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Improved phylogenetic resolution of toxic and non-toxic *Alexandrium* strains using a concatenated rDNA approach

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ABSTRACT

Dinoflagellates of the genus *Alexandrium* are known producers of paralytic shellfish toxins. Species within the genus have similar phenotypes making morphological identification problematical. The use of *Alexandrium* rDNA sequence data is therefore increasing, resulting in the improved resolution of evolutionary relationships by phylogenetic inferences. However, the true branching pattern within *Alexandrium* remains unresolved, with minimal support shown for the main phylogentic branch. The aim of this study is to improve phylogenetic resolution via a concatenated rDNA approach with a broad sample of taxa, allowing inference of the evolutionary pattern between species and toxins. 27 *Alexandrium* strains from 10 species were tested with HPLC for PSP toxin presence and additionally sequenced for 18S, ITS1, 5.8S, ITS2 and 28S rDNA before being phylogenetically inferred together with all available orthologous sequences from NCBI. The resulting alignment is the largest to date for the genus, in terms of both inferred characters and taxa, thus allowing for the improved phylogenetic resolution of evolutionary patterns there in. No phylogenetic pattern between PSP producing and non-producing strains could be established, however the terminal *tamarense* complex was shown to produce more PSP analogues than basal clades. Additionally, we distinguish a high number of polymorphic regions between the two copies of *A. fundyense* rDNA, thus allowing us to demonstrate the presence of chimeric sequences within GenBank, as well as a possible over estimation of diversification within the *tamarense* complex.

1. Introduction

The dinoflagellate genus *Alexandrium* is a known producer of neurotoxic saxitoxin (STX) commonly known as paralytic shellfish toxins (PSTs). A reported 57 STX analogues are known to date (Anderson et al., 2002; Kellmann et al., 2010; Sellner et al., 2003; Wiese et al., 2010), with the genes required for saxitoxin-synthesis in *Alexandrium* recently being identified (Stüken et al., 2011). These toxins can cause severe symptoms upon consumption of vector species, including filter feeding invertebrates such as shellfish, crustaceans, mollusks, gastropods and planktivorous fish (Deeds et al., 2008). PSTs work by reversibly binding voltage-gated Na+ channels, blocking the neural pore, resulting in the occasionally fatal paralytic shellfish poisoning (PSP) (Catterall, 1980; Catterall et al., 1979; Cestele and Catterall, 2000). Marine STX also occurs external to the *Alexandrium* genus, with both *Gymnodinium catenatum* (Oshima et al., 1987) and *Pyrodinium bahamense* var. *compressum* (Harada et al., 1982) known dinoflagellate PST producers. However, it is the genus *Alexandrium* that are the most abundant and widespread STX producers, responsible for harmful algal blooms (HABs) or red tides in subarctic, temperate, and tropical locations (Anderson et al., 1994; Hallegraeff, 2005; Taylor et al., 1995). The majority of toxic blooms have been caused by the species *A. catenella*, *A. tamarense*, and *A. fundyense* (Cembella, 1998); together comprising the *tamarense* species complex (Balech, 1985). However, of the 30 known species within the genus (Balech, 1995) only 8 are documented PST producers (Cembella, 1998; Kodama, 2000)., *A. acatenella* (Cembella et al., 1987), *A. andersoni* (Ciminiello et al., 2000), *A. catenella* (Hallegraeff and Lucas, 1988), *A. fundyense* (Anderson et al., 1990), *A. minutum* (Chang et al., 1997; Mascarenhas et al., 1995), *A. ostenfeldii* (MacKenzie et al., 2004), *A. tamarense* (Buckley et al., 1976), and *A. tamiyavanichi* (Hashimoto et al., 2002).

Alexandrium species have similar phenotypes making identification by morphological characters an expert task. The main criteria for morphological identification are the shape of the apical

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pore complex, the posterior sulcal plate and the first apical 'rhomb' plate (Balech, 1985, 1995; Fukuyo, 1985; Steidinger, 1990). Presence or absence of a ventral pore is the key morphological feature differentiating species within the *tamarense* complex, with presence in *A. tamarense* and absence in *A. catenella* and *A. fundyense*. A consequence of this is that sequence data, in parallel with morphological characters, has become an invaluable tool for the identification of *Alexandrium* species. The ever-increasing availability of DNA sequence data has helped improve our understanding of the *Alexandrium* genus through phylogenetic inference. For example, inferred rDNA sequence alignments have recently given support to the *tamarense* complex, additionally placing both *A. affine* and *A. tamiyavanichi* as sister groupings to this (Leaw et al., 2005; Masseret et al., 2009; Penna et al., 2008; Sebastian et al., 2005). *A. satoanum, A. taylori, A. hiranoi, A. pseudogonyaulax* and *A. monilatum* have all recently been placed within phylogenies (Kim et al., 2005; Rogers et al., 2006). However, despite these advances the true branching pattern remains unresolved, with minimal support shown for the main phylogentic branch (backbone). The most basal *Alexandrium* speceis also remains elusive with long-branching, fast evolving speceis often being recovered basally (John et al., 2003b; Kim et al., 2005; Leaw et al., 2005; Montresor et al., 2004; Rogers et al., 2006), a possible artefact of undersampling of either taxa and/or characters (Philippe and Germot, 2000; Philippe et al., 2000). A "correct" phylogentic inference of the genus is important for the improved identification of species and any evolutionary relationships they share. Furthermore, a well-resolved phylogeny can be used to infer if additional relationships between taxa exist, for example, any possible connection between the evolution of the genus, PST production and analogues. The aim of this study is to infer a well resolved phylogeny of *Alexandrium* in order to adress the genus evolution and possible relationship to PST production. Previously, studies have focused on specific questions, taxa and/or genes when inferring *Alexandrium* evolution (Alpermann et al., 2010; Penna et al., 2008; Pitcher et al., 2007; Sebastian et al., 2005). However, sampling a broad range of both taxa and characters in a concatenated phylogenetic approach will decrease alignment bias and/or artifacts that result in the basal placement of fast evolving species (Philippe and Germot, 2000). To achieve this, a large number of *Alexandrium* strains and species have been sequenced for the entire rDNA region (18S-ITS1-5.8S-ITS2-28S) and available database sequence data for this region additionally acquired, before inference with newest phylogenetic methods. Additionally, strains have been analyzed for PST presence, with all available toxicity data for inferred strains being acquired.

2. Materials and methods

2.1. Culturing

The 27 *Alexandrium* strains (10 species) used in this study are listed in Table 1. All strains were grown in L1 media (Guillard and Hargraves, 1993) at 16 °C or 19 °C with a 12:12 h light–dark photoperiod and a photon irradiance of ~100 mmol photons m^{-2} s^{-1}. Strains were not maintained axenic.

2.2. DNA isolation, PCR amplification, sequencing and assembly

Genomic DNA was isolated from 20 ml culture in the exponential growth phase, centrifuged for 2 min at 12,000 × *g* before using Invitrogen Genomic DNA plant Chargeswitch kit (Invitrogen, USA) in accordance with supplied protocol. DNA quality was subsequently checked using a NanoDrop spectrophotometer (ThermoScientic, Wilmington, DE, USA). DNA template was PCR amplified into 3 separate overlapping products of ~1.8–2.0 kb using the primer pairs: F8+R0-ITS, F1574+28R1318 and F3475+R5298 (see Supplementary Table 1). Together, the amplicons covered ~5.2 kb of the rDNA and were amplified together with BD Advantage 2 polymerase (Clonetech, USA) with the following PCR conditions in a Master Cycler (Eppendorf): an initial 60 s 94 °C denaturing before 30 cycles of (1) 15 s 94 °C denaturing, (2) 30 s 56 °C annealing, and (3) 120 s 68 °C extension, with a final 180 s extension at the same temperature. PCR products were purified using ExoSAP-IT (GE Healthcare, USA) before direct sequencing with an ABI3730 DNA analyzer (Applied Biosystems,

Table 1
Species and strains of *Alexandrium* used in this study.

Species	Strain	Origin	Supplier/isolator
A. affine	AABB01/01	Bell Bay, Tasmania	CSIRO (AUS)
A. affine	AABB01/02	Bell Bay, Tasmania	CSIRO (AUS)
A. affine	CCMP112	Ria de Vigo, Spain	CCMP (USA)
A. andersoni	CCMP1597	Massachusetts, USA	CCMP (USA)
A. andersoni	CCMP1718	Massachusetts, USA	CCMP (USA)
A. andersoni	CCMP2222	Naples, Italy	CCMP (USA)
A. fraterculus	AF0307MIE01	Mie, Japan	Kindly provided by S. Nagai
A. fraterculus	AF0307MIE07	Mie, Japan	Kindly provided by S. Nagai
A. fundyense	CCMP1719	New Hampshire, USA	CCMP (USA)
A. fundyense	CCMP1846	Massachusetts, USA	CCMP (USA)
A. fundyense	CCMP1978	Bay of Fundy, Canada	CCMP (USA)
A. fundyense	CCMP1979	Bay of Fundy, Canada	CCMP (USA)
A. fundyense	CCMP1980	Bay of Fundy, Canada	CCMP (USA)
A. fundyense	IMR_S_182008	Flodevigen, Norway	Kindly provided by L. Naustvoll
A. insuetum	CCMP2082	Japan	CCMP (USA)
A. minutum	ALSP01	Ria de Vigo, Spain	CSIRO (AUS)
A. minutum	ALSP02	Ria de Vigo, Spain	CSIRO (AUS)
A. minutum	AMAD16	Adelaide, Australia	CSIRO (AUS)
A. minutum	CCMP113	Ria de Vigo, Spain	CCMP (USA)
A. minutum	CCMP1888	Laguna Obidos, Portugal	CCMP (USA)
A. ostenfeldii	CCMP1773	Kattegat, Denmark	CCMP (USA)
A. ostenfeldii	IMR_V_062007	Flodevigen, Norway	Kindly provided by L. Naustvoll
A. pseudogonyaulax	OF2AUG09	Oslo Fjord, Norway	W. Eikrem
A. tamarense	ATBB01	Bell Bay, Tasmania	CSIRO (AUS)
A. tamarense	CCMP115	Plymouth, England	CCMP (USA)
A. tamarense	CCMP1493	Hongkong Island, China	CCMP (USA)
A. tamiyavanichi	AT0112F06	Japan	Kindly provided by S. Nagai

CCMP (USA): Provasoli-Guillard National Centre for Culture of Marine Phytoplankton. CSIRO (AUS): Australian National Algae Culture Collection.