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Genetic relatedness of Japanese isolates of *Alexandrium ostenfeldii* with global isolates

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Abstract

In recent years, blooms of toxic *Alexandrium ostenfeldii* have been reported from around the world. In 2013, the species formed a red tide in a shallow lagoon in western Japan, which was the first report of the species in the area. To investigate the genetic relatedness of Japanese *A. ostenfeldii* and global isolates, the full-length SSU, ITS and LSU sequences were determined and phylogenetic analyses were conducted for isolates from western and northern Japan and from the Baltic Sea. Genotyping and microsatellite sequence comparison were performed to estimate the divergence and connectivity between the populations from western Japan and the Baltic Sea. In all phylogenetic analyses, the isolates from western Japan grouped together with global isolates from shallow and low saline areas, such as the Baltic Sea, estuaries on the east coast of U.S.A. and from the Bohai Sea, China. The isolates from northern Japan formed a well-supported separate group in the ITS and LSU analyses, indicating differentiation between the Japanese populations. This was further supported by the notable differentiation between the sequences of western and northern Japanese isolates, whereas the lowest differentiation was found between the western Japanese and Chinese isolates. Microsatellite genotyping revealed low genetic diversity in the western Japanese

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population, possibly explained by a recent colonization of the lagoon from where it was detected. The red tide recorded from the shallow lagoon followed notable changes in the salinity of the waterbody and phytoplankton composition, potentially facilitating the bloom of *A. ostenfeldii*.

Keywords

Alexandrium ostenfeldii; western Japan; northern Japan; Baltic Sea; phylogenetic analysis; genotyping

1. Introduction

About half of the species belonging to the genus *Alexandrium* are recognized as harmful either due to toxin production or mass occurrences related to fish mortality (Anderson et al., 2012). Blooms of the toxic *Alexandrium* species can have a negative influence on higher trophic levels directly (Sopanen et al., 2011) or through toxin accumulation and transfer in the food web (e.g. Jester et al., 2009; Setälä et al., 2014). *Alexandrium ostenfeldii* (Paulsen) Balech and Tangen, 1985, is a globally distributed marine planktonic dinoflagellate (e.g. Mackenzie et al., 1996; John et al., 2003; Gribble et al., 2005; Kaga et al., 2006; Kremp et al., 2009; Almandoz et al., 2014; Tillmann et al., 2014), which produces three different types of neurotoxins: paralytic shellfish toxins, spirolides and gymnodimines (e.g. Cembella et al., 2000; Van de Waal et al., 2015; Harju et al., 2016). The type of toxin produced differs between the strains and their geographic origin (Lim and Ogata, 2005; Kremp et al., 2009; Otero et al., 2010; Touzet et al., 2011; Salgado et al., 2015). Although salinity varies between and within those localities, it does not change the toxin profile, which might be genetically determined (Suikkanen et al., 2013). At the same time, salinity influences the growth rate and cellular toxin concentrations of *A. ostenfeldii* (Lim and Ogata, 2005; Suikkanen et al., 2013; Martens et al., 2016). Elevated temperature and pCO₂ levels also have an effect on *A. ostenfeldii* toxin composition and growth rate, which may lead to increased toxic bloom events in the future (Kremp et al., 2012).

Until recently *A. ostenfeldii* was considered to be a background species present in low abundances (e.g. Hansen & Moestrup, 1992; John et al., 2003; Gribble et al., 2005), however, dense blooms have been reported from the coastal areas of Finland (Kremp et al., 2009), the Netherlands (Burson et al., 2014), Italy (Ciminiello et al., 2006), and the U.S.A. (Borkman et al., 2012). In the northern Baltic Sea and in a brackish creek in southwestern Netherlands, the blooms of *A. ostenfeldii* have become a reoccurring phenomenon explained by high sea surface temperatures (>15°C), low DIN:DIP ratios, and grazer densities and high cyst abundances (Hakanen et al., 2012; Brandenburg et al., 2017). In Japan, *A. ostenfeldii* (approx. 3×10^5 cells L⁻¹) first bloomed together with *Levanderina fissa* (approx. 1.246×10^6 cells L⁻¹) in a shallow semi-enclosed lagoon in autumn 2013. The strains isolated during the bloom produced paralytic shellfish toxins (H. Oikawa unpubl. data). This was also the first record of the species in western Japan, as *A. ostenfeldii* has been previously only been present in low abundances (10 - 320 cells L⁻¹) along the Pacific coast of Japan and in the Seto Inland Sea (Kaga et al., 2006; Yuki and Yoshimatsu, 2012).

Alexandrium ostenfeldii produces resting cysts as a part of its life cycle (Mackenzie et al., 1996). These can accumulate in the sediments and provide an inoculum for blooms (e.g. Genovesi et al., 2009; Anderson et al., 2014; Mardones et al., 2016), while also facilitating species dispersal via ships' ballast water (Hallegraeff and Bolch, 1991) and/or waterfowl (Tesson et al., 2018). However, before blooming, the non-native species have to pass through several stages to achieve colonization and community maintenance. Thus a sudden bloom might not reflect a recent introduction but rather a change in the environment favoring particular species (Smayda, 2007). This is exemplified by a bloom of *Gymnodinium catenatum* in New Zealand after a La Nina event, which was also the first record of the species in that region, although based on resting cysts, it was already present two decades earlier (Irwin et al., 2003).

In previous phylogenetic studies utilizing various rDNA regions, global *A. ostenfeldii* isolates clustered into six groups representing differences in plate morphology, toxin composition, ecophysiology and geographic distribution (Kremp et al., 2014). The analyses also included strains from northern Japan that grouped separately from other *A. ostenfeldii* isolates (Kremp et al., 2014; Tillmann et al., 2014) and were thus suggested to represent a distinct East Asian genotype (Kremp et al., 2014). This differentiation has been explained by the geographic isolation as the similar pattern was also seen for the isolates from New Zealand (Kremp et al., 2014). At the same time, another *A. ostenfeldii* strain from the Asian region, the Bohai Sea, China, grouped close to the isolates from shallow and mesohaline coastal environments, suggesting a closer relationship with global isolates than with the northern Japanese *A. ostenfeldii* (Gu, 2011; Kremp et al., 2014).

In this study, full-length sequences of the small subunit (SSU), internal transcribed spacer (ITS) and large subunit (LSU) ribosomal DNA regions were obtained from strains originating from western and northern Japan and from the Baltic Sea. The sequences were used for phylogenetic analyses together with *A. ostenfeldii* sequences available in GenBank to further investigate the phylogenetic relationship between Japanese and global *A. ostenfeldii* isolates. In addition, strains from western Japan and from the Baltic Sea were genotyped and the flanking regions and the number of microsatellite repeats compared between both localities to gain further insight to the connectivity between the *A. ostenfeldii* populations from western Japan and the Baltic Sea.

2. Methods

2.1 Study areas and establishment of cultures

Strains from western Japan were established from vegetative cells isolated during a bloom in 2013 from the Lake Koyama-ike, Tottori Prefecture (35° 29' 54.7" N, 134° 09' 20.2" E, Fig. 1) with a surface area of 7 km² and an average depth of 2.8 m (Nomura et al., 2009). During the time of isolation, the salinity was 11.44, temperature 26.23 °C and Chl *a* 156.78 µg/L. The cells from northern Japan originate from the Funka Bay, Hokkaido (42° 22' 24.4" N, 140° 35' 19.1" E), that has an average depth of 59 m (Takahashi et al., 2005) and where *A. ostenfeldii* usually appears during spring, when the average surface salinity is around 33 (Kudo and Matsunaga, 1999). Despite several attempts, it was not possible to establish cultures and thus single cells were used for further study. The single cells from

northern Japan were collected during 2008 and 2009 and sequences from the majority of the isolates have been previously used in a report by Nagai, et al. (2010). The strains from the Baltic Sea originate from Föglö archipelago, Åland, Finland (60° 05' 45.8" N, 20° 31' 28.4" E) from a shallow sound (< 3 m) with summer sea surface salinities around 6-7. The sampling site and establishment of cultures are described in more detail by Kremp et al. (2009) and Hakanen, et al. (2012).

2.2 DNA extraction and number of strains used

DNA was extracted from cultures grown in a 25mL F2 medium (Guillard, 1975) in the exponential phase (strains from Finland and western Japan) and from single cells (northern Japan). The extraction was done by using 5% Chelex buffer as described by Nagai, et al. (2012). Eight strains from western Japan, eight single cells from northern Japan and four strains from the Baltic Sea were used to investigate their phylogenetic position in comparison with the global isolates. For genotyping and sequence comparison additional 29 strains from western Japan and 41 strains from the Baltic Sea were used.

2.3 PCR amplification and sequencing for phylogenetic analyses

To determine the full-length SSU, ITS, and LSU rDNA sequences, forward and reverse primers developed by Adachi et al. (1994), Takano and Horiguchi (2004) Ki and Han (2007) were used as well as primers targeting the end of LSU developed by Nagai et al. (2010). PCR amplification was performed on 20 µL reaction volume consisting of 2 µL of PCR buffer, 2 µL of dNTP, 1.2 µL of MgSO₄, 0.4 µL of each primer (50 µM), 0.4 µL of enzyme (KOD-Plus-Ver. 2), 12.1 µL of sterile MQ water and 1.5 µL of DNA. The PCR amplification conditions consisted of one denaturation step for 3 min at 94°C, followed by 30 cycles of 15 sec at 94°C, 30 sec at 55°C and 40 sec at 68°C. The amplified DNA was directly sequenced in both directions using the BigDye™ Terminator v.3.1 Cycle Sequencing Kit and an ABI3730xl DNA Analyzer (Applied Biosystems). The sequences were edited manually by using MEGA version X (Kumar et al., 2018) and are available from Genbank (Table S1).

2.4 Microsatellite genotyping and genetic variation

The primer pairs and the PCR conditions used are described by Nagai et al. (2015). All PCR products were electrophoresed on an ABI 3730xl DNA Analyzer (Applied Biosystems). Allele sizes were determined using a 600 LIZ size standard (Applied Biosystems) and GeneMapper ver. 4.0 (ABI). MS tools (Park, 2001) was used to estimate the number of alleles, allelic frequency, and gene diversity (Nei, 1987). Allele numbers at the 10 loci ranged from 2 to 12 with an average of 5.3, and estimates of gene diversity (Nei, 1987) varied between 0.10 and 0.92, suggesting that these microsatellites have a good potential to characterize genetic structure of *A. ostentfeldii* at the population level.

2.5 Sequence comparison of microsatellite regions

Two microsatellite loci (*Aosten13* and *Aosten296*, Nagai et al., 2015) were amplified following the PCR conditions given by Nagai et al. (2015). Amplified fragments were cloned into the pGEM-T Easy Vector Systems (Promega) and transformed into *Escherichia coli* following the manufacturer's protocol (Promega, 2010). Microsatellite sequences were

determined using the BigDye™ Terminator v.3.1 Cycle Sequencing Kit and an ABI3730xl DNA Analyzer (Applied Biosystems). The sequences were edited manually in MEGA version X (Kumar et al., 2018).

2.6 Phylogenetic tree constructions

The sequences from the SSU region were aligned with 26 *A. ostenfeldii* and 13 sequences from different *Alexandrium* species downloaded from the GenBank (Table S1). For the ITS alignment, 22 *A. ostenfeldii* and 10 other *Alexandrium* species sequences were added (Table S1). For the LSU sequences, two different alignments were made to include as many sequences from different geographic locations as possible (shorter alignment) and to explore phylogenetic relationships by using the full-length LSU sequences (longer alignment). For the shorter LSU alignment, 36 *A. ostenfeldii* sequences and 10 other *Alexandrium* species sequences were downloaded from the GenBank (Table S1). For the full-length alignment, 4 *A. ostenfeldii* and 10 other *Alexandrium* species sequences from the GenBank were added (Table S1). The total length of the alignments used for phylogenetic analysis was 1680 base pairs (bp) (SSU), 546 bp (ITS), 599 bp (LSU short alignment) and 3474 bp (LSU full-length alignment) including insertions/deletions. Phylogenetic trees were constructed by using maximum likelihood analysis in MEGA version X (Kumar et al., 2018). The models used for the analysis were as follows: TN93 + G (SSU, LSU both alignments) and HKY + I (ITS). The models for the SSU and LSU were selected based on the lowest Bayesian Information Criterion scores, whereas for the ITS the models with lowest scores did not show a reasonable phylogeny as *A. ostenfeldii* strains from northern Japan were placed on a higher phylogenetic level than other *Alexandrium* species and other *A. ostenfeldii* strains. Thus, several models were tested and the first model producing a more conventional phylogeny was selected. All sites were used for analyses. Tree topologies were supported by bootstrap values calculated with 100 replicates.

2.7 Modelling the origin of northern Japanese isolates

A high resolution (1/50 degree) ocean model covering all coastal regions around Hokkaido, northern Japan, (Kuroda et al., 2014) was used to investigate origin of *A. ostenfeldii* isolates in the spring 2008 and 2009 as the species was detected from the Funka Bay during those years (K. Baba, personal comm.). To model the potential origin of the vegetative cells a backward particle-tracking model configured for Regional Ocean Modelling System (Shchepetkin and McWilliams, 2003; 2005) was used. During 2008 and 2009, *A. ostenfeldii* cells were detected from the 5 and 30 m depth, and thus 5 m, 10 m, 20 m and 30 m depths were used for the backward particle-tracking simulations. The simulation period was 60 days, covering the period from the middle of January to middle of March.

3. Results

3.1 Phylogenetic analyses

The strains from the Baltic Sea and western Japan had identical SSU and ITS sequences within each geographic location. In the LSU sequences, there was one bp difference between the strains from the Baltic Sea and western Japan. The strains from northern Japan displayed 3 bp differences in the SSU sequences, no differences between the ITS, and 5 bp differences

in the LSU sequences. There was a 2 bp difference for the SSU, no differences for the ITS and a 3 bp difference for the LSU region between the northern Japanese strains from different years. Comparison between the sequences of different geographical origin showed the presence of a low number of insertions/deletions in the ITS sequences, but no insertions/deletions were found in the SSU or LSU sequences (Table 1). The number of substitutions was the lowest in the SSU sequences and highest in the ITS sequences. The highest number of substitutions in all amplified rDNA regions was found between the sequences from western and northern Japan, whereas the lowest number of substitutions was present between the sequences from western Japan and from the Bohai Sea, China (Table 1).

For comparisons among strains originating from the Baltic Sea and western Japan, no identical SSU sequences were found using GenBank data, and the sequence similarity was 99 % with the most similar sequence (1794/1799 bp for all the strains, *A. ostentfeldii*, AB538439, Funka Bay, northern Japan). No identical SSU sequences were found for the strains from northern Japan, and the highest similarity was 99 % (1666/1669 bp for AOFUN0801 and 1667/1669 for AOFUN0802 and AOFUN803) with *A. ostentfeldii* strain KJ362003 from Palamos, Spain. For ITS, the Baltic Sea sequences matched 100% with several other *A. ostentfeldii* sequences from the Baltic Sea, e.g. 519/519 bp, KY783632, Gotland, Sweden. For the western Japanese strains, no identical ITS sequences were found and the similarity with the most similar sequence was 99% (517/519 bp, *A. ostentfeldii*, JN173268, Bohai Sea, China). No identical ITS sequences were found for strains from northern Japan, and the highest similarity was 96% with several strains previously identified as *A. peruvianum* (now considered as *A. ostentfeldii*), e.g. 497/519 bp for all the strains, JX841267, Spain.

For the full-length LSU, the most similar sequence in GenBank to both Baltic Sea sequences (99 %, 3955/4004 bp for BS405 and 3956/4004 bp for BS424) was *A. ostentfeldii* sequence from the Funka Bay, northern Japan (AB538439). This was also the most similar LSU sequence for all the sequences from western Japan (99%, 3958/4003 bp). For strains from northern Japan, the most similar sequence was the *A. ostentfeldii* sequence JF521637 from Flodevigen Norway (99%, 3136/3256 bp for AOFUN0801, 3180/3219 bp for AOFUN0901, 3179/3219 bp for AOFUN0902, 3180/3219 bp for AOFUN0903).

In the phylogenetic tree based on SSU sequences, the two strains from the Baltic Sea and four strains from western Japan grouped together with *A. ostentfeldii* strains from different locations in the Baltic Sea, one strain from Palamos, Spain (Mediterranean Sea) and a strain from Bohai Sea, China (Fig. 2). The strains from northern Japan, Hokkaido, formed a separate group from the other *A. ostentfeldii* strains, however this was not supported by a bootstrap value. In a phylogenetic tree based on the ITS sequences, two strains from the Baltic Sea grouped together with the other *A. ostentfeldii* strains from the Baltic Sea, one strain from the Netherlands and one strain from the U.S.A. (Fig. 3). This group also included two strains of *A. peruvianum* (now considered as *A. ostentfeldii*) from the U.S.A. and the clustering was supported by a high bootstrap value (100 %). The *A. ostentfeldii* strains from western Japan formed a separate group, which was close to the *A. ostentfeldii* strain from the Bohai Sea, China. The strains from northern Japan, Hokkaido, formed a separate group from all the other strains, which was also supported by a high bootstrap value (100 %).

In a phylogenetic tree based on the LSU short alignment (Fig. 4), the strains from the Baltic Sea grouped together with the *A. ostenfeldii* strains from the Baltic Sea, the Netherlands and the New River, North Carolina, U.S.A. The same group also included a strain of *A. peruvianum* (now considered as *A. ostenfeldii*) from the New River, North Carolina, U.S.A. The *A. ostenfeldii* strains from western Japan formed a separate group under a lower node and the *A. ostenfeldii* strain from the Bohai Sea, China grouped close to the strains from western Japan. The *A. ostenfeldii* strains from northern Japan, Hokkaido, grouped separately from the other *A. ostenfeldii* strains, supported by a high bootstrap value (99 %). In the phylogenetic tree based on the LSU full-length alignment (Fig. 5), the strains from the Baltic Sea grouped together with another *A. ostenfeldii* strain from the Baltic Sea and the strains from western Japan formed a separate group, which was not supported by a bootstrap value. The strains from northern Japan formed a separate group supported by a high bootstrap value (93 %). The strains within this group also showed differentiation, which was supported by bootstrap values (66%).

3.2 Genotyping by microsatellites and sequence variation in flanking regions

From ten microsatellite loci, two loci (*Aosten13* and *Aosten 296*) were successfully amplified for strains from both locations. Both loci were polymorphic in the Baltic Sea strains, whereas in the strains from western Japan, only the locus *Aosten13* was polymorphic. The number of alleles varied from 1 - 6 (Fig. 6) with the average number of alleles for western Japanese strains 1.5 ± 0.71 (S.D.) ($n = 29$) and for the strains from the Baltic Sea the average was 4 with S.D. of 2.83 ($n = 29$). The Nei's unbiased genetic diversity (Nei, 1987) was 0.074 for the western Japanese strains (*Aosten13*) and 0.792 (*Aosten13*) and 0.520 (*Aosten296*) for the Baltic Sea strains.

In locus *Aosten13*, the 5' flanking region was identical between the strains from the Baltic Sea and the published microsatellite sequence (AB971375) (Fig. 7). At the same time, sequences of western Japanese strains showed on average 6 substitutions and 2 insertions/deletions in this region compared to strains from the Baltic Sea and the published sequence. The number of microsatellite repeats was lower (3+15 CT repeats) for one of the strains and higher for another strain from the Baltic Sea (3+18) compared to the number of repeats in the published sequence (3+17). The sequences of western Japanese isolates had on average 7 CT repeats and contained up to 17 substitutions and 4 insertions/deletions in the microsatellite region, whereas the sequences from the Baltic Sea did not have any substitutions, although insertions/deletions were present. In the 3' flanking region, the strains from the Baltic Sea had 2 substitutions, whereas in western Japanese sequences the number of substitutions varied between 5 and 6. No insertions/deletions were present in the 3' flanking region for all the strains.

In *Aosten296*, the 5' flanking region contained a number of substitutions between the published sequence and sequences from the Baltic Sea and western Japan as well as within the sequences from the Baltic Sea and western Japan (Fig. 8). In general, two main sequence types were present both within the Baltic Sea and western Japanese sequences. Insertions/deletions were present in 6 bases of the sequences, 4 of them in single sequences. In the microsatellite region, 4 substitutions and 13 insertions/deletions were found. The two

sequences from western Japan contained 6 and 8 CG repeats and in the Baltic Sea sequences, the number of repeats ranged from 5 to 8, whereas the published sequence contained 12 CG repeats. In the 3' flanking region, 2 substitutions were present in sequences from both locations and one substitution was found only from a single sequence from the Baltic Sea (BS4 42). Only 2 insertions/deletions were present in this region.

3.3. Modelled origin of the northern Japanese isolates

In 2008, hydrodynamic particle tracking modeling showed that the majority of the particles observed in Funka Bay would have been transported there along the north-east coast of Hokkaido, originating from the Sea of Okhotsk or further north along the Pacific coast of the Kurili Islands (Fig. 9). In 2009, most of the particles also originated from the north, transported along the north-eastern coast of Hokkaido (Fig. 9), whereas some particles also originated from the Sea of Japan.

4. Discussion

In this phylogenetic analyses of different rDNA regions, *A. ostenfeldii* strains from the Baltic Sea and western Japan mainly clustered with the strains originating from shallow and productive coastal areas with low salinity (group 1) (Kremp et al., 2014 and references therein), as both the Baltic Sea and western Japanese strains originate from similar conditions. Interestingly, the establishment of cultures was also easier for the isolates from western Japan and the Baltic Sea, whereas culturing was unsuccessful (after several trials) for isolates from northern Japan that grouped separately from *A. ostenfeldii* from other locations as in previous studies (Kremp et al., 2014; Tillmann et al., 2014). High uncorrected P-value ranges based on the ITS sequences have been reported between the isolates from northern Japan and from other locations (> 0.04) (Kremp et al., 2014). The value above 0.04 has been considered to indicate a species-level divergence in some dinoflagellates (Litaker et al., 2007), however, no indication for reproductive isolation has been found between the phylogenetic groups of *A. ostenfeldii*, including the group representing the northern Japanese isolates (Kremp et al., 2014). Thus, the separate grouping was suggested to indicate geographical isolation (Kremp et al., 2014), which is supported by the average surface current patterns between the sampling locations (Rosa et al., 2009; Ito et al., 2014). The model-simulated transport for two spring seasons showed a northern origin for the majority of particles transported to Funka Bay, supporting the geographic isolation hypothesis. In addition, the environmental conditions at the northern and western Japan sampling locations are very different, with average salinity around 33 (Kudo and Matsunaga, 1999) and temperature $< 3^{\circ}\text{C}$ to 5°C in Funka Bay during spring, and average salinity of 14.45 (Okamoto and Miyamoto, 2016) and temperature $1 - 30^{\circ}\text{C}$ in the Lake Koyama-ike during summer-autumn, potentially indicating different environmental preferences.

Genotyping was successful only in two out of ten microsatellite loci in western Japanese samples due to lack of PCR amplification, suggesting a mutation in the primer regions. The low average number of alleles and low genetic diversity of the western Japanese strains compared to the Baltic Sea strains suggests a recent introduction of *A. ostenfeldii* to western Japan. The comparison of microsatellite flanking regions between western Japanese and the

Baltic Sea revealed a number of differences in the sequence,s and the microsatellite repeat numbers also varied, indicating high divergence and low connectivity between the populations. Interestingly, the potential introduction of *A. ostenfeldii* from the Baltic Sea to China has been discussed due to the close grouping of the Chinese strain with the strains from the Baltic Sea and the first occurrence in China in an international port located in an estuary (Gu, 2011). In phylogenetic trees based on the ITS and LSU short alignments, western Japanese strains clustered closely together with the *A. ostenfeldii* non-toxic strain from the Bohai Sea, China (Gu, 2011). In the SSU phylogeny, the Chinese strain was placed within the same group with strains from the Baltic Sea and western Japan. Thus, the close grouping of western Japanese strains and the strain from the Bohai Sea might indicate a potential introduction of *A. ostenfeldii* to western Japan from China.

However, as *A. ostenfeldii* has only been reported once from the Chinese coastal water based on a single cyst, this species might not be very abundant and thus the natural dispersal to western Japan over approximately 2000 km, following the annual mean surface current patterns (Lie et al., 1998; Ichikawa and Beardsley, 2002), may not be plausible. At the same time, anthropogenic introduction of *A. ostenfeldii* from China to western Japan cannot be excluded as the Chinese *A. ostenfeldii* strain originates from near the Port of Tianjin, which has a cargo exchange with the Port of Busan, South Korea, (Busan Port Authority, 2016) and gene flow between the *Margalefidinium polykrikoides* (previously *Cochlodinium polykrikoides*) populations from the coast of South Korean and western coast of the Sea of Japan has been reported (Nagai et al., 2009). Furthermore, the Port of Busan has also a cargo exchange with the Port of Kitakyushu, western Japan, (Kitakyushu Seaport and Airport Bureau, 2017) and thus the anthropogenic introduction of *A. ostenfeldii* to the west coast of Japan might be possible. Further transport along the coast might be facilitated by surface currents (Lee and Niiler, 2005; Onitsuka, et al. 2010) or human activities, such as transport of shellfish, as is potentially the case for *A. tamarense*, North American clade (now *A. catenella*) (Scholin et al., 1995), from the western and eastern coasts of Japan (Nagai et al., 2007; Matsuyama et al., 2008).

The first red tide of *A. ostenfeldii* appeared in te Lake Koyama-ike, Tottori Prefecture in October 2013 after the opening of floodgates in March 2012. As this was the first report of *A. ostenfeldii* in the region, it is not known if the bloom was based on local seed population or instead reflects a more recent introduction. *Alexandrium ostenfeldii* produces resting cysts as a part of its life cycle (Mackenzie et al., 1996), which may provide a seed population for the bloom. Thus, the bloom might have been a response of the local population, previously present as hidden flora (Smayda, 2002), to favorable environmental conditions, such as change in salinity due to the opening of floodgates (Okamoto and Miyamoto, 2016). However, no *A. ostenfeldii* or other *Alexandrium* species had been recorded from the lagoon before the change in salinity (Okamoto and Miyamoto, 2016), perhaps due to the unfavorable salinity conditions (< 3.25) (Nomura et al., 2009; Okamoto and Miyamoto, 2016) as *A. ostenfeldii* from a brackish creek in the Netherlands died at salinity of 3 (Martens et al., 2016). Transport of cysts or vegetative cells from the Sea of Japan to the lagoon was presumably limited, as prior to 2012 the floodgates were closed from spring to late autumn (Nomura et al., 2009). Therefore, the formation of red tide after the opening of the floodgates probably results from a recent colonization of the lagoon by *A.*

ostenfeldii responding to suitable environmental conditions such as lower summer surface salinity compared to the Sea of Japan (summer salinity of > 32, (Kim et al., 2004)). Recent colonization is further supported by the low genetic diversity present in the western Japanese population. Obtaining more *A. ostenfeldii* strains from different coastal areas of Japan and utilizing high-resolution genetic markers such as microsatellites or RAD/MIG-sequencing could provide further information on the origin and genetic relatedness of different *A. ostenfeldii* populations in Japan.

5. Conclusion

Phylogenetic analyses revealed a distant relationship between *A. ostenfeldii* isolates from western and northern Japan that could be explained by the differences in habitats as well as the geographic isolation of the northern Japanese population. The isolates from western Japan were more similar with global isolates from shallow and brackish habitats; for example the western Japanese isolates were more similar to an isolate from China than to the northern Japanese isolates. Genotyping of western Japanese isolates revealed low genetic diversity, which might be explained by its sudden mass appearance facilitated by the notable change in salinity as well as by a recent introduction to the area. A further study employing more isolates from coastal areas of Japan may clarify the origin of western and northern Japanese *A. ostenfeldii* populations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Sampling locations in western and northern Japan and in the Baltic Sea, sampling locations are indicated by squares

Figure 2.

A maximum-likelihood tree based on the SSU alignment (1679 bp, incl. insertion/deletions) including 50 sequences and *Fragilidium subglobosum* serving as an outgroup. Sequences from this study are indicated as bold. Bootstrap values > 50% are shown. Group assignments based on Kremp, et al. (2014).

SSU tree from file SSU 190718 (.mas;.meg;.fas;.mtsx)

From the beginning 62 bp were deleted, total length of the alignment is 1679 bp (incl. indels)(total length of amplified SSU is 2098 bp). Model:TN93+G

Figure 3.

A maximum-likelihood tree based on the ITS alignment (525 bp, incl. insertion/deletions) including 46 sequences and *Alexandrium catenella* serving as an outgroup. Sequences from this study are indicated as bold. Bootstrap values > 50% are shown. Group assignments based on Kremp, et al. (2014).

ITS tree from file: ITS_180718_SN_SS (.mas;.meg;.fas;.mtsx)

Length 546 bp (incl. indels), cut based on sequence AB538439 (ITS 1804-2323 bp), total length of ITS 525 bp, 20 bp cut from the end. Model: HKY+I

Figure 4.

A maximum-likelihood tree based on the LSU short alignment (599 bp, incl. insertion/deletions) including 56 sequences and *Prorocentrum micans* serving as an outgroup. Sequences from this study are indicated as bold. Bootstrap values > 50% are shown. Group assignments based on Kremp, et al. (2014).

LSU short alignment based on file LSU170818_1 (.mas;.meg;.fas;.mtsx)

Length of the final alignment is 599 bp (incl. indels) and model used for tree is TN93+G

ITS was cut out based on sequence AB538439 (LSU starts from 2324 bp), 124 bp were deleted from the beginning of the LSU to have more strains from different geographic origin.

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Figure 5. A maximum-likelihood (ML) tree based on the LSU full-length alignment (3474 bp, incl. insertion/deletions) including 26 sequences and *Prorocentrum micans* serving as an outgroup. Sequences from this study are indicated as bold. Bootstrap values > 50% are shown. Group assignments based on Kremp, et al. (2014).
LSU long alignment from file LSU130718_P.micans (.mas;.meg;.fas;.mtsx)

Length 3474 bp (incl. indels) (3459 bp if a full length JS sequence is added), to remove the ITS region the sequences were cut based on sequence AB538439, from the end 25 bp were cut (without cutting, the length of the LSU sequence would be 3475 bp). Model: TN93+G

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Figure 6. Allele frequencies in loci *Aosten13* (A) and *Aosten296* (B). Black bars represent allele frequencies in western Japanese and grey bars the Baltic Sea populations

BS4_49	1	ACACCACCTCGTGTACGATATGCAAAACGCTTGGGCCATGCACGTTTGGATATC---GCCTCCACACATGGGGCGGCGACTGGCACACAAAGAGAAGGCC	97
BS4_56	1	ACACCACCTCGTGTACGATATGCAAAACGCTTGGGCCATGCACGTTTGGATATCAACGCCTCCACACATGGGGCGGCGACTGGCACACAGGAGAAGGCC	100
BS4_40	1	ACACCACCTCGTGTACGATATGCAAAACGCTTGGGCCATGCACGTTTGGATATCAACGCCTCCACACATGGGGCGGCGACTGGCACACAAAGAGAAGGCC	100
BS4_62	1	ACACCACCTCGTGTACGATATGCAAAACGCTTGGGCCATGCACGTTTGGATATCAACGCCTCCACACATGGGGCGGCGACTGGCACACAAAGAGAAGGCC	100
BS4_67	1	ACACCACCTCGTGTACGATATGCAAAACGCTTGGGCCATGCACGTTTGGATATCAACGCCTCCACACATGGGGCGGCGACTGGCACACAAAGAGAAGGCC	100
BS28	1	ACACCACCTCGTGTACGATATGCAAAACGCTTGGGCCATGCACGTTTGGATATCAACGCCTCCACACATGGGGCGGCGACTGGCACACAAAGAGAAGGCC	100
BS6	1	ACACCACCTCGTGTACGATATGCAAAACGCTTGGGCCATGCA-GTTTGGATATCAACGCCTCCACACATGGGGCGGCGACTGGCACACAAAGAGAAGGCC	99
BS5	1	ACACCACCTCGTGTACGATATGCAAAACGCTTGGGCCATGCACGTTTGGATATCAACGCCTCCACACATGGGGCGGCGACTGGCACACAAAGAGAAGGCC	100
JP_S7	1	ACACCACCTCGTGTACGATATGCAAAACGCTTGGGCCATGCACGTTTGGATATCAACGCCTCCACACATGGGGCGGCGACTGGCACACAAAGAGAAGGCC	100
BS4_25	1	ACACCACCTCGTGTACGATATGCAAAACGCTCGGGCCATGCACGTGCGGATATCAACGCCTCCCA-ACATGGGGCGGCGACTGGCACACAGAGAATGCC	99
BS4_42	1	ACACCACCTCGTGTACGATATGCAAAACGCTCGGGCCATGCACGTGCGGATATCAACGCCTCCCA-GCATGGGGCGGCGACTGGCACACAGAGAAGGCC	99
AB971380	1	ACACCACCTCGTGTACGATATGCAAAACGCTCGGGCCATGCACGTGCGGATATCAACGCCTCCCA-ACATGGGGCGGCGACTGGCACACAGAGAAGGCC	99
BS4_49	98	CAAGCCAAACACGCCACACCGCGCAACAAAGCAAATGACCAACTGCGTCAACAATAAGGTGGTGGCTCCTGTGTGCATAGCCTTAA-CGCCGTGTATTGTTT	196
BS4_56	101	CAAGCCAAACACGCCACACCGCGCAACAAAGCAAATGACCAACTGCGTCAACAATAAGGTGGTGGCTCCTGTGTGCATAGCCTTAA-CGCCGTGTATTGTTT	199
BS4_40	101	CAAGCCAAACACGCCACACCGCGCAACAAAGCAAATGACCAACTGCGTCAACAATAAGGTGGTGGCTCCTGTGTGCATAGCCTTAA-CGCCGTGTATTGTTT	199
BS4_62	101	CAAGCCAAACACGCCACACCGCGCAACAAAGCAAATGACCAACTGCGTCAACAATAAGGTGGTGGCTCCTGTGTGCATAGCCTTAA-CGCCGTGTATTGTTT	199
BS4_67	101	CAAGCCAAACACGCCACACCGCGCAACAAAGCAAATGACCAACTGCGTCAACAATAAGGTGGTGGCTCCTGTGTGCATAGCCTTAA-CGCCGTGTATTGTTT	199
BS28	101	CAAGCCAAACACGCCACACCGCGCAACAAAGCAAATGACCAACTGCGTCAACAATAAGGTGGTGGCTCCTGTGTGCATAGCCTTAA-CGCCGTGTATTGTTT	199
BS6	100	CAAGCCAAACACGCCACACCGCGCAACAAAGCAAATGACCAACTGCGTCAACAATAAGGTGGTGGCTCCTGTGTGCATAGCCTTAA-CGCCGTGTATTGTTT	197
BS5	101	CAAGCCAAACACGCCACACCGCGCAACAAAGCAAATGACCAACTGCGTCAACAATAAGGTGGTGGCTCCTGTGTGCATAGCCTTAA-CGCCGTGTATTGTTT	199
JP_S7	101	CAAGCCAAACACGCCACACCGCGCAACAAAGCAAATGACCAACTGCGTCAACAATAAGGTGGTGGCTCCTGTGTGCATAGCCTTAA-CGCCGTGTATTGTTT	199
BS4_25	100	CACACCAACACGCCACACCGTGCACCAACACCATGACCAACCGCGGCAACAATAAGGTGGTGGCTACCGTGTGCATAGCCTTAAACGCCGTGGATGTTTT	199
BS4_42	100	CACACCAACACGCCACACCGTGCACCAACACCATGACCAACCGCGGCAACAATAAGGTGGTGGCTACCGTGTGCATAGCCTTAAACGCCGTGGATGTTTT	199
AB971380	100	CACACCAACACGCCACACCGTGCACCAACACCATGACCAACCGCGGCAACAATAAGGTGGTGGCTACCGTGTGCATAGCCTTAAACGCCGTGGATGTTTT	198
BS4_49	197	GCATGGGGAGTCTGTCGCGCGCGCGCGCA-----TAACCCCGAGGGGCACATGGCAAGAGAAGAGCGAC	260
BS4_56	200	GCATGGGGAGTCTGTCGCGCGCGCGCGCA-----TAACCCCGAGGGGCACATGGCAAGAGAAGAGCGAC	263
BS4_40	200	GCATGGGGAGTCTGTCGCGCGCGCGCGCTG-----CATACCCCGATGGCGCAATGGCAAGAGAAGAGCGAC	265
BS4_62	200	GCATGGGGAGTCTGTCGCGCGCGCGCGCTG-----CATACCCCGATGGCGCAATGGCAAGAGAAGAGCGAC	265
BS4_67	200	GCATGGGGAGTCTGTCGCGCGCGCGCGCTGCA-----TAACCCCGATGGCGCAATGGCAAGAGAAGAGCGAC	263
BS28	200	GCATGGGGAGTCTGTCGCGCGCGCGCGCTGCA-----TAACCCCGATGGCGCAATGGCAAGAGAAGAGCGAC	263
BS6	198	GCATGGGGAGTCTGTCGCGCGCGCGCTG-A-----TAACCCCGATGGCGCAATGGCAAGAGAAGAGCGAC	260
BS5	200	GCATGGGGAGTCTGTCGCGCGCGCGCGCTG-----TAACCCCGATGGCGCAATGGCAAGAGAAGAGCGAC	263
JP_S7	200	GCATGGGGAGTCTGTCGCGCGCGCGCGCTG-----ATAACCCCGATGGCGCAATGGCAAGAGAAGAGCGAC	265
BS4_25	200	GCATGGGGAGTCTGCGCGCGCGCGCGCTG-----CATACCCCGAGGGCGCAATGGCAAGAGAAGAGCGAC	265
BS4_42	200	GCATGGGGAGTCTGCGCGCGCGCGCGCTG-----CATACCCCGAGGGGTGCAATGGCAAGAGAAGAGCGAC	263
AB971380	199	GCATGGGGAGTCTGCGCGCGCGCGCGCGCGCATACCCCGAGGGCGCAATGGCAAGAGAAGAGCGAC	270

Figure 8.
Comparison between the flanking regions and microsatellite sequences from western Japan, Baltic Sea and the published sequence (AB971380) in locus *Aosten296*, horizontal bars indicate primer regions

Figure 9.
Backward particle-tracking simulations from 5-30 m depth during 2008 and 2009 covering 60-day periods, the Funka Bay is indicated by a square, an arrow indicates the Hidaka Bay

Table 1.

Comparison of the number of substitutions (S) and insertions/deletions (I/D) between sequences from strains of different geographic origin: BS – Baltic Sea, wJP –western Japan, nJP –northern Japan, BSC – Bohai Sea, China; asterisk indicates comparison between short LSU sequences; only substitutions present in all strains from one geographic location were taken into an account

	BS vs wJP		wJP vs nJP		BS vs BSC		wJP vs BSC	
	S	I/D	S	I/D	S	I/D	S	I/D
SSU	0	0	3	0	1	0	1	0
ITS	11	0	23	2	10	1	1	1
LSU	7	0	20	0	3*	0*	5*	0*