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Sympatric occurrence of two *Azadinium poporum* ribotypes in the Eastern Mediterranean Sea

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ABSTRACT

The marine dinoflagellate *Azadinium poporum* produce azaspiracids (AZA) and has been recorded widely in the world. However, information on its biogeography is still limited, especially in view of the fact that *A. poporum* comprises several genetically differentiated groups. A total of 18 strains of *A. poporum* were obtained from the Eastern Mediterranean area by incubating surface sediment collected from Ionian Sea of Greece. The morphology of these strains was examined with light microscopy and scanning electron microscopy. Small subunit ribosomal DNA (SSU rDNA), large subunit ribosomal DNA (LSU rDNA) and internal transcribed spacer (ITS) sequences were obtained from all cultured strains. Molecular phylogeny based on concatenated SSU, LSU and ITS sequences confirmed three ribotypes within *A. poporum* and revealed two subclades within ribotypes A and C. Greek strains of *A. poporum* ribotype A were nested within ribotype A2 together with strains from Western Mediterranean Sea and French Atlantic, and Greek strains of *A. poporum* ribotype C were nested within ribotype C2 together with a strain from the Gulf of Mexico. Growth experiments on four selected strains revealed that ribotypes A and C from Greece differed in their growth at higher temperatures, indicating that they are physiologically differentiated. Azaspiracid profiles were analyzed for 15 cultured *A. poporum* strains using LC–MS/MS and demonstrate that the *A. poporum* ribotype A from Greece produce low level or no AZA and *A. poporum* ribotype C from Greece produces predominantly AZA-40 (9.6–30.2 fg cell^{-1}) followed by AZA-2 (2.1–2.6 fg cell^{-1}). The first record of AZA-40 producing *A. poporum* from the Mediterranean suggests that this species is a potential source for azaspiracid contaminations in shellfish from the Eastern Mediterranean Sea.

1. Introduction

The planktonic dinophyte *Azadinium spinosum* Elbrächter & Tillmann is the type species of the genus *Azadinium* Elbrächter & Tillmann, which for most species is characterized by a plate pattern of Po, cp, X, 4′, 3a, 6″, 6C, 5S, 6‴, 2⁗ (Tillmann et al., 2009). Slightly varying epithecal plate pattern were found in two other species, i.e. in *Azadinium dalianense* Z.Luo, H.Gu & Tillmann possessing three apical and two anterior intercalary plates (3′, 2a) (Luo et al., 2013) and in *Azadinium zhuanum* Z.Luo, H.Gu & Tillmann possessing four apical and two anterior intercalary plates (4′, 2a) (Luo et al., 2017a). In the molecular phylogeny however, *Azadinium* is monophyletic, forming the family Amphidomataceae together with the genus *Amphidoma* Stein (Tillmann et al., 2012; Luo et al., 2017a).

The toxigenic species *Azadinium poporum* has been recorded more widely than any other *Azadinium* species but still its presence in many areas has not been examined yet. It was originally described from the North Sea (North Atlantic) off Denmark (Tillmann et al., 2011) and now is known to comprise three genetically different but morphologically indistinguishable ribotypes (Gu et al., 2013). Strains from Denmark, French Atlantic, French Mediterranean, Chile, Pacific USA and New Zealand have been classified within ribotype A (Kim et al., 2017; Luo et al., 2017a; Tillmann et al., 2017b), whereas strains from Asian Pacific, Argentina, Gulf of Mexico were classified within ribotypes C (Tillmann et al., 2016; Luo et al., 2017a). Ribotype B was only reported in Chinese waters (Gu et al., 2013). Sympatric occurrence of two ribotypes of *A. poporum* was only reported in Chinese waters (ribotypes B and C, Gu et al., 2013). The presence of different ribotypes in the same area, however, might get unnoticed for other locations when only a limited number of strains are available.

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Compared to other *Azadinium* species, many more strains of *A. poporum* have been sequenced, but with new sequences from other localities additional ribotypes might be revealed. Some *A. poporum* strains of the same ribotype were found to share nearly identical LSU and ITS sequences although they originated from distant locations. For instance, *A. poporum* from New Zealand shared identical LSU sequences with those from Denmark (Smith et al., 2016). On the other hand, strains collected from close-by localities such as the French Atlantic and Danish Atlantic coasts may show some level of genetic differentiation even within the same ribotype (ribotype A) (Luo et al., 2017a). Whether further subdivisions of the ribotypes are justified remains to be determined.

Motile cells of *A. poporum* are relatively small ranging from 10 to 20 µm in cell length. Such small cells are easily overlooked in routine monitoring. To study the biogeography of *Azadinium*, molecular detection is promising, but specific probes have been designed for only a few species yet (Toebe et al., 2013) and they are only occasionally used (Smith et al., 2016; Kim et al., 2017). To evaluate the species specificity of previous molecular probes, it is essential to reveal genetic differentiation among strains from different area.

The first *Azadinium* species shown to produce azaspiracids (AZA) was *Azadinium spinosum* (Tillmann et al., 2009), and in Ireland this is the species responsible for cases of human intoxication via mussel consumption. Later, two other *Azadinium* species (*A. poporum* and *A. dexteroporum*) and one *Amphidoma* species (*Amphidoma languida*) were found to produce AZA, too (Krock et al., 2012; Gu et al., 2013; Rossi et al., 2017; Tillmann et al., 2017a). Initially, *Azadinium poporum* was described as a non-toxigenic species (Tillmann et al., 2011), but later it turned out that the type strain in fact produces a novel AZA (Krock et al., 2012), and that new strains of *A. poporum* from China produce diverse AZA, e.g. AZA-2, AZA-11, AZA-36, AZA-40 and AZA-41 (Krock et al., 2014). For *A. poporum* strains outside China, the AZA profiles are relatively uniform. AZA-37 is the only AZA reported yet in European Atlantic strains (Krock et al., 2012), whereas only AZA-2 (and low levels of AZA-2 phosphate) were detected in strains from Gulf of Mexico, Corsica of France and Argentina (Luo et al., 2016; Tillmann et al., 2016; Luo et al., 2017a). Likewise, only AZA-11 (and two phosphorylated AZA) were detected in Chilean strains (Tillmann et al., 2017b), and AZA-59 was the sole AZA detected in strains from Washington State, USA (Kim et al., 2017).

In the Mediterranean Sea, only two *Azadinium* species have been recorded. One strain of *Azadinium dexteroporum* has been described from the Gulf of Naples (Percopo et al., 2013), and was shown to produce seven (six of which were new to science) different AZA (Rossi et al., 2017). One strain of *A. poporum* was recovered in Corsica which is able to produce AZA-2 (Luo et al., 2017a), and an unidentified *Azadinium* species was encountered in water samples collected in Fangar Bay (Catalan coast, NW Mediterranean) (Busch et al., 2016), suggesting that higher diversity and wider distribution of *Azadinium* can be expected in the Mediterranean Sea. In addition, AZA-2 was detected in mussels collected in the Adriatic Sea in 2012 and 2013 (Bacchiocchi et al., 2015), but the responsible species has not been identified yet.

In ongoing attempts to complement *Azadinium* diversity and biogeography, 18 strains of *A. poporum* from the Ionian Sea of Greece were established. Cultured strains were examined for their morphology, and were analyzed for the presence of AZA. In addition, small subunit ribosomal DNA (SSU rDNA), partial large subunit ribosomal DNA (LSU rDNA) and internal transcribed spacer (ITS) sequences were determined for the cultured strains and molecular phylogeny was inferred using concatenated SSU, ITS and LSU rDNA sequences.

2. Material and methods

2.1. Sample collection and treatment

Sediment samples were collected from the Gulf of Amvrakikos (station 21) and Igoumenitsa (station 24) in 2014 using a grab (Table 1, Fig. S1). The Amvrakikos Gulf is a large (405 km^2), semi-enclosed embayment, located in Western Greece. It is connected to the open Ionian Sea through a narrow, elongated channel, the Preveza Straits, which is approximately 6 km long, 0.8 to 2.5 km wide and 20 m deep (Naeher et al., 2012). The mean depth of the gulf is 26 m, while the maximum depth of 63 m has been recorded in its eastern part (Kehayias and Aposporis, 2014). The Ionian Sea is an elongated bay of the Mediterranean Sea, south of the Adriatic Sea.

Station 21 is in its southern part of Amvrakikos gulf, near to Amfilochia town, and station 24 is located in the Igoumenitsa gulf (Epirus, Northern Greece), a sheltered enclosed mass of water in the Ionian Sea, with a shoreline of approximately 1.4 km in length (Beza et al., 2014). In the easternmost part of Igoumenitsa gulf is located the port of Igoumenitsa, one of the largest passenger ports in the Eastern Mediterranean Sea basin, which handles around one million passengers per year for international destinations.

The top 2 cm were sliced off and stored in the dark at 4 °C until further treatment. Approximately 5 g of wet sediment was mixed with 20 mL of 0.22 µm filtered seawater and stirred vigorously to dislodge detrital particles. The settled material was subsequently sieved through 120 µm and 10 µm filters. The 10–120 µm fractions were rinsed with f/2-Si medium (Guillard and Ryther, 1962) and transferred into a 96-well culture plate. The culture plate was incubated at 20 °C, 90 µmol quanta m^{-2} s^{-1} under a 12:12 h light: dark cycle (hereafter called "standard culture conditions"). Cells of *Azadinium* are characterized by swimming at low speed, interrupted by short, high-speed 'jumps' in various directions (Tillmann et al., 2009). Cells exhibiting such a characteristic swimming behavior were isolated with a micropipette under an inverted microscope Leica DMi1 (Leica, Wetzlar, Germany) and established in clonal cultures under standard culture conditions.

2.2. Light microscopy (LM)

Live cells were examined and photographed using a Zeiss Axio Imager microscope (Carl Zeiss, Göttingen, Germany) equipped with a Zeiss Axiocam HRc digital camera. Cell size of thirty cells was measured using Axiovision (4.8.2 version) software at ×1000 magnification. To observe the shape and location of the nucleus, cells were stained with 1:100 000 SYBR Green (Sigma Aldrich, St. Louis, USA) for 1 min, and photographed using the Zeiss fluorescence microscope with a Zeiss-38 filter set (excitation BP 470/40, beam splitter FT 495, emission BP 525/50).

Table 1
Azadinium poporum strains examined in the present study, including the ribotype, collection locality, coordinates, collection date and water depth.

Species	Strains	Ribotypes	Latitude (N)	Longitude (E)	Location	Collection date	Station	Depth (m)
A. poporum	TIO420-425	A	38°55'18.12"	21°05'42.36"	Amvrakikos/Ionian Sea	2014.09.27	21	42.5
A.poporum	TIO433-438	A	38°55'18.12"	21°05'42.36"	Amvrakikos/Ionian Sea	2014.09.27	21	42.5
A.poporum	TIO431, 432	A	39°30'13.68"	20°15'01.08"	Igoumenitsa/Ionian Sea	2014.09.28	24	22.9
A.poporum	TIO427-429	C	39°30'13.68"	20°15'01.08"	Igoumenitsa/Ionian Sea	2014.09.28	24	22.9
A. poporum	TIO452	C	39°30'13.68"	20°15'01.08"	Igoumenitsa/Ionian Sea	2014.09.28	24	22.9

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