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

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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) Bütschli is found in low abundances throughout the open Baltic (Hällfors, 2004; Mertens *et al.*, 2012), while *Lingulodinium polyedrum* (Stein) Dodge is only encountered in the southern parts of the Baltic (Hällfors, 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 ºC 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 *lcf*. We chose these primers because they amplify the region at the beginning of *lcf* which is more diverse than central regions, and give longer sequences than more conservative primers (Baker *et al.*, 2008). PCR reactions were performed in 25 µl reaction volume in PCR beads (Illustra PuReTaq Ready-to-go-PCR-beads, GE Healthcare) consisting of 16 - 22 µl sterile ultrapure water, 2 µl of each primer and 1 to 5 µl of DNA (about 100 ng). PCR reactions were performed following Baker *et al.* (2008) as follows: 5 min at 95 ºC, 35 cycles of 45 s at 95 ºC, 30 s at 62 ºC and 30 s at 68 ºC and a final extension step of 10 min at 68 ºC. Presence of *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 *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 ºC. 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 ± 6 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 ºC for 20 min and mixed by vortexing every 5 min. Samples were then centrifuged at 4000 × *g* for 1 min to sediment particles. The supernatant was transferred to a clean Eppendorf tube and centrifuged again at 13000 × *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 ºC 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 to 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~~ and 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

317 (WW516 and WW517 from UK and LSA06 and LSE05 from Ireland) from which
318 bioluminescence production was also not observed. These four strains belong to a distinct
319 phylogenetic rDNA clade, group 2 of *A. ostenfeldii* (Kremp *et al.*, 2014). The fact that
320 these strains are closely related according to rDNA suggests that they all have either lost
321 the *lcf* or the gene has mutated so that it is not functional anymore. This is supported by
322 the fact that their close relatives, Spanish IEO-VGOAMD12 and IEO-VGOAM10C
323 belonging to the same phylogenetic clade, possess the gene, but lack the function. Other
324 studies have found that in some dinoflagellate species, e.g. *C. horrida* and *A. tamarense*,
325 both bioluminescent and non-bioluminescent strains co-occur but also the non-
326 bioluminescent strains always have *lcf* (Valiadi *et al.*, 2012). Similar findings have been
327 reported from *V. cholerae* in which both bioluminescent and non-bioluminescent strains
328 had *lcf* (Palmer & Colwell, 1991).
329 It is possible to draw parallels with the saxitoxin gene, another functional
330 gene providing a similar defensive mechanism as bioluminescence, but more widely
331 studied in *Alexandrium* species. The intra-species variation in saxitoxin production is
332 similar to what has been observed in bioluminescence production. For example,
333 *Alexandrium minutum* Halim, *A. ostenfeldii* and strains from the *A. tamarense* species
334 complex contain both saxitoxin producing and non-saxitoxin producing strains (Touzet *et*
335 *al.*, 2007; Orr *et al.*, 2011; Murray *et al.*, 2012; Suikkanen *et al.*, 2013). Several studies
336 show that non-saxitoxin producing strains still have the *sxtA* genes necessary for STX
337 production (Stüken *et al.*, 2011; Murray *et al.*, 2012), whereas others have found that
338 non-saxitoxin producing strains lack essential genes of the cluster (Suikkanen *et al.*,
339 2013). It is worth recalling that the primers used in the present study targeted the most
340 variable region of *lcf*: the N-terminal region and the start of the first domain (Baker *et al.*,
341 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-VGOAMD12 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

391 other genes (Tahvanainen *et al.*, 2012; Tillmann *et al.*, 2014; Kremp *et al.*, 2014). In fact,
392 the partial *lcf* phylogeny revealed by our analysis largely reflects the general topology of
393 rDNA phylogenies obtained for *A. ostenfeldii* (Tahvanainen *et al.*, 2012; Tillmann *et al.*,
394 2014; Kremp *et al.*, 2014). The phylogenetic groups identified by Kremp *et al.* (2014) are
395 reproduced by *lcf* phylogeny shown here with the Baltic Sea isolates representing group
396 1, the Spanish and North Sea strains representing group 2 and 6 respectively. As in
397 Kremp *et al.* (2014), the position of clade 2 was poorly resolved (ML 52%, BI 0.65).
398 When compared to an ITS phylogeny of the strains analyzed for *lcf* (Fig. S1) the
399 grouping and branching of both phylogenies are nearly identical. The main difference is
400 the lack of subclusters inside Baltic Sea and North Sea clades in the ITS tree.
401 Phylogenetic analysis of the ITS alignment revealed only one subgroup formed by four
402 Kalmar strains, but this subgroup was not recovered by partial *lcf* analyses. It is
403 interesting that a functional gene and ribosomal ITS rDNA phylogenetic analysis produce
404 the same main result at the species level with differences being minor and limited to the
405 lowest hierarchical clusters. A high congruence of *lcf* and rDNA phylogenies has been
406 shown for dinoflagellates at genus level: Valiadi *et al.* (2012) found that *lcf* phylogeny
407 resembles the SSU rDNA phylogeny (Gómez *et al.*, 2010) for example in *Alexandrium*
408 spp., *Ceratium, Ceratocorys horrida* Stein and *P. reticulatum* and Baker *et al.* (2008)
409 found that *lcf* phylogeny was similar to 18S rDNA (Scholin *et al.*, 1995) and LSU D1-D2
410 (Persich *et al.*, 2006) phylogenies in *Alexandrium catenella* (Whedon and Kofoid) Balech
411 and *Alexandrium fundyense* Balech. *Lcf* phylogeny by Valiadi *et al.* (2012) also closely
412 represents the newly published luciferin binding protein phylogeny (Valiadi & Iglesias-
413 Rodriguez, 2014).

414 Functional genes are expected to be less conserved and evolve faster than
415 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.

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Tables

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.

Origin		Phylogenetic group (Kremp *et al.* 2014)	Luciferase gene (detected / number of strains)	Bioluminescence (detected / number of strains)	Strain codes
Baltic Sea	Föglö, Åland, Finland	1	10/10	10/10	**AOF0905, AOF0909, AOF0915, AOF0919,** AOF0923**, AOF0930, AOF0938,** AOF0940**, AOF0957, AOTVA4**
	Kökar, Åland, Finland	1	11/11	11/11	**AOK1006, AOK1007, AOK1009, AOK1013, AOK1014, AOK1020, AOK1028, AOK1032, AOK1037,** AOK1038**, AOK1045**
	Sandviken, Åland, Finland	1	9/9	9/9	**AOS1001, AOS1002, AOS1004, AOS1006,** AOS1011**, AOS1013, AOS1014,** AOS1017**, AOS1020**
	Öresund, Denmark	1	1/1	1/1	**K1354**
	Hel, Poland	1	10/10	10/10	**AOPL0902, AOPL0906, AOPL0909, AOPL0914, AOPL0918, AOPL0924, AOPL0930, AOPL0945, AOPL0961, AOPL0967**
	Gotland, Sweden	1	10/10	9/10	**AOVA0901, AOVA0903, AOVA0904, AOVA0906,** AOVA0907**, AOVA0910, AOVA0923, AOVA0924, AOVA0929, AOVA0931**
	Kalmar, Sweden	1	10/10	10/10	**AOKAL0902, AOKAL0909,**

					AOKAL0913, AOKAL 0916, AOKAL0918**, AOKAL0919, AOKAL0923,** AOKAL0925**, AOKAL0927, AOKAL0928**
North Sea	Lough Swilly, Ireland	2	0/2	0/2	LSA06, LSE05
	Fal River, UK	2	0/2	0/2	WW516, WW517
	North Sea, Scotland	6	3/3	3/3	**CCAP1119/45, CCAP1119/47, S6P12E11**
	North Sea, Norway	6	1/1	1/1	**NCH85**
	Oslofjord, Norway	6	1/1	1/1	**AONOR4**
Other	Bohai Sea, China	1	1/1	1/1	ASBHO1
	Palamos, Spain	2	2/2	0/2	**IEO-VGOAMD12,** IEO-VGOAM10C
	Saanich, Canada	6	1/1	1/1	AOPC1

745

746 **Table 2.** Results of the transect data for luciferase gene (*lcf*) presence, *A. ostenfeldii* cell
747 numbers and bioluminescence observations (averaged over a 2-minute observation
748 period). Station numbers correspond to the map of the study area (Fig. 1)
749

Station No.	***Lcf* detected**	***A. ostenfeldii* cells l $^{-1}$**	**Bioluminescence (pW cm^{-2})**
1	Yes	40	83.93
2	No	120	68.37
3	Yes	18 289	232.49
4	Yes	24 705	271.65
5	Yes	35 890	371.29
6	Yes	38 089	1618.97
7	Yes	2 800	164.37
8	No	-	177.22
9	Yes	120	65.78
10	Yes	40	75.35

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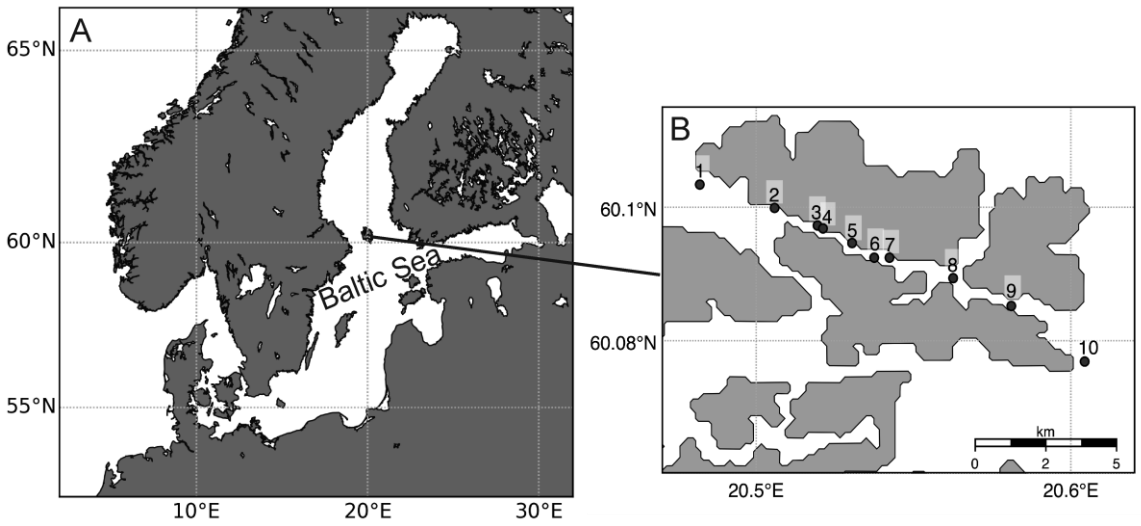
Figure legends

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.

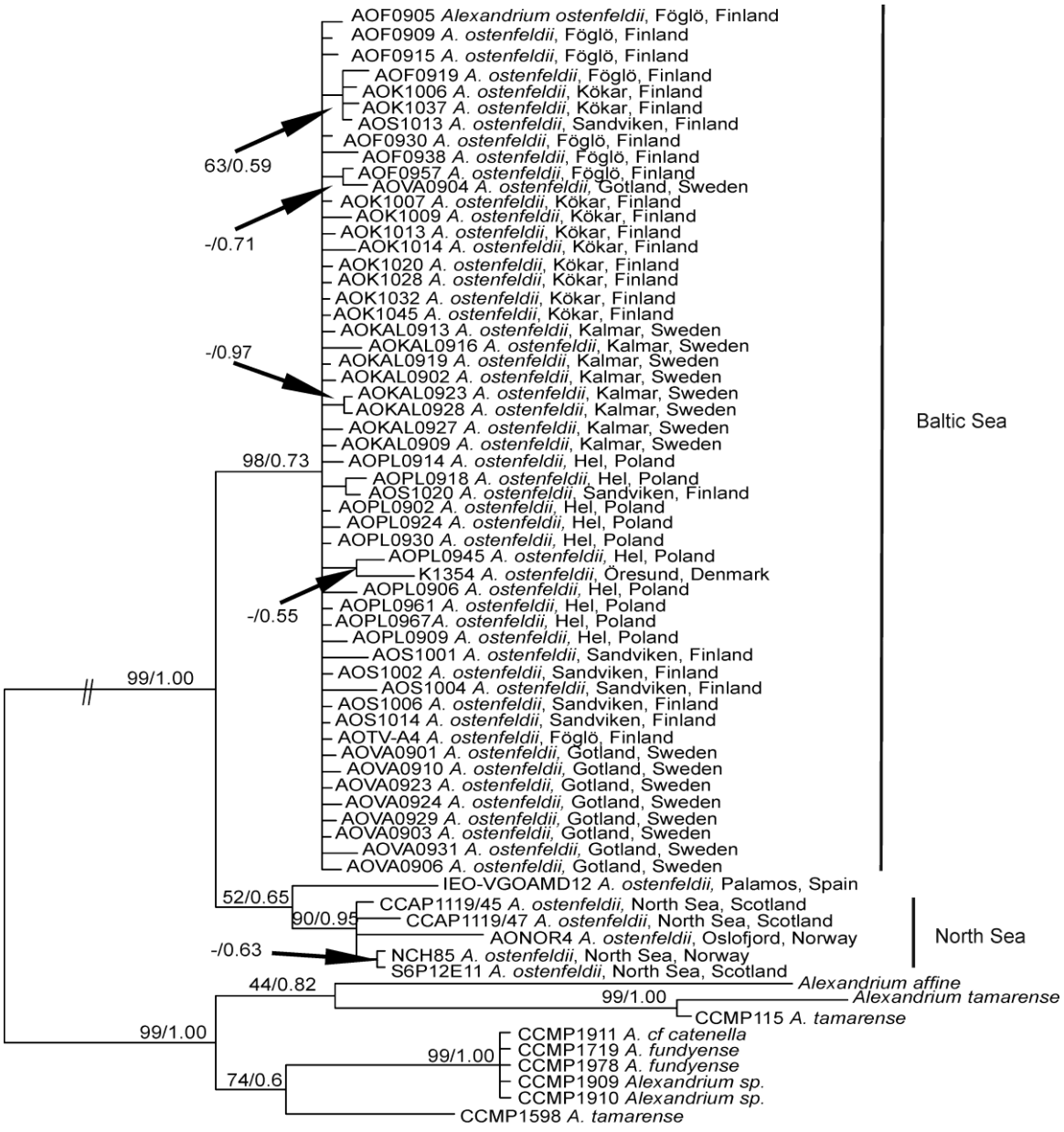
769 **Figures**



770

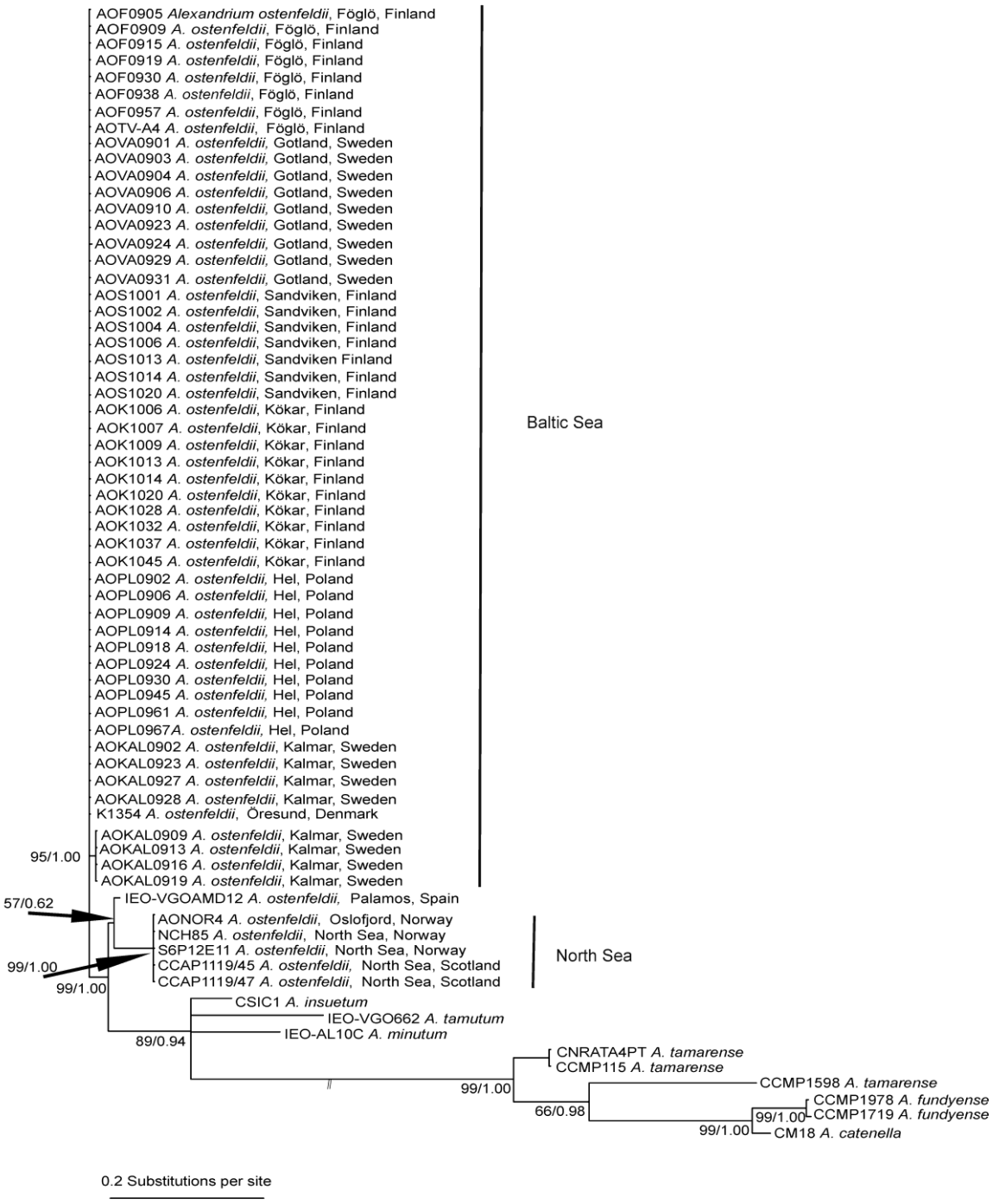
771 Figure 1.

772



773

774 Figure 2.



775

776 Figure S1.