Diversity of luciferase sequences and bioluminescence production in Baltic Sea *Alexandrium ostenfeldii*

Anniina H. Le Tortorec$^{1,2}$, Pia Tahvanainen$^{1,2}$, Anke Kremp$^1$, Stefan G. H. Simis$^{3,1}$

$^1$Finnish Environment Institute SYKE, Marine Research Centre, Erik Palménin aukio 1, 00560 Helsinki, Finland

$^2$University of Helsinki, Tvärminne Zoological Station, J.A. Palmenin tie 260, Hanko 10900, Finland

$^3$Plymouth Marine Laboratory, Prospect Place, The Hoe, Plymouth PL1 3DH, United Kingdom

**Corresponding author:**

Anniina Le Tortorec

Finnish Environment Institute

Marine Research Centre

Erik Palménin aukio 1

00560 Helsinki, Finland

Email. anniina.lindroos@helsinki.fi

Author self-archiving copy - to be cited as:

Abstract

The toxic dinoflagellate *Alexandrium ostenfeldii* is the only bioluminescent bloom forming phytoplankton in coastal waters of the Baltic Sea. We analysed partial luciferase gene (*lcf*) sequences and bioluminescence production in Baltic *A. ostenfeldii* bloom populations to assess the distribution and consistency of the trait in the Baltic Sea, and to evaluate applications for early detection of toxic blooms. *Lcf* was consistently present in 61 Baltic Sea *A. ostenfeldii* strains isolated from six separate bloom sites. All Baltic Sea strains except one produced bioluminescence. In contrast, the presence of *lcf* and the ability to produce bioluminescence did vary among strains from other parts of Europe. In phylogenetic analyses, *lcf* sequences of Baltic Sea strains clustered separately from North Sea strains, but variation between Baltic Sea strains was not sufficient to distinguish between bloom populations. Clustering of the *lcf* marker was similar to internal transcribed spacer (ITS) sequences with differences being minor and limited to the lowest hierarchical clusters, indicating a similar rate of evolution of the two genes. In relation to monitoring, the consistent presence of *lcf* and close coupling of *lcf* with bioluminescence suggests that bioluminescence can be used to reliably monitor toxic bloom-forming *A. ostenfeldii* in the Baltic Sea.

Key words: *Alexandrium ostenfeldii*, bioluminescence, dinoflagellate, harmful algal bloom, internal transcribed spacer, luciferase gene, phylogeny, paralytic shellfish poisoning toxins
Introduction

Bioluminescence has independently evolved at least 40 times in oceans and terrestrial environments among taxa ranging from bacteria and unicellular microalgae to large fish and squids (Haddock et al., 2010; Widder, 2010). The majority of bioluminescent organisms occur in oceans, with around 80% of the more than 700 genera containing bioluminescent species found in marine environments (Widder, 2010). In the oceans bioluminescence occurs from surface waters to the deep sea, serving a range of functions from self-defense to camouflage, reproduction and intra-species communication (Widder, 2010; Haddock et al., 2010). Virtually all bioluminescence in surface waters of oceans originates from dinoflagellates and they are responsible for the sparkling lights that can be seen at night (Marcinko et al., 2013). Dinoflagellates consist of 117 described genera of which 17 have bioluminescent members (Valiadi & Iglesias-Rodriguez, 2013). Bioluminescence is present in a large number of bloom-forming and/or toxic dinoflagellate species (Valiadi et al., 2012; Cusick & Widder, 2014). In the genus *Alexandrium*, one of the major harmful algal bloom genera, bioluminescence occurs in 89% of screened species (Valiadi et al., 2012). It has therefore been suggested that bioluminescence could be used as an indicator of harmful dinoflagellate blooms (Kim et al., 2006; Haddock et al., 2010; Le Tortorec et al., 2014).

Bioluminescence in dinoflagellates is considered to be a defensive mechanism against grazing, possibly attracting the predator’s predators (Buskey et al., 1983; Buskey & Swift, 1985; Abrahams & Townsend, 1993; Fleisher & Case, 1995) or functioning as an aposematic signal (Cusick & Widder, 2014). The bioluminescent system of dinoflagellates is uniquely based in specific cellular organelles, scintillons (DeSa & Hastings, 1968). These contain a luciferase enzyme, a light-emitting luciferin
substrate and, in many species, a luciferin-binding protein (Knaust et al., 1998; Akimoto et al., 2004; Valiadi & Iglesias-Rodriguez, 2014). To date, the luciferase gene (lcf) has been fully sequenced from eight dinoflagellate species (Liu & Hastings, 2007; Valiadi & Iglesias-Rodriguez, 2013) and partial sequences are available from species belonging to genera Alexandrium, Ceratium, Ceratocorys, Gonyaulax, Fragilidium and Protoperidinium (Valiadi et al., 2012; Valiadi et al., 2014). Previous studies have included up to 4 - 6 partial sequences from single species (Baker et al., 2008; Valiadi et al., 2012). In most species lcf consists of three tandemly repeated domains, each consisting of a highly conserved central region that encodes a catalytically active site (Li et al., 1997; Li & Hastings, 1998; Liu et al., 2004) and is bordered by more variable N- and C- terminal regions (Okamoto et al., 2001; Liu et al., 2004). In general, within-species differences between lcf domains are larger than between-species differences of the same domain (Okamoto et al., 2001; Liu et al., 2004). In addition, there are differences in the untranslated region sequences and in the length of these regions between species (Okamoto et al., 2001; Liu et al., 2004).

There is also intra-species variation in bioluminescence production. For example, Valiadi et al. (2012) found that the species Ceratocorys horrida Stein and Alexandrium tamarense (Lebour) Balech contained both bioluminescent and non-bioluminescent strains even though all strains possessed lcf. Also, the bacteria Vibrio cholerae has been reported to have both bioluminescent and non-bioluminescent strains that all have lcf (Palmer & Colwell, 1991). Similar findings have been obtained from the more widely studied functional gene for saxitoxin, where many strains from the A. tamarense species complex contain the gene but not all produce saxitoxin (Orr et al., 2011; Murray et al., 2012). The relationship between presence of lcf and production of
bioluminescence is not well known in dinoflagellates, and very little information exists on intra-species variation of lcf sequences from dinoflagellate species in general.

Alexandrium ostenfeldii (Paulsen) Balech and Tangen is a toxin-producing and bioluminescent dinoflagellate with a wide geographic distribution in temperate and Arctic waters (e.g. Moestrup & Hansen, 1988; Mackenzie et al., 1996; John et al., 2003; Gribble et al., 2005; Almandoz et al., 2014; Tillmann et al., 2014). In oceans, A. ostenfeldii typically occurs at low abundance (Moestrup & Hansen, 1988; John et al., 2003) but dense blooms of the species have been increasingly observed during the last decade in coastal areas of the Atlantic USA (Borkman et al., 2012; Tomas et al., 2012), Italy (Ciminiello et al., 2006), The Netherlands (Burson et al., 2014) and the Baltic Sea (Kremp et al., 2009). These blooms have the potential to be harmful as A. ostenfeldii can produce paralytic shellfish toxins (PSTs), spirolides and gymnodimines (Van Wagoner et al., 2011; Tomas et al., 2012; Kremp et al., 2014; Tillmann et al., 2014). For example, PSTs can accumulate in benthic and littoral food webs and affect higher trophic levels (Campbell et al., 2005; Jester et al., 2009; Anderson et al., 2012; Setälä et al., 2014).

This study focuses on the Baltic Sea where three dinoflagellate species capable of bioluminescence have been recorded. Protoceratium reticulatum (Claparéde & Lachmann) Büttschli is found in low abundances throughout the open Baltic (Hållfors, 2004; Mertens et al., 2012), while Lingulodinium polyedrum (Stein) Dodge is only encountered in the southern parts of the Baltic (Hållfors, 2004). Currently A. ostenfeldii is the only bioluminescent dinoflagellate known to form dense blooms in the coastal areas of central and northern Baltic Sea (Hakanen et al., 2012; Le Tortorec et al., 2014). The distribution and consistency of bioluminescence in Baltic A. ostenfeldii is of interest to evaluate applications for early detection of toxic blooms. Therefore, we examine here: a) if separate bloom populations of A. ostenfeldii have lcf, b) if lcf is uniformly present
throughout bloom populations, and c) if it is consistently paired with bioluminescence production. Subsequently, we analyse the diversity of lcf sequences among A. ostenfeldii bloom populations to determine whether lcf can be used to distinguish local Baltic bloom populations. Finally, we present results from a field survey where lcf presence, bioluminescence production and abundance of A. ostenfeldii cells are provided in order to evaluate the use of lcf and bioluminescence for early detection of A. ostenfeldii blooms.

Materials and methods

A. ostenfeldii cultures

Included in this study are cultured strains isolated from six bloom sites around the Baltic Sea with known A. ostenfeldii bloom occurrences, nine strains originating from the North Sea, and four strains originating from Canada, China and Spain (Table S1). All Baltic strains were established from resting cysts isolated from sediment samples, while the other strains were grown from cells collected from the water column. Detailed information on the isolation and establishment of the used isolates is given in Tahvanainen et al. (2012) and Kremp et al. (2014). All strains were grown in 40 ml batch cultures in f/2-Si medium (Guillard & Ryther, 1962) adjusted to native salinities (6 psu for Baltic Sea strains and 30 psu for all other strains) in vented 50 ml polycarbonate tissue culture flasks at 16 °C on a 12:12 light dark cycle at 100 µmol photons m⁻² s⁻¹.

Bioluminescence test

Dense A. ostenfeldii cultures in their late exponential to early stationary phase were visually tested for bioluminescence production by shaking the culture bottles in a dark room during their scotophase. If no bioluminescence was observed, 2.7 ml of culture was
transferred into a 3.7 ml optical glass cuvette. The cuvette was placed inside a cuvette holder in a Varian Cary Eclipse Spectrofluorometer. Bioluminescence was chemically stimulated by adding 400 µl of 0.1 M HCl (Hamman & Seliger, 1972) using plastic tubing and a pipette. The intensity of bioluminescence was measured at 470 nm. If bioluminescence was still not observed, the procedure was repeated at least five times for separate culture bottles of the same strain, over a three-month period. Bioluminescence tests were always carried out at the same time of the scotophase, examining all cultures within a one hour period.

DNA extraction of cultured A. ostenfeldii

Clonal cultures in their exponential growth stage were used for DNA extraction. 30 ml of culture was transferred to a 50 ml Falcon tube and concentrated by centrifuging at 4000 × g for 30 min. After discarding most of the supernatant the pellet was resuspended into the remaining liquid and pipetted into two 1.5 ml Eppendorf tubes. The samples were subsequently centrifuged at 14000 × g for 15 min. The supernatant was then discarded, and cell pellets were disrupted using a motor pestle (Pellet Pestle Cordless Motor, Kontes Glass Company, Kimble). DNA was extracted using a Plant Mini Kit (Qiaqen) and purified with a PCR Template Purification Kit (Roche) according to manufacturer instructions. DNA purity and concentration were measured using NanoDrop ND-1000 (Thermo Scientifec). DNA samples were stored at -80 ºC until further processing.

PCR reaction

All DNA samples were amplified using the “universal” lcf primers for dinoflagellates (LcfUniCHF3: TCCAGGTTGCACGGCTTCGAGCNGCNTGGC and LcfUniCHR4: GGGTCTTGTCGCCGTAGTCAAANCCYTTRCA) developed by Baker et al. (2008).
These primers target the non-homologous N-terminal region and the start of the first domain of the \textit{lcf}. We chose these primers because they amplify the region at the beginning of \textit{lcf} which is more diverse than central regions, and give longer sequences than more conservative primers (Baker \textit{et al.}, 2008). PCR reactions were performed in 25 \(\mu\)l reaction volume in PCR beads (Illustra PuReTaq Ready-to-go-PCR-beads, GE Healthcare) consisting of 16 - 22 \(\mu\)l sterile ultrapure water, 2 \(\mu\)l of each primer and 1 to 5 \(\mu\)l of DNA (about 100 ng). PCR reactions were performed following Baker \textit{et al.} (2008) as follows: 5 min at 95 °C, 35 cycles of 45 s at 95 °C, 30 s at 62 °C and 30 s at 68 °C and a final extension step of 10 min at 68 °C. Presence of \textit{lcf} was confirmed by running the PCR products on to a 2% TBE gel. The expected size of the PCR product was 500 - 550 bp. All cultured strains that produced a clear PCR product on gel were sequenced to confirm that the correct product had been amplified and to allow further phylogenetic analysis. The internal transcribed spacer (ITS-1 and ITS-2) and 5.8 rDNA sequences were generated as described in Tahvanainen \textit{et al.} (2012).

**Sequencing**

Purified PCR products were used as templates in sequencing reactions, which were carried out with the same forward or reverse primers as the PCR reaction. Sequencing was done according to the protocol of Applied Biosystems with BigDyeH Terminator v3.1 Cycle Sequencing Kit, and purified in a Biomek® NXP Laboratory Automation Workstation (Beckman Coulter) according to the Agencourt® CleanSEQ kit protocol, and sequenced in an Applied Biosystems ABI3130XL Genetic Analyzer (16-capillaries) or ABI3730 DNA Analyzer (48-capillaries).

**Phylogenetic analysis**
The lcf sequences were assembled and edited as necessary in Chromas Pro 1.7.6. The DNA sequences were aligned using ClustalW (Thompson et al., 1994) in MEGA6 (Tamura et al., 2013) and minor manual adjustments to final alignment were performed. The data set consisted of 59 ingroup sequences with a total alignment length of 525 bp. GenBank was searched for known Alexandrium lcf sequences for the region amplified by LcfUniCHF3 and LcfUniCHR4. All nine found sequences were used as outgroups. These included A. tamarense, A. catenella, A. fundyense and Alexandrium sp.

ITS sequences were assembled and manually edited where needed in Chromas Pro 1.5. Multiple sequence alignment was carried out in MAFFT (Multiple Alignment with Fast Fourier Transform, Katoh et al., 2009) in SeaView (Gouy et al., 2010) using default settings. The data set consisted of 59 ingroup sequences, and had a total length of 581 bp. Alexandrium minutum, A. tamutum, A. tamarense, A. fundyense, A. catenella and A. insuetum were used as outgroups. The resulting alignments are available upon request.

**Statistical analysis**

Phylogenetic analyses were performed using MrBayes v3.2 (Ronquist & Huelsenbeck, 2003). For lcf data Bayesian inference (BI), with substitution model GTR + G (Rodríguez et al., 1990), selected under the Bayesian Information Criterion (BIC) with jModelTest 0.1.1. (Posada, 2008) was used. Lacking specific knowledge on parameter priors, default settings for prior distributions were used in all analyses. Two runs with four chains (one cold and three incrementally heated chains) were run for 15 million generations, sampling every 500 trees. In each run, the first 25% of samples were discarded as the burn-in phase. The stability of model parameters and the convergence of the two runs were confirmed using Tracer v1.5 (Rambaut & Drummond, 2007). A maximum likelihood
(ML) phylogenetic tree was calculated in MEGA6 (Tamura et al., 2013), using an evolutionary model GTR + G + I, selected under the Akaike Information Criteria (AIC) with jModelTest 0.1.1 (Posada, 2008). Gaps and missing data were treated as complete deletion. Tree topology was supported with bootstrap values calculated with 2000 replicates.

For the BI of ITS-1, ITS-2 and 5.8 rDNA sequences, the substitution model, GTR + G (Rodríguez et al., 1990) based on the BIC in jModelTest 0.1.1. (Posada, 2008) was used. The BI analyses of ITS sequences were done with the default settings for prior distributions. Two runs with four chains (one cold and three incrementally heated chains) were run for 15 million generations, sampling every 500 trees. In each run, the first 25% of samples were discarded as the burn-in phase. The stability of model parameters and the convergence of the two runs were confirmed using Tracer v1.5 (Rambaut & Drummond, 2007). For ITS-1, ITS-2 and 5.8 rDNA sequences, the ML phylogenetic tree was calculated in MEGA6 (Tamura et al., 2013), using an evolutionary model GTR + G selected under the AIC with jModelTest 0.1.1 (Posada, 2008). Gaps and missing data were treated as complete deletion. Tree topology was supported with bootstrap values calculated with 2000 replicates.

**Field survey**

Seawater samples for *lcf* detection were collected in the Åland archipelago situated between Finland and Sweden from 9 to 11 of August 2011 (Fig. 1). A 7 km transect through a known bloom site of *A. ostenfeldii* was sampled at 10 locations. On each sampling occasion, between 5 to 10 l of seawater from 0.5 m depth was sequentially filtered through 76- and 25-μm sieves. The 25- to 76- μm fraction was retained and washed into a 50 ml Falcon tube. The concentrated sample was filtered onto a Whatman
GF/F glass fibre filter (Ø 25 mm), transported in liquid nitrogen and stored at -80 °C.

Stimulated bioluminescence was recorded at night using a sensitive submersible light sensor (GlowTracka, Chelsea Technologies Group, West Molesey, UK) that was lowered to a depth of 0.5 - 1.0 m at each location for approximately 2 min. The noise floor of the instrument was $50 \pm 6 \text{ pW cm}^{-2}$. Because of this low noise floor, no threshold was used to define the presence of a bioluminescence signal. Water samples for cell counts were collected simultaneously with lcf samples. A detailed description of study site and procedures is given in Le Tortorec et al. (2014).

For lcf detection, DNA was extracted from filters as follows: Filters were placed into a 2 ml safe lock Eppendorf tube with 0.2 ml glass beads (Ø 0.5 mm, Mobio) added beforehand. 600 µl of 5% Chelex buffer was added and tubes were vortexed at 4 min in a Mobio vortex adapter for Genie2 to disrupt dinoflagellate theca and release the DNA. Tubes were incubated at 99 °C for 20 min and mixed by vortexing every 5 min. Samples were then centrifuged at $4000 \times g$ for 1 min to sediment particles. The supernatant was transferred to a clean Eppendorf tube and centrifuged again at $13000 \times g$ for 1 min. The supernatant containing extracted DNA was transferred to a new Eppendorf tube and refrigerated for 12 – 24 h for DNA to dissolve. DNA samples were stored at -80 °C before PCR reactions were carried out as described above. PCR products from field samples were not sequenced.

**Results**

**Amplification of lcf and bioluminescence production**

*lcf* was uniformly present in all *A. ostenfeldii* strains isolated from the Baltic Sea (Table 1). We were able to detect lcf from all North Sea *A. ostenfeldii* strains, with the exception
of two Irish (LSA06 and LSE05) and two UK strains (WW515 and WW517). *Lcf* was also present in strains from Canada, China and Spain. Bioluminescence production was observed in 60 out of 61 strains from the Baltic Sea. Only AOVA0924 from Gotland (Sweden) did not produce bioluminescence. All North Sea strains from Norway and Scotland produced bioluminescence but no bioluminescence production was observed in strains from Ireland and the UK. Isolates from Canada and China both produced bioluminescence but no detectable bioluminescence was observed from the two Spanish strains (IEO-VGOAMD12 and IEO-VGOAM10C).

**Lcf diversity and phylogenetic structure in A. ostenfeldii**

Phylogenetic analyses of the partial *lcf* alignment, using both BI and ML methods, revealed that *A. ostenfeldii lcf* sequences formed a cluster that was distinct from other *Alexandrium lcf* sequences (BI 1.00, ML 99%). Within *A. ostenfeldii*, two major, well-supported geographic clades were identified (Fig. 2), separating sequences of Baltic and North Sea isolates. Baltic and North Sea isolates differed by 11 - 22 (mean = 16) nucleotides. Within the North Sea clade the BI divided strains into two subgroups (BI 0.63) but these groups were not recognised by ML analysis. The difference between North Sea isolates was 0 - 8 nucleotides (mean = 4), with sequences of NCH85 and S6P12E11 being identical and with strain AONOR4 from Oslo fjord differing most (7 – 8 nucleotides) from others. Strain IEO-VGOAMD12 from the Spanish Mediterranean formed a separate branch between Baltic Sea and North Sea clades, with 14 - 23 nucleotide difference to Baltic Sea isolates and 14 - 20 nucleotide difference to North Sea isolates. However, the position of this strain in phylogeny was weakly resolved, with low bootstrap and posterior probability values (BI 0.65, ML 52%). Baltic Sea strains of *A. ostenfeldii* formed a monophyletic group (BI 0.73, ML 98%). Both BI and ML revealed
one subcluster formed by four strains from Åland area (BI 0.59, ML 63%). Three additional subclusters were revealed by BI (BI 0.97, 0.71, 0.55) but these were not recovered by ML. Generally, nucleotide differences within the Baltic *A. ostenfeldii* strains were minor within and among Baltic bloom populations occurred between strains from the same bloom population (Föglö: 1 - 5 nucleotides, Kökar: 1 - 5, Sandviken: 2 - 9, Gotland: 1 - 6, Kalmar: 1 - 7, Poland: 1 - 6). On average, the within-site nucleotide difference was 3 nucleotides. Between bloom sites, slightly larger differences (1 - 10 nucleotides, mean = 4) were observed. Even though subclusters and nucleotide differences occurred among Baltic strains, they did not reveal any geographic patterns were not evident within the Baltic Sea clade.

**Presence of lcf in natural samples**

Bioluminescence was observed at all 10 locations along the investigated transect in the Föglö archipelago (Fig. 1). Bioluminescence intensities ranged from 65.78 - 1618.97 pW cm⁻². *A. ostenfeldii* cells were detected from 9 locations (Table 2), and lcf was detected in 8 out of 10 locations. The PCR signal was absent from 2 samples (stations 2 and 8), of which station 2 contained a low abundance (120 cells L⁻¹) of *A. ostenfeldii* cells. No *A. ostenfeldii* cells were detected at station 8.

**Discussion**

It is interesting to observe that lcf and ability to produce bioluminescence were uniformly present in the Baltic Sea *A. ostenfeldii*, in contrast to strains from other parts of Europe where these properties did vary. Lcf was detected in all studied strains except four strains
(WW516 and WW517 from UK and LSA06 and LSE05 from Ireland) from which bioluminescence production was also not observed. These four strains belong to a distinct phylogenetic rDNA clade, group 2 of *A. ostenfeldii* (Kremp et al., 2014). The fact that these strains are closely related according to rDNA suggests that they all have either lost the *lcf* or the gene has mutated so that it is not functional anymore. This is supported by the fact that their close relatives, Spanish IEO-VGOAM12 and IEO-VGOAM10C belonging to the same phylogenetic clade, possess the gene, but lack the function. Other studies have found that in some dinoflagellate species, e.g. *C. horrida* and *A. tamarense*, both bioluminescent and non-bioluminescent strains co-occur but also the non-bioluminescent strains always have *lcf* (Valiadi et al., 2012). Similar findings have been reported from *V. cholerae* in which both bioluminescent and non-bioluminescent strains had *lcf* (Palmer & Colwell, 1991).

It is possible to draw parallels with the saxitoxin gene, another functional gene providing a similar defensive mechanism as bioluminescence, but more widely studied in *Alexandrium* species. The intra-species variation in saxitoxin production is similar to what has been observed in bioluminescence production. For example, *Alexandrium minutum* Halim, *A. ostenfeldii* and strains from the *A. tamarense* species complex contain both saxitoxin producing and non-saxitoxin producing strains (Touzet et al., 2007; Orr et al., 2011; Murray et al., 2012; Suikkanen et al., 2013). Several studies show that non-saxitoxin producing strains still have the *sxA* genes necessary for STX production (Stüken et al., 2011; Murray et al., 2012), whereas others have found that non-saxitoxin producing strains lack essential genes of the cluster (Suikkanen et al., 2013). It is worth recalling that the primers used in the present study targeted the most variable region of *lcf*: the N-terminal region and the start of the first domain (Baker et al., 2008). Valiadi *et al.* (2012) showed that primers designed for these variable regions may
not always give a positive signal for lcf, even if it is present, potentially due to too many nucleotide differences at the primer binding sites. Therefore, based on the results observed here it is not possible to say if the lcf is modified or truly absent in the respective strains.

In addition to intra-species variation, the lack of bioluminescence in lcf positive strains might be a result of prolonged cultivation. Loss of bioluminescence in cultures over time has been observed in previous studies (Sweeney, 1986; von Dassow et al., 2005; Valiadi et al., 2012). Like toxin production, bioluminescence is a costly trait (e.g. Latz & Jeong, 1996; Wang et al., 2012) to maintain in monoclonal cultures where it brings no advantage. The Spanish strains IEO-VGOAM12 and IEO-VGOAM10C have been in culture for more than ten years, hence this might indeed be considered as an alternative explanation for lack of light emission in these strains despite presence of lcf.

As the gene has most likely not been expressed in culture where bioluminescence serves no immediate purpose, mutations may have accumulated that inhibit the function. Detection limits might explain some of the negative bioluminescence observations as it has been suggested that detection of the gene without the obvious function (e.g. saxitoxin production) may also be due to detection limits of methods used (e.g. Negri et al., 2003).

Baker et al. (2008) found that a strain of Gonyaulax spinifera (Claparède & Lachmann) Diesing produced bioluminescence at such low levels that it was undetectable to the human eye and only detectable with a sensitive light meter. Some strains of V. cholerae were also found to emit light in low levels undetectable to the human eye (Palmer & Colwell, 1991). However, we considered that the sensitivity of the detection method in the present study, using acid addition in a spectrometer equipped with a sensitive photomultiplier, was sufficient to detect bioluminescence of relatively few individual cells. Therefore, detectability is unlikely to have been an issue here.
The 59 new partial lcf sequences of *A. ostenfeldii* generated here provide a first insight into intraspecific diversity of lcf. So far, lcf sequences have mostly been compared among dinoflagellate species (Baker *et al*., 2008; Valiadi *et al*., 2012). Our study revealed relatively low levels of sequence variation for *A. ostenfeldii*. Sequences were particularly conserved in isolates from the same geographic region, comparable to ITS rDNA. Previously reported values for lcf similarities in dinoflagellates range from 37.3% to 100% (Baker *et al*., 2008), with highest similarities between *L. polyedrum* strains. *Pyrocystis lunula* strains were found to be only 96.4% identical and members of the *Alexandrium* genus had an average sequence identity of 94.4%. In the present study, we observed large similarities between Baltic Sea strains (99.2% sequence identity). Strains from the North Sea also showed little to no variation (99.2% identity, with NCH85 and S6P12E11 sequences being identical). Valiadi *et al.* (2012) found multiple non-identical copies of lcf within some dinoflagellate strains, with variation up to ca. 9% among sequences of an *A. fundyense* strain. Large variation among gene copies is common in dinoflagellates and the degree of variation in lcf is in line with other studies (Tanikawa *et al*., 2004; Kim *et al*., 2011; Valiadi & Iglesias-Rodriguez, 2014). However, polymorphisms among gene copies are often species-specific and have been observed for other genes particularly in *A. fundyense* (Miranda *et al*., 2012). In this study only two sequences were obtained per strain, and copy variation was not addressed. Sequence differences among lcf copies exist in *A. ostenfeldii* strains and they are negligible as indicated by the high sequence similarity and consistent clustering in phylogenetic analyses of the many Baltic isolates sequenced here.

The Baltic Sea *A. ostenfeldii* form a divergent phylogenetic lineage that is clearly separated from North Sea populations, which is in line with previous studies using
other genes (Tahvanainen et al., 2012; Tillmann et al., 2014; Kremp et al., 2014). In fact, the partial lcf phylogeny revealed by our analysis largely reflects the general topology of rDNA phylogenies obtained for A. ostenfeldii (Tahvanainen et al., 2012; Tillmann et al., 2014; Kremp et al., 2014). The phylogenetic groups identified by Kremp et al. (2014) are reproduced by lcf phylogeny shown here with the Baltic Sea isolates representing group 1, the Spanish and North Sea strains representing group 2 and 6 respectively. As in Kremp et al. (2014), the position of clade 2 was poorly resolved (ML 52%, BI 0.65). When compared to an ITS phylogeny of the strains analyzed for lcf (Fig. S1) the grouping and branching of both phylogenies are nearly identical. The main difference is the lack of subclusters inside Baltic Sea and North Sea clades in the ITS tree. Phylogenetic analysis of the ITS alignment revealed only one subgroup formed by four Kalmar strains, but this subgroup was not recovered by partial lcf analyses. It is interesting that a functional gene and ribosomal ITS rDNA phylogenetic analysis produce the same main result at the species level with differences being minor and limited to the lowest hierarchical clusters. A high congruence of lcf and rDNA phylogenies has been shown for dinoflagellates at genus level: Valiadi et al. (2012) found that lcf phylogeny resembles the SSU rDNA phylogeny (Gómez et al., 2010) for example in Alexandrium spp., Ceratium, Ceratocorys horrida Stein and P. reticulatum and Baker et al. (2008) found that lcf phylogeny was similar to 18S rDNA (Scholin et al., 1995) and LSU D1-D2 (Persich et al., 2006) phylogenies in Alexandrium catenella (Whedon and Kofoid) Balech and Alexandrium fundyense Balech. Lcf phylogeny by Valiadi et al. (2012) also closely represents the newly published luciferin binding protein phylogeny (Valiadi & Iglesias-Rodriguez, 2014).

Functional genes are expected to be less conserved and evolve faster than neutral genetic markers as they should be under greater evolutionary pressure and
therefore should give a better resolution for phylogenetic studies at lower taxonomic level. Lcf primers used in this study were chosen because they amplify the region at the beginning of lcf, which is more diverse than central regions and gives longer sequences than more conserved primers (Baker et al., 2008; Valiadi et al., 2012). Therefore, sequences produced by these primers should reveal most of the variation present in lcf. Nevertheless, the evolution rate of the lcf marker did not resolve population-level structures in Baltic Sea A. ostenfeldii but was similar to ITS. Valiadi et al. (2012) suggested that lcf remains largely unmuted, based on their finding that lcf is conserved and still detectable among nonbioluminescent strains of dinoflagellates. This could be due to a number of reasons. One prominent explanation is that the ability to produce bioluminescence is very important to dinoflagellates. It has been reported that dinoflagellates allocate energy to bioluminescence before growth but after the ability to swim (Latz & Jeong, 1996) and therefore there would be only a small evolutionary pressure acting on lcf. An alternative or additional explanation for the observed pattern in the Baltic Sea is the generally low genetic diversity observed in Baltic A. ostenfeldii (Tahvanainen et al., 2012). The Baltic Sea is an ecologically extreme and geologically marginal habitat, with low salinity and large temperature differences between seasons, resulting in low genetic diversity in many Baltic Sea macroorganisms (e.g. Reusch et al., 1999; Nikula & Väinölä, 2003; Olsson et al., 2011, 2012).

We were able to amplify lcf sequences from water samples collected along a transect that passed through a known bloom site of A. ostenfeldii. Presence of lcf, A. ostenfeldii cells and bioluminescence coincided at the majority of locations. At station 8 a weak bioluminescence signal was measured but lcf and A. ostenfeldii cells were not detected. This location is next to a strait that opens to the sea and is thus influenced by currents. Cell migration and currents may both have contributed to the differences in
observations, with bioluminescence measurements carried out at night and water sampling activities during the day. Since only a small volume of water was analysed for *lcf* and *A. ostenfeldii* cells it is possible that small numbers of bioluminescent cells were present *in situ* but not included in the sample. At station 2, bioluminescence and *A. ostenfeldii* cells were found while *lcf* was not detected. This sample was collected outside the main bloom area and only a low number of *A. ostenfeldii* cells (120 cells l\(^{-1}\)) were present. Therefore it is possible that not enough material was collected to detect *lcf*. Alternatively, the possibility of a processing issue (e.g. DNA extraction) cannot be fully excluded, despite repeat analysis, since *lcf* was detected from stations 1, 9 and 10, where cell numbers were in the same order of abundance (40 - 120 cells l\(^{-1}\)). Therefore, sensitivity of *lcf* detection is considered sufficient for detection of non-bloom abundance.

The distribution of *A. ostenfeldii* based on *lcf* and bioluminescence measurements reflects the distribution of *A. ostenfeldii* resting cysts (Hakanen et al., 2012), which defines the occurrence of the species in the area. The distribution of *A. ostenfeldii* based on *lcf* and bioluminescence measurements was found to be slightly wider than based on toxin measurements (Hakanen et al., 2012). This suggests that bioluminescence is a more sensitive indicator for the presence of *A. ostenfeldii* than toxicity. Our study shows good agreement of *lcf* and bioluminescence signals. In a field study conducted in surface waters of the Patagonian Shelf where the presence of *lcf* and bioluminescence signal were compared, Valiadi *et al.* (2014) found that bioluminescence measurements comparatively underestimated the presence of bioluminescent dinoflagellates. This result may have been due to presence of different dinoflagellate species with different bioluminescent properties and co-occurrence of bioluminescent zooplankton. In the coastal Baltic Sea where the bioluminescent plankton community
consists entirely of *A. ostenfeldii*, detecting bioluminescence can be considered as reliable an indicator of the presence of this bioluminescent dinoflagellate as detecting *lcf*.

From the perspective of monitoring toxic *A. ostenfeldii* blooms the consistent presence of *lcf* and co-occurrence of *lcf* with bioluminescence suggests that bioluminescence can be used to reliably monitor the presence of *A. ostenfeldii* in the Baltic Sea. The optical detection of bioluminescence provides an immediate result, however it is limited to observing known bloom sites and to the dark period (Le Tortorec *et al.*, 2014). The complementary use of optical monitoring of bioluminescence and molecular detection of *lcf* could be a promising direction for integrated monitoring of the environmental risks of toxic bloom-forming *A. ostenfeldii* in coastal areas of the Baltic Sea. Other molecular methods, for example species specific DNA-based molecular probe methods, DNA barcoding and microarrays (e.g. McCoy *et al.*, 2013; Taylor *et al.*, 2014; Comtet *et al.*, 2015) also exist and provide an effective tool for cheap and precise monitoring. In the Northern Baltic Sea, monitoring harmful algal blooms based on light microscopy, rDNA sequences or *lcf*, is challenging, because the coastline with its thousands of islands is vast as is the number of shallow bays that favor *A. ostenfeldii* blooms (Hakanen *et al.*, 2012; Le Tortorec *et al.*, 2014). The link between *lcf*, bioluminescence and *A. ostenfeldii* cells demonstrated here points to the potential of bioluminescence observations by eye, e.g. by citizens, to record toxic *A. ostenfeldii* blooms with the spatial coverage required by the geography of the area.

**Acknowledgements**

The authors wish to thank Johan and Helene Franzén for their help and hospitality during field work and Johanna Oja for helping with data collection and culture maintenance.
Tilman Alpermann, Isabel Bravo, Haifeng Gu, Linda Percey and Nicolas Touzet are acknowledged for providing additional cultures for this study. The work was supported by the Academy of Finland (grants number 128833 and 251564 to A.K., 132409 to A.H.L. and S.G.H.S.), Emil Aaltonen foundation (grant to A.H.L), Onni Talas foundation (grant to A.H.L) and Walter and Andrée de Nottbeck foundation (grant to P.T.).

References


and paralytic shellfish toxins in finfish, shellfish and rock crabs in Monterey Bay, California, USA. Marine Biology, 156: 493–504.


luciferases, enzymes with three catalytic domains in a single polypeptide.


phylogenetic resolution of toxic and non-toxic *Alexandrium* strains using a concatenated rDNA approach. *Harmful Algae, 10*: 676–688.


Tamura, K., Stecher, G., Peterson, D., Filipski, A. & Kumar, S. (2013). MEGA6:


Quantitative proteomic analysis of differentially expressed proteins in the toxicity-
lost mutant of *Alexandrium catenella* (Dinophyceae) in the exponential phase.
*Journal of Proteomics*, *75*: 5564–5577.

Widder, E.A. (2010). Bioluminescence in the ocean: origins of biological, chemical, and
Table 1. Results for the presence of the luciferase gene and production of bioluminescence for all *Alexandrium ostenfeldii* strains used in this study. Strains used in phylogenetic analyses are given in bold.

<table>
<thead>
<tr>
<th>Origin</th>
<th>Phylogenetic group (Kremp <em>et al.</em> 2014)</th>
<th>Luciferase gene (detected / number of strains)</th>
<th>Bioluminescence (detected / number of strains)</th>
<th>Strain codes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Föglö, Åland, Finland</td>
<td>1</td>
<td>10/10</td>
<td>10/10</td>
<td>AoF0905, AoF0909, AoF0915, AoF0919, AoF0923, AoF0930, AoF0938, AoF0940, AoF0957, AoTVA4</td>
</tr>
<tr>
<td>Kökar, Åland, Finland</td>
<td>1</td>
<td>11/11</td>
<td>11/11</td>
<td>AOK1006, AOK1007, AOK1009, AOK1013, AOK1014, AOK1020, AOK1028, AOK1032, AOK1037, AOK1038, AOK1045</td>
</tr>
<tr>
<td>Sandviken, Åland, Finland</td>
<td>1</td>
<td>9/9</td>
<td>9/9</td>
<td>AOS1001, AOS1002, AOS1004, AOS1006, AOS1011, AOS1013, AOS1014, AOS1017, AOS1020</td>
</tr>
<tr>
<td>Öresund, Denmark</td>
<td>1</td>
<td>1/1</td>
<td>1/1</td>
<td>K1354</td>
</tr>
<tr>
<td>Hel, Poland</td>
<td>1</td>
<td>10/10</td>
<td>10/10</td>
<td>AoPL0902, AoPL0906, AoPL0909, AoPL0914, AoPL0918, AoPL0924, AoPL0930, AoPL0945, AoPL0961, AoPL0967</td>
</tr>
<tr>
<td>Gotland, Sweden</td>
<td>1</td>
<td>10/10</td>
<td>9/10</td>
<td>Aова0901, Aова0903, Aова0904, Aова0906, Aова0907, Aова0910, Aова0923, Aова0924, Aова0929, Aова0931</td>
</tr>
</tbody>
</table>
| Kalmar, Sweden           | 1                                        | 10/10                                         | 10/10                                         | AOKAL0902, AOKAL0909, }
<table>
<thead>
<tr>
<th>Location</th>
<th>Replicas</th>
<th>Sampling</th>
<th>Isolation</th>
<th>Codes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lough Swilly, Ireland</td>
<td>2</td>
<td>0/2</td>
<td>0/2</td>
<td>LSA06, LSE05</td>
</tr>
<tr>
<td>Fal River, UK</td>
<td>2</td>
<td>0/2</td>
<td>0/2</td>
<td>WW516, WW517</td>
</tr>
<tr>
<td>North Sea, Scotland</td>
<td>6</td>
<td>3/3</td>
<td>3/3</td>
<td>CCAP1119/45, CCAP1119/47, S6P12E11</td>
</tr>
<tr>
<td>North Sea, Norway</td>
<td>6</td>
<td>1/1</td>
<td>1/1</td>
<td>NCH85</td>
</tr>
<tr>
<td>Oslofjord, Norway</td>
<td>6</td>
<td>1/1</td>
<td>1/1</td>
<td>AONOR4</td>
</tr>
<tr>
<td>Bohai Sea, China</td>
<td>1</td>
<td>1/1</td>
<td>1/1</td>
<td>ASBHO1</td>
</tr>
<tr>
<td>Palamos, Spain</td>
<td>2</td>
<td>2/2</td>
<td>0/2</td>
<td>IEO-VGOAMD12, IEO-VGOAM10C</td>
</tr>
<tr>
<td>Saanich, Canada</td>
<td>6</td>
<td>1/1</td>
<td>1/1</td>
<td>AOPC1</td>
</tr>
</tbody>
</table>
Table 2. Results of the transect data for luciferase gene (lcf) presence, *A. ostenfeldii* cell numbers and bioluminescence observations (averaged over a 2-minute observation period). Station numbers correspond to the map of the study area (Fig. 1).

<table>
<thead>
<tr>
<th>Station No.</th>
<th><em>Lcf</em> detected</th>
<th><em>A. ostenfeldii</em> cells</th>
<th>Bioluminescence (pW cm⁻²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Yes</td>
<td>40</td>
<td>83.93</td>
</tr>
<tr>
<td>2</td>
<td>No</td>
<td>120</td>
<td>68.37</td>
</tr>
<tr>
<td>3</td>
<td>Yes</td>
<td>18 289</td>
<td>232.49</td>
</tr>
<tr>
<td>4</td>
<td>Yes</td>
<td>24 705</td>
<td>271.65</td>
</tr>
<tr>
<td>5</td>
<td>Yes</td>
<td>35 890</td>
<td>371.29</td>
</tr>
<tr>
<td>6</td>
<td>Yes</td>
<td>38 089</td>
<td>1618.97</td>
</tr>
<tr>
<td>7</td>
<td>Yes</td>
<td>2 800</td>
<td>164.37</td>
</tr>
<tr>
<td>8</td>
<td>No</td>
<td>-</td>
<td>177.22</td>
</tr>
<tr>
<td>9</td>
<td>Yes</td>
<td>120</td>
<td>65.78</td>
</tr>
<tr>
<td>10</td>
<td>Yes</td>
<td>40</td>
<td>75.35</td>
</tr>
</tbody>
</table>
**Figure legends**

**Fig 1.** (A) The Finnish Archipelago Sea in the northern Baltic Sea showing the location of the Föglö islands in the Åland islands group. (B) Detail of the islands around the study site showing station numbers along the sampled transect.

**Fig 2.** Phylogenetic tree of *Alexandrium ostenfeldii* isolates from the Baltic Sea, the North Sea and Spain based on a nucleotide alignment of a partial sequence of the luciferase gene as derived from Bayesian inference. Node labels correspond to posterior probabilities from Bayesian inference (BI) and bootstrap values from maximum likelihood (ML) analyses shown as ML/BI.

**Fig S1.** Phylogenetic tree of *Alexandrium ostenfeldii* isolates from the Baltic Sea, the North Sea and Spain based on a nucleotide alignment of internal transcribed spacer (ITS-1 and ITS-2) and 5.8 rDNA sequences as derived from Bayesian inference. Node labels correspond to posterior probabilities from Bayesian inference (BI) and bootstrap values from maximum likelihood (ML) analyses shown as ML/BI.
Figure 1.
Figure 2.