Published in final edited form as:

J Appl Phycol. 2016 June ; 28(3): 1677–1681. doi:10.1007/s10811-015-0681-7.

CHARACTERIZATION OF 17 NEW MICROSATELLITE MARKERS FOR THE DINOFLAGELLATE *ALEXANDRIUM FUNDYENSE* (DINOPHYCEAE), A HARMFUL ALGAL BLOOM SPECIES

Taylor Sehein,

Woods Hole Oceanographic Institution, Biology Department, 266 Woods Hole Road, MS#32, Woods Hole, MA 02543

Mindy L. Richlen²,

Woods Hole Oceanographic Institution, Biology Department, 266 Woods Hole Road, MS#32, Woods Hole, MA 02543

Satoshi Nagai,

National Research Institute of Fisheries Science, 2-12-4 Fukuura, Kanazawa, Yokohama Kanagawa, 236-8648, Japan

Motoshige Yasuike,

National Research Institute of Fisheries Science, 2-12-4 Fukuura, Kanazawa, Yokohama Kanagawa, 236-8648, Japan

Yoji Nakamura, and

National Research Institute of Fisheries Science, 2-12-4 Fukuura, Kanazawa, Yokohama Kanagawa, 236-8648, Japan

Donald M. Anderson

Woods Hole Oceanographic Institution, Biology Department, 266 Woods Hole Road, MS#32, Woods Hole, MA 02543

Abstract

Alexandrium fundyense is the toxic marine dinoflagellate responsible for “red tide” events in temperate and sub-arctic waters worldwide. In the Gulf of Maine (GOM) and Bay of Fundy in the Northwest Atlantic, blooms of *A. fundyense* recur annually, and are associated with major health and ecosystem impacts. In this region, microsatellite markers have been used to investigate genetic structure and gene flow; however, the loci currently available for this species were isolated from populations from Japan and the North Sea, and only a subset are suitable for the analysis of *A. fundyense* populations in the Northwest Atlantic. To facilitate future studies of *A. fundyense* blooms, both in this region and globally, we isolated and characterized 17 polymorphic microsatellite loci from 31 isolates collected from the GOM and from the Nauset Marsh System, an estuary on Cape Cod, MA, USA. These loci yielded between two and 15 alleles per locus, with an average of 7.1. Gene diversities ranged from 0.297 to 0.952. We then analyzed these same 31 isolates using previously published markers for comparison. We determined the new markers are

²Corresponding author; Email: mrichlen@whoi.edu, PH: (508) 289-2552, FAX: (508) 457-2027

sufficiently variable and better suited for the investigation of genetic structure, bloom dynamics, and diversity in the Northwest Atlantic.

Key index words

Alexandrium fundyense; dinoflagellate; harmful algal bloom; microsatellite; Paralytic Shellfish Poisoning

INTRODUCTION

Alexandrium fundyense Balech is a toxic marine dinoflagellate associated with persistent harmful algal blooms (HABs), also known as “red tides”, which cause the widespread HAB poisoning syndrome known as Paralytic Shellfish Poisoning (PSP). In North America, blooms of *A. fundyense* represent a newly described species comprising certain geographic groups within the “*tamarensis* species complex”, *A. catenella*, *A. fundyense*, and *A. tamarensis*, distinguished by the hypervariable D1-D2 region of the large subunit (LSU) rRNA gene (John *et al.*, 2014). Blooms of this species have expanded dramatically in frequency and geographic distribution over the past few decades and recently were documented for the first time in the Arctic, including the Chukchi Sea (Gu *et al.*, 2013, Natsuike *et al.*, 2013) and Greenland (Baggesen *et al.*, 2012), potentially representing a climate-driven range expansion from adjacent temperate and sub-arctic areas.

Microsatellite markers have been used to study dispersal pathways and connectivity among *Alexandrium* populations in Japanese coastal waters (Nagai *et al.*, 2007), the Baltic Sea (Tahvanainen *et al.*, 2012), and populations along the French coast (Dia *et al.*, 2014), and have also been used to investigate the effects of sexual reproduction. In the Northwest Atlantic, microsatellite markers were used to examine the population structure of *Alexandrium* blooms in both coastal waters (Erdner *et al.*, 2011) and enclosed embayments (Richlen *et al.*, 2012). These studies utilized published microsatellite loci that were previously isolated from populations from Japan (Nagai *et al.*, 2004) and the North Sea (Alpermann *et al.*, 2006). However, only a subset of these loci were suitable for the analysis of *A. fundyense* blooms in the GOM based on amplification success and number of alleles observed (see Erdner *et al.*, 2011), possibly due to regional differences in the genetic composition of blooms. This limitation prompted the current effort to identify new microsatellite markers from regional populations in the Northwest Atlantic. Here, we present 17 new polymorphic microsatellite loci developed for *A. fundyense* that can be used to investigate spatial and temporal bloom diversity, assess connectivity across geography, and investigate questions regarding dispersal events and range expansion.

MATERIALS AND METHODS

Microsatellite isolation by next-generation sequencing

Total genomic DNA was extracted from a cell pellet of an exponentially growing culture using the Power Soil kit (MoBio Laboratories Inc., Carlsbad, CA, USA). Extracted DNA was fragmented into c.a. 300–800 bps, and adaptor sequences were ligated to each end of the

fragments. Resulting DNA library was subjected to shotgun sequencing on GS FLX Titanium picotiterplates using the Roche GS FLX 454 system. A total of 25,569 sequences were obtained with total bases of 136 Mb. Assembly of these sequencing reads was performed using the Newbler Assembler software ver. 2.6 (Roche Applied Sciences, Indianapolis, IN, USA) under the default settings. Subsequent contigs and singletons were screened using a Perl pipeline coupling Tandem Repeats Finder ver. 4.0.4 (Benson 1999) and PRIMER 3 ver. 2.2.2 beta (Rozen and Skaletzky 2000), to design primers for the potential microsatellite loci (Nakamura et al. 2013; Nagai et al. 2014). A total of 2,334 pairs of primers with di-, tri-, tetra-, penta-, hexa-, hepta-, and octa-nucleotides motif loci were designed, of which 52 primer sets were selected for initial amplification trials.

Cultured strains

A total of 31 isolates of North American *A. fundyense* were selected from the culture collection maintained by Dr. Donald Anderson's lab at the Woods Hole Oceanographic Institution, and used to screen candidate microsatellite loci (Table 1). Cultures were established over the span of several years, either from single cell isolations from bloom populations (as in Erdner *et al.* 2011 and Richlen *et al.* 2012), or from germinated cysts (see Nagai *et al.* 2007). For the latter, cysts were isolated from sediment cores collected in the Gulf of Maine (GOM), Bay of Fundy (BOF), and the Nauset Marsh System (NMS); after germination, single vegetative cells were isolated by micropipetting and established in culture. One isolate "hybrid" was established during cyst formation experiments and represents a cross between *A. fundyense* strains from the NMS and BOF. Vegetative cells were isolated from blooms in the NMS and GOM. Isolates were confirmed to be *A. fundyense* via amplification with species-specific primers (Scholin *et al.*, 1994, Dyhrman *et al.*, 2006).

Microsatellite genotyping and analysis

To investigate the characteristics of the 17 candidate microsatellite loci, DNA was extracted from concentrated cell pellets of each culture (~2 mL) using the Power Soil kit (MoBio) following the manufacturer's instructions. Amplifications were performed following a nested PCR method described by Schuelke (2000). Each PCR reaction mixture (10 µl) contained approximately 5ng template DNA, 1× PCR buffer, 2.5mM MgCl₂, 0.2mM dNTP, 0.5mM FAM-labeled universal M13 primer (5' – TGT AAA ACG ACG GCC AGT – 3'), 0.5mM of designed reverse primer, 0.1mM of designed forward primer, and 0.1U Ampli Taq Gold (Applied Biosystems, Foster City, CA, USA). PCR amplifications were performed in an Eppendorf Mastercycler Nexus thermal cycler (Eppendorf, Hamburg, Germany) using the following cycling conditions: 94°C (5 min), then 30 cycles at 94°C (30 s), 56°C (45 s), 72°C (45 s), followed by 8 cycles at 94°C (30 s), 53°C (45 s), and a final extension at 72°C for 10 minutes. Amplification products were screened using gel electrophoresis, then products were diluted 1:50 in nuclease-free water, dried at 60°C for one hour in the thermocycler, and analyzed using an ABI 3730x1 DNA Analyzer (Eurofins MWG Operon, Huntsville, AL, USA). Allele sizes were determined using Peak Scanner software v.1.0 (Applied Biosystems) and confirmed by visualizing the trace files in Geneious Pro 6.1.2 (Biomatters, Auckland, New Zealand). Dinucleotide alleles were rounded to the nearest even number and trinucleotide alleles were rounded to the nearest whole number before performing statistical

analyses. Microsatellite loci allele frequency and gene diversity were calculated using Arlequin v. 3.5.1.3 (Excoffier & Lischer, 2010).

Each culture was also analyzed using a subset of nine published microsatellite loci for *A. fundyense* (Nagai *et al.* 2004, Alpermann *et al.* 2006) that were previously determined to be most suitable for the analysis of blooms in the GOM region (Erdner *et al.*, 2011, Richlen *et al.*, 2012). These analyses were carried out as described above, using the locus-specific PCR cycling conditions in Nagai *et al.* (2004) and Alpermann *et al.* (2006).

RESULTS AND DISCUSSION

The characterizations of the 17 newly identified microsatellite loci are listed in Table 2. Loci were named in the order that primers were tested, with the addition of the expected size (ES) to the nomenclature to simplify future fragment analyses. All loci yielded bright, single bands when visualized on agarose gels. Additionally, all loci, with the exception of Afun2_ES165 and Afun6_ES269, occasionally had non-amplifying PCR products, suggesting null alleles at these loci. Isolates with non-amplifying PCR product varied by locus, however, one isolate from the NMS (Eastham, MA) yielded non-amplifying PCR product for 9 of the 17 loci. The isolate was included in the loci characterization because the remaining eight loci provided informative alleles. The 17 polymorphic loci had between two and 15 alleles per locus, with an average of 7.1. Gene diversities ranged from 0.297 to 0.952, with an average of 0.712.

In comparison, previously published loci were not as well suited for the Northwest Atlantic isolates. The same characterizations were performed for these markers, which are described in Table 3. Several of the markers failed to amplify one-third or more of the strains, and one isolate did not contain enough alleles for analysis (n=30). The number of alleles ranged from 2 to 9, with an average of 5.7. Additionally, diversities ranged from 0.402 to 0.855, with an average of 0.682. These markers have previously been used to study populations in the Northwest Atlantic; however, based on our comparison, use of these markers may be limited by the varying degrees of compatibility (i.e., amplification failure) with some of the populations in this region.

The 17 polymorphic microsatellite loci offer an updated resource to study the genetic structure of *A. fundyense* blooms in the Northwest Atlantic and adjacent coastal embayments, and for assessments of global connectivity. Previous studies successfully used markers designed with Japanese and North Sea isolates to assess the bloom dynamics and population connectivity of *A. fundyense* in these regions (Erdner *et al.*, 2011, Richlen *et al.*, 2012); however, only a subset of the primers was suitable based on amplification success and the number of alleles observed. These new markers successfully amplified *A. fundyense* isolates established over the span of several years, and from several different geographic locations in the GOM region of the Northwest Atlantic, including the BOF and NMS. Our expanded library of markers provides informative loci to study external factors that contribute to bloom diversity, test range expansion hypotheses, and assess additional spatial and temporal diversity questions to better understand the population dynamics of this important HAB species.

Acknowledgments

We thank David Kulis and Alexis Fischer for isolating some of the cysts and strains used in this study. Support for this study was provided by the Woods Hole Center for Oceans and Human Health through National Science Foundation (NSF) Grant OCE-1314642, National Institute of Environmental Health Sciences (NIEHS) Grant 1-P01-ES021923-01, and the research grant of international collaboration projected in Fisheries Research Agency of Japan in 2014.

Abbreviations

GOM	Gulf of Maine
HAB	harmful algal bloom
NGS	next-generation sequencing
PSP	paralytic shellfish poisoning

REFERENCES

- Alpermann TJ, John U, Medlin LK, Edwards KJ, Hayes PK, Evans KM. Six new microsatellite markers for the toxic marine dinoflagellate *Alexandrium tamarense*. *Molecular Ecology Notes*. 2006; 6:1057–1059.
- Baggesen C, Moestrup Ø, Daugbjerg N, Krock B, Cembella AD, Madsen S. Molecular phylogeny and toxin profiles of *Alexandrium tamarense* (Lebour) Balech (Dinophyceae) from the west coast of Greenland. *Harmful Algae*. 2012; 19:108–116.
- Dia A, Guillou L, Mauger S, Bigeard E, Marie D, Valero M, Destombe C. Spatiotemporal changes in the genetic diversity of harmful algal blooms caused by the toxic dinoflagellate *Alexandrium minutum*. *Molecular Ecology*. 2014; 23:549–560. [PubMed: 24330231]
- Dyrhman ST, Erdner D, Du JL, Galac M, Anderson DM. Molecular quantification of toxic *Alexandrium fundyense* in the Gulf of Maine using real-time PCR. *Harmful Algae*. 2006; 5:242–250.
- Erdner DL, Richlen M, McCauley LAR, Anderson DM. Diversity and dynamics of a widespread bloom of the toxic dinoflagellate *Alexandrium fundyense*. *PloS one*. 2011; 6:e22965. [PubMed: 21829565]
- Excoffier L, Lischer HEL. Arlequin suite ver 3.1.1.3: a new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources*. 2010; 10:564–567. [PubMed: 21565059]
- Gu H, Zeng N, Xie Z, Wang D, Wang W, Yang W. Morphology, phylogeny, and toxicity of *Atama* complex (Dinophyceae) from the Chukchi Sea. *Polar Biol*. 2013; 36:427–436.
- John U, Litaker RW, Montresor M, Murray S, Brosnahan ML, Anderson DM. Formal Revision of the *Alexandrium tamarense* Species Complex (Dinophyceae) Taxonomy: The Introduction of Five Species with Emphasis on Molecular-based (rDNA) Classification. *Protist*. 2014; 165:779–804. [PubMed: 25460230]
- Nagai S, Lian C, Hamaguchi M, Matsuyama Y, Itakura S, Hogetsu T. Development of microsatellite markers in the toxic dinoflagellate *Alexandrium tamarense* (Dinophyceae). *Molecular Ecology Notes*. 2004; 4:83–85.
- Nagai S, Lian C, Yamaguchi S, Hamaguchi M, Matsuyama Y, Itakura S, Shimada H, Kaga S, Yamauchi H, Sonda Y, Nishikawa T, Kim C-H, Hogetsu T. Microsatellite markers reveal population genetic structure of the toxic dinoflagellate *Alexandrium tamarense* (Dinophyceae) in Japanese coastal waters. *Journal of Phycology*. 2007; 43:43–54.
- Natsuike M, Nagai S, Matsuno K, Saito R, Tsukazaki C, Yamaguchi A, Imai I. Abundance and distribution of toxic *Alexandrium tamarense* resting cysts in the sediments of the Chukchi Sea and the eastern Bering Sea. *Harmful Algae*. 2013; 27:52–59.

- Richlen ML, Erdner DL, McCauley LAR, Libera K, Anderson DM. Extensive genetic diversity and rapid population differentiation during blooms of *Alexandrium fundyense* (Dinophyceae) in an isolated salt pond on Cape Cod, MA, USA. *Ecology and Evolution*. 2012; 2:2588–2599. [PubMed: 23145343]
- Scholin CA, Herzog M, Sogin M, Anderson DM. Identification of group- and strain-specific genetic markers for globally distributed *Alexandrium* (Dinophyceae). II. Sequence analysis of a fragment of the the LSU rRNA gene. *Journal of Phycology*. 1994; 30:999–1011.
- Schuelke M. An economic method for the fluorescent labeling of PCR fragments. *Nature biotechnology*. 2000; 18:233–234.
- Tahvanainen P, Alpermann TJ, Figueroa RI, John U, Hakanen P, Nagai S, Blomster J, Kremp A. Patterns of post-glacial genetic differentiation in marginal populations of a marine microalga. *PloS one*. 2012; 7:e53602. [PubMed: 23300940]

Table 1

Details regarding isolates used to characterize the 17 microsatellite markers; GOM=Gulf of Maine, NMS=Nauset Marsh System.

Isolate	Isolation location	Year	Isolation method
GT7	Bay of Fundy	1976	Single vegetative cell
GTCA28	GOM	1985	Single cell from cyst germination
38-3	GOM	1993	Single vegetative cell
CB-601	GOM	1997	Single cell from cyst germination
2000-CB-08	GOM	2000	Single vegetative cell
GOM D2	GOM	2005	Single vegetative cell
GOM F14	GOM	2005	Single vegetative cell
GOM H15	GOM	2005	Single vegetative cell
17C8C	Hybrid	1987	Single cell from cyst germination
ATSP7-D9	NMS	2009	Single vegetative cell
N5-MP3	NMS	2012	Single vegetative cell
N6-SP3	NMS	2012	Single vegetative cell
28 Jan D10-E3	NMS	2014	Single cell from cyst germination
22 Dec D7-C5	NMS	2014	Single cell from cyst germination
3Feb_Am_C7	NMS	2014	Single cell from cyst germination
6Mar_Am_B8	NMS	2014	Single cell from cyst germination
6Mar_Am_D11	NMS	2014	Single cell from cyst germination
21May_Ch2_C2	NMS	2014	Single cell from cyst germination
21May_Ch2_B6	NMS	2014	Single cell from cyst germination
21May_Ch2_D6	NMS	2014	Single cell from cyst germination
21May_Ch2_D7	NMS	2014	Single cell from cyst germination
21May_Ch2_D9	NMS	2014	Single cell from cyst germination
21May_Ch2_C11	NMS	2014	Single cell from cyst germination
21May_Ch2_B10_2	NMS	2014	Single cell from cyst germination
6May_Am_C2	NMS	2014	Single cell from cyst germination
6May_Am_B3	NMS	2014	Single cell from cyst germination
6May_Am_C3	NMS	2014	Single cell from cyst germination
6May_Am_C7	NMS	2014	Single cell from cyst germination
6May_Am_B8	NMS	2014	Single cell from cyst germination
6May_Am_B9	NMS	2014	Single cell from cyst germination

Microsatellite primers for the 17 polymorphic loci designed for *Alexandrium fundyense* and select characteristics of the loci; T_a = annealing temperature.

Table 2

Locus	Primer sequence (5'-3')	Motif	T _a (°C)	Number of non- amplifying samples	Number of Alleles	Size Range	Genetic Diversity
Afun1_ES190	F (GGAACCTTTCTTAGGGGTCTGTG) R (AGCCCCAAGAACTCATACCATA)	(CCG) ₉	60	1	7	188–204	0.717
Afun2_ES165	F (GAGAGCACAAAGGAAGGAAAGAA) R (GCACAACAAGAAAGGATGAACA)	(CA) ₂₀	60	0	2	152–154	0.503
Afun3_ES116	F (GCATTGATATCACCATGCAATC) R (CTTGCTTTGTCGGTAGCTATGTG)	(CA) ₁₅	60	1	10	118–138	0.882
Afun4_ES261	F (GCGACCATGACTGAATAATGAA) R (CGTCATGTCCATTGCATCTTAT)	(GAT) ₈	60	2	5	274–281	0.685
Afun5_ES252	F (TCGTCCAAAACCTCTGACTGAAGA) R (CTCGGTGTGTGCACTAGTCTC)	(TCG) ₉	60	1	4	234–262	0.609
Afun6_ES269	F (CGAGGACCATGAGAAAGAAAGT) R (CCCAGGTCAATCACTACTGACA)	(AGC) ₉	60	0	4	273–277	0.297
Afun7_ES182	F (GTGTCTGTCTTTTCCCTCAAC) R (GGAACGGACAGATAAATACGC)	(TGC) ₉	60	3	7	198–244	0.825
Afun8_ES136	F (ATCGATGATTCCTCAAAACGACT) R (GCGATTTTCTGTAATTTGACCC)	(GCT) ₈	60	2	4	144–150	0.569
Afun9_ES394	F (AACACAAAACAGCATACAACGC) R (AAATGGCACCTCTGAAAGTAT)	(CA) ₁₃	60	3	7	394–404	0.778
Afun10_ES203	F (GTGTGGGATGAGAGTTGCAG) R (GTCTCCGCATGCTGACTGATT)	(CA) ₁₂	60	2	11	170–240	0.892
Afun11_ES285	F (AATTCATTCTTCCCAAGCAGAAAG) R (TGGTCAGGTATTTGTTGACAGC)	(CA) ₁₃	60	3	5	288–308	0.630
Afun12_ES207	F (GTGTGCTATGCTATGCCCTCAAG) R (GACCCGTAAGGATTAACGTCG)	(GT) ₁₃	60	8	2	202–204	0.403
Afun13_ES156	F (GGCCACACACTAAAACACAAGA) R (TTTGCAATGTGCAGTATTTGA)	(CA) ₁₃	60	7	11	150–202	0.891
Afun14_ES321	F (ATCCACGTCGTAAACCTGAGTG) R (ACCGTAATTATGATGGCGTT)	(CA) ₄₂	60	4	15	302–344	0.952
Afun15_ES350	F (AAGTAGACGATCGAAGCACAGC) R (ATGCTGACTTGAGCAACTGTGT)	(CAG) ₉	60	1	7	351–368	0.832
Afun16_ES282	F (TTGTGTGGTGGTCTCAGTATC) R (GTGTTTACTTCAAGCAGTGGGC)	(GT) ₂₉	60	1	13	238–300	0.931
Afun17_ES120	F (GCATTGCTTATTAATGGCCTC) R (CAGAAAGCAGTGGGAACAAAAA)	(AT) ₁₂	60	3	7	116–142	0.709

Characterization of a subset of nine previously published microsatellite loci using 30 isolates of *A. fundyense* identified as most suitable for the analysis of *A. fundyense* populations from the northwestern Atlantic.

Table 3

Locus	Source	Number of non-amplifying samples	Number of Alleles	Size Range	Genetic Diversity
ATF1	Alpermann <i>et al.</i> , 2006	2	9	152–194	0.735
Atama 4	Nagai <i>et al.</i> , 2004	14	2	116–118	0.525
ATD8	Alpermann <i>et al.</i> , 2006	0	8	265–278	0.855
Atama 15	Nagai <i>et al.</i> , 2004	0	4	241–263	0.402
Atama 16	Nagai <i>et al.</i> , 2004	8	5	150–162	0.788
Atama 17	Nagai <i>et al.</i> , 2004	5	3	132–140	0.507
Atama 23	Nagai <i>et al.</i> , 2004	10	6	174–196	0.784
Atama 27	Nagai <i>et al.</i> , 2004	10	6	152–164	0.747
Atama 39	Nagai <i>et al.</i> , 2004	12	8	120–152	0.791