning to emerge. Further efforts to fully sequence *lcf* in genetically distant organisms are essential to understand the evolution of bioluminescence within dinoflagellates. The *lcf* PCR primers will undoubtedly make this task easier since some initial sequence information is now easy to obtain.

6.2 Detection and molecular phylogeny of the luciferin binding protein gene in marine gonyaulacoid dinoflagellates

This study on *lbp* presence was initially intended to be as comprehensive as the study of the luciferase gene in Chapter 2. However, because *lbp* sequences differ between organisms more than *lcf*, it was not possible to create “universal” PCR primers for this gene. Nevertheless, detection was possible in several ecologically important bioluminescent dinoflagellate genera and led to the first identification of *lbp* in the genera *Ceratocorys*, *Gonyaulax* and *Protoceratium* and confirmed that its presence is consistent within *Alexandrium*. The long sequences that were obtained from this gene allowed for a phylogenetic analysis which supported the results of the *lcf* phylogeny presented in Chapter 2 and further highlighted the differences of *Alexandrium monilatum* to the rest of the *Alexandrium* species that were tested. Initial evidence of the importance of *lbp* in maintaining the integrity of the bioluminescence system was revealed by finding only pseudogenes in the dim bioluminescent *G. spinifera* strain. The role of *lbp* in the integrity of the bioluminescence system should be further investigated by sequencing more copies of *G. spinifera* and comparing them to species with fully functional bioluminescence systems.

The most important finding of this study was that *lbp* is widespread in many bioluminescent species which resulted in challenging the appropriateness of *Pyrocystis lunula*, an organism with a likely uncommon bioluminescence system, as a model species for the chemistry of the

dinoflagellate bioluminescence. The current model of the origin of luciferin from chlorophyll has already been disputed in the heterotrophic species *Protoperidinium crassipes*. It is thus important to further characterise which bioluminescent species contain *lbp* but methods alternative to PCR will have to be used, such as expressed sequenced tags (ESTs) or transcriptomics to enable non targeted *de novo* gene discovery. This information of which species contain *lbp* is highly necessary for evolutionary analyses of the bioluminescence systems, for understanding the mechanism of bioluminescence and for selecting model species for physiological and chemical studies of bioluminescence.

6.3 Bioluminescent dinoflagellates of the Patagonian Shelf during early austral summer 2008

This study represented the first application of a molecular protocol in studying natural populations of bioluminescent dinoflagellates. The synergistic use of molecular detection of *lcf*, sequences of *lcf* and single cell PCR together with microscopy counts and bioluminescence measurements allowed for the first investigation of these organisms in relation to their natural environment. The detection of *lcf* was superior over optical bioluminescence measurements in revealing the distribution of bioluminescent dinoflagellates and led to a critical evaluation of the use of bioluminescence measurements in ecological studies. Sequence information and single cell PCR allowed for bioluminescent dinoflagellates to be identified and enumerated and the composition of their population to be determined. When these data were compared to environmental parameters, the population composition was shown to be determined by water mass characteristics and their interactions at the SBF created an important environment that decreased the diversity and increased the abundance of both bioluminescent and non bioluminescent dinoflagellate populations. This study also led to the recognition that the inherently complex ecology and patchy nature of dinoflagellate populations requires biological sampling at a higher resolution and complementary data of more biotic and abiotic factors that might be catalytic for dinoflagellate growth. Studies of the ecology of bioluminescent dinoflagellate species and particularly their differences to non bioluminescent species would initially be easier over smaller spatial scales with fewer large environmental gradients or alternatively could be based on seasonal monitoring at one location.

While the *lcf* detection formed the basis of this novel study, it would be preferable to be able to directly quantify *lcf* in the water column so to avoid the heavy reliance on microscopy to identify and quantify bioluminescent cells which requires several assumptions to be made about potential bioluminescent species. The differences in the number of domains contained or amplified in each organism and the necessity for degenerate primers in "universal" species detection, makes this protocol unsuitable for quantitative or semi quantitative PCR of either DNA or RNA. These gene features also preclude any quantitative analysis of community composition based on amplified gene sequences. One way to improve the classification of bioluminescent dinoflagellates counted by microscopy would be to employ extensive single cell PCR tests on the sampled populations. Ethanol was found to be a poor fixative for this purpose. On the other hand, methanol, that has been shown to be superior for this purpose (Godhe et al. 2002a), is problematic in air transport from remote locations. Therefore the isolation of single cells would have to be done immediately on board to completely avoid the use of fixatives. The best way to overcome these difficulties would be to design a suite of *lcf* genus or species specific primers for use with quantitative PCR on DNA and RNA, to quantify the number of bioluminescent cells and the levels of gene expression, respectively. However, this requires complete *lcf* sequences from all major bioluminescent dinoflagellate taxa which are lacking at present and therefore progress is needed on this front.

6.4 Functional diversity among geographic varieties of *Noctiluca scintillans*: A comparative molecular and cellular study of the bioluminescence system

The primary purpose of this study was to investigate the reasons for the absence of bioluminescence in a regional variety of *N. scintillans* that blooms off the west coast of the USA. Using the primers developed in Chapter 2 together with microscopy, it was shown that the bioluminescence system in this variety is present but has been "switched off". Furthermore, the hydrography of the area suggested that this variety may be excluded from regions where bioluminescent *N. scintillans* is present. The conclusion from these results was that the bioluminescent and non bioluminescent varieties originate from a bioluminescent population that became spatially segregated. This gave the first indication of external selection pressures acting on bioluminescence leading either to its maintenance or to its demise.

After these results, a decision was taken to look further into the sequences of bioluminescence and ribosomal genes of the two varieties to investigate their potential differences and to confirm that these varieties are in fact the same species, respectively. This part of the study is still ongoing although an initial finding of a new single *lbp* in this species was reported in this thesis. This finding showed that *lcf* and *lbp* occur as separate genes even in *N. scintillans* and also highlighted that it is important to know the composition of the bioluminescence system not only for evolutionary studies but also to monitor the changes that occur effectively; current experiments are now investigating the sequences of the two bioluminescence genes separately (hybrid *lcf*/*lbp* and single *lbp*). Ongoing experiments are also focused on retrieving the sequences of the LSU and possibly ITS regions of the rDNA gene. Nevertheless, the partial SSU rDNA sequences reported in this thesis have already shown distinct differences between the studied varieties, strongly indicating that they are different species. This has important implications as it leads to the question of whether observed intraspecific differences in bioluminescence, such as the findings reported in Chapter 2, are in fact interspecific differences.

One of the species for which intraspecific variability in bioluminescence but not in *lcf* was previously reported in Chapter 2, was *A. tamarense*. The *A. tamarense* "species complex" (*A. catenella*, *A. fundyense* and *A. tamarense*) has been phylogenetically defined based on LSU gene sequences as a group containing 5 phylogenetic species (Groups I-V) that do not correspond to their current names and only two of these species produce toxins (Lilly et al. 2007). Reproductive incompatibility between two of these species supports the phylogenetic interpretation (Brosnahan et al. 2010). A very recent study reporting the newly discovered saxitoxin gene, showed that this gene was present in 5 strains of *A. tamarense,* 4 of which did not produce detectable saxitoxin (Stüken et al. 2011) and therefore should belong to the non toxic species. These reports suggest that genes encoding non housekeeping functional traits such as bioluminescence and toxicity may remain as a detectable remnant in species that have recently diverged. This would be in agreement with the observations of this study in the possible speciation *N. scintillans*, where one provisional species lost its bioluminescence but a bioluminescence gene remains. Further research should compare the functional characteristics and corresponding genes of strains of the same "species" with concomitant sequencing of rDNA regions that are useful for phylogenetic reconstructions. This will be essential in interpreting functional diversity in the context of speciation. Another important conclusion of

these findings with implications for Chapter 3, is that detecting *lcf* in the genomic pool of field populations does not mean that bioluminescence is necessarily expected. Therefore, the next field studies will have to use the *lcf* PCR on the expressed genes (i.e. RNA converted to cDNA) to get a more representative description of the processes occurring at the time.

6.5 Outlook and final remarks

This thesis represents the first holistic investigation of fundamental aspects of dinoflagellate bioluminescence at the molecular level. The studies reported in this thesis have provided considerable insight into bioluminescent dinoflagellate diversity, phylogeny and some aspects of their ecology, as well as a powerful molecular tool for further investigating evolutionary and ecological aspects of this process. However, these studies have also highlighted the infant status of our knowledge of the evolution, mechanism and controls on bioluminescence, by raising several new questions that need to be investigated. One of the research priorities should be to determine the structure and origin of luciferin, preferably in a number of organisms with phylogenetically distinct *lbp* as determined in Chapter 4. This will provide important insights into how the bioluminescence system may be linked to other metabolic functions and enable informed hypotheses to be made for testing in physiological experiments. Physiological experiments are themselves critically lacking at present. In the absence of information about luciferin, a good place to start would be to investigate changes in bioluminescence and gene/protein expression in response to changing environmental conditions such as nutrient availability, particularly phosphate whose depletion has already been shown to increase the levels of expressed *lbp* (Erdner and Anderson 2006). This will start to give clues of the environmental factors that shape bioluminescence in dinoflagellates and link into the ecological characteristics of bioluminescent dinoflagellates. Finally, full sequences of *lcf* from a variety of organisms are needed for several reasons: to enable tracing the evolutionary origin of bioluminescence, to assess how the composition of the bioluminescence system has changed over time in relation to differential regulatory requirements or selection pressures on each gene and its variants, and to enable the creation of specific/genus PCR primers that can be used to quantify *lcf* gene copies or transcripts of different taxa in natural samples. To this end, the *lcf* primers can facilitate the retrieval of the initial partial sequences from transcriptomes or metatranscriptomes that are needed for the complete sequencing of these genes.

Establishing the biological function of bioluminescence is still a distant goal but chemical, genetic, physiological and ecological information will work in concert to finally understand the significance of this process. Even though the immediate importance of bioluminescence in dinoflagellates cannot yet be defined but only implied, I hope that the sheer beauty of this phenomenon will motivate curiosity driven research on this fascinating and awe inspiring phenomenon that lights up our oceans.

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