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Isolation of 12 microsatellite markers following a pyrosequencing procedure and cross-priming in two invasive cryptic species, *Alexandrium catenella* (group IV) and *A. tamarense* (group III) (Dinophyceae)

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ABSTRACT

Alexandrium catenella (group IV) and *Alexandrium tamarense* (group III) (Dinophyceae) are two cryptic invasive phytoplankton species belonging to the *A. tamarense* species complex. Their worldwide spread is favored by the human activities, transportation and climate change. In order to describe their diversity in the Mediterranean Sea and understand their settlements and maintenances in this area, new microsatellite markers were developed based on Thau lagoon (France) samples of *A. catenella* and *A. tamarense* strains. In this study twelve new microsatellite markers are proposed. Five of these microsatellite markers show amplifications on *A. tamarense* and ten on *A. catenella*. Three of these 12 microsatellite markers allowed amplifications on both cryptic species. Finally, the haplotypic diversity ranged from 0.000 to 0.791 and 0.000 to 0.942 for *A. catenella* and *A. tamarense* respectively.

1. Introduction

Proliferation phenomena of harmful phytoplankton are known as Harmful Algal Blooms (HABs). The worldwide spread of the HAB events is worrisome. Forty percent of harmful species are toxic and cause different syndromes associated with neurological or gastric disorders in humans after consumption of contaminated aquatic organisms (Zingone and Enevoldsen, 2000). Anthropogenic pressures (environmental/climate changes, shellfish transplantation, shipping) have broken the ecological barriers which had driven isolation and evolution of original areas (Smayda, 2007). Health and socio-economic impacts of HABs prove dramatic effects on commercially exploitable resources (Hoagland and Scatasta, 2006). Since 1970, 5 times more areas are affected by HAB type Paralytic Shellfish Poisoning (Glibert et al., 2005). The cosmopolite *Alexandrium* genus (Dinophyceae) regroups 31 toxic and non toxic species (Anderson et al., 2012). The two cryptic species *Alexandrium catenella* and *Alexandrium tamarense* belonging to the *A. tamarense* complex, spreading on the world coastal waters were classified as invasive species by Molnar et al. (2008). *A. catenella* (group VI-Temperate Asian clade) and *A. tamarense* (group III-Western European clade) have been recorded in the Mediterranean Sea coastal waters of Spain, France, Italy, Tunisia and Algeria (Lilly et al., 2002, 2007; Frehi et al., 2007; Turki et al., 2007; Penna et al., 2008; Genovesi et al., 2011). Microsatellite markers have already been developed from *A. tamarense* strains (group I-North American clade) and from *A. catenella* (IV-Temperate Asian clade) isolated from Scottish and Japanese strains (Nagai et al., 2004, 2006; Alpermann et al., 2006; Nishitani et al., 2007). However, these markers did not work on *A. tamarense* (III) (data not shown) (Nagai et al., 2007) or appeared to be poorly polymorphic inside the French *A. catenella* (IV) population (Masseret et al., 2009).

The goal of this study is to develop new highly polymorphic microsatellite markers from Thau lagoon strains in order to describe the genetic diversity within Thau lagoon populations and among the Mediterranean populations in present and upcoming studies. This will help the understanding of the successful settlements and maintenances of these two planktonic invasive species under local environmental selective pressures and widely

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in the Mediterranean Sea. Thus, we describe here the development of twelve microsatellite markers and their cross-species application on both *A. catenella* and *A. tamarense*.

2. Methods

Two monoclonal strains isolated in 2007 from each *A. catenella* and *A. tamarense* species of Thau lagoon have been used to extract total genomic DNA following standard phenol–chloroform protocol (Sambrook et al., 1989; for strain isolation refer to Genovesi et al., 2011). The total genomic DNA was then sent to Genoscreen private company (Lille, France) to produce a coupling multiplex microsatellite enrichment isolation with the 454 GS-FLX Titanium pyrosequencing platforms, using the method described in Malaussa et al. (2011). From these microsatellite markers, the most promising were selected for their high repetitions and their absence of compound repeat to test their PCR amplification effectiveness.

In order to test the selected microsatellite markers, a total of 50 individuals of the *A. tamarense* species complex from the Thau lagoon (Mediterranean coast, France) were isolated: 26 individuals of *A. catenella* in autumn 2007 and 24 individuals of *A. tamarense* in spring 2007. Cultures were grown in enriched natural sea water (Harrison et al., 1980) at 20 °C, on a 12 h:12 h light: dark cycle, using a photon flux density of 100 μmol m^{-2} s^{-1}. Total genomic DNA was extracted from the 50 individuals following standard phenol–chloroform protocol (Sambrook et al., 1989).

PCR amplifications were performed on an Eppendorf Mastercycler® in a reaction mixture (10 μl) containing 20 ng of DNA, 2.5 mM/μl of $MgCl_2$, 2 μl of 5× buffer (Promega™), 0.5 mM/μl of each new primer, 0.2 mM/μl for each dNTPs, 0.25 U of Taq (Promega™) and ultra-pure water quality. The primer cycling conditions were as follows: 10 min at 95 °C, 39 cycles of 30 s at 95 °C, 30 s at primer specific annealing (see Table 1), 30 s at 72 °C and a final elongation of 5 min at 72 °C. Pre-tests on selected microsatellite markers were produced to visualize allelic variation with non labelled primers on a new generation submarine electrophoresis system (ORIGINS by Elchrom™), using El600 gels to ensure high resolution on large ranged marker size (100–300 bp) on 8 individuals (4 *A. catenella and* 4 *A. tamarense*). Microsatellite markers that show allelic variation at least between species were then amplified again using primers labelled with FAM or CY5 followed by a migration on a denaturing 8% acrylamide gel on all 50 individuals. The gels were scanned on a FMBIO® fluorescent imaging system (HITACHI) and scored using GeneMapper® v4.0 (Applied Biosystems). The haplotypic diversity and the number of alleles were calculated respectively with FSTAT software v1.2 (Goudet, 1995) and CONVERT software v1.31 (Glaubitz, 2004).

3. Results

Two libraries of 1179 microsatellite markers for *A. catenella* and 1325 for *A. tamarense* were obtained from 454 GS-FLX Titanium pyrosequencing. From these libraries, 48 microsatellite markers (26 from *A. catenella* and 22 from *A. tamarense*) were selected according to their high repetitions and their absence of compound repeat. Of the 48 microsatellite markers checked from each library, 18 from *A. catenella* and 18 from *A. tamarense* libraries were rejected for unsuccessful PCR amplifications, scoring problems or lack of allelic variation. Of the twelve microsatellite markers remaining, five showed successful amplification for *A. tamarense* (R2M3-Atam05, R2M3-Atam09, R2M3-Atam15, R2M3-Atam16 and R2M3-Acat22) and ten for *A. catenella* (R2M3-Atam05, R2M3-Atam09, R4M8-Acat02, R4M8-Acat05, R4M8-Acat09, R4M8-Acat19, R4M8-Acat20, R4M8-Acat21, R4M8-Acat22 and R4M8-Acat 23). The characteristics of the twelve microsatellite markers selected (8 from *A. catenella* and 4 from *A. tamarense* libraries) are given in Table 1. The haplotypic diversity ranged from 0.000 to 0.791 and 0.000 to 0.942 for *A. catenella* and *A. tamarense* respectively.

Three microsatellite markers worked on both species (R2M3-Atam05, R2M3-Atam09 and R4M8-Acat22). However, R4M8-Acat22 showed difficulties in genotyping due to non-target bands in *A. catenella* electrophoregram (21 of 26). Furthermore, only R4M8-Acat09 showed no variation inside both *Alexandrium* species. Excluding this potential intra-species monomorphic loci, a total of 11 new microsatellite markers were evidenced to be polymorphic and applicable for at least one of the two species analysed in this study.

4. Discussion

As observed recently in other studies (Laporte et al., 2012; Froufe et al., 2013), second-generation sequencing of microsatellite-enriched libraries is useful to develop new microsatellite markers in non-model organisms in a shorter time and at relatively low expense compared with traditional methods. The new microsatellite markers developed in this study will be particularly important in order to understand the settlement and maintenance of two non-model invasive species in the Mediterranean Sea. Invasive species are recognized as one of the major causes of biodiversity loss in addition to changes in ecosystem functioning and services (Millennium Ecosystem Assessment, 2005). HAB events involve approximately 2% of known marine species of which most (75%, 45–60 taxa) are Dinophyceae (Smayda, 1997). *Alexandrium* genus is among the most studied owing to the severe toxic events and because it exhibits enhanced fitness parameters in new colonized habitats (Jauzein et al., 2008; Anderson et al., 2012; Hadjadji et al., 2012). Microsatellite markers, commonly used as tools to describe population genetics of macroorganisms (Jarne and Lagoda, 1996) are now considered tools of choice for describing microorganisms' ecology and evolution (Pettay and LaJeunesse, 2013). Therefore, developing microsatellite markers to describe this toxic and invasive genus with the most precision as possible should be a priority.

At this point, population genetics studies performed across different geographic scales have highlighted a complex intraspecific diversity within the *Alexandrium* genus (Nagai et al., 2007; Masseret et al., 2009; Erdner et al., 2011; Casabianca et al., 2012; Richlen et al., 2012). However, the microsatellite markers presently available show lack of success in describing genetic diversity within the *A. catenella* (IV) population from Thau lagoon (Masseret et al., 2009) and does not amplify in *A. tamarense* (III). The new microsatellite markers developed in this study would help us explore (i) genetic diversity linked to reproduction modes (vegetative and asexual reproduction) and local selection processes, and (ii) population structure within both species' populations at different scales, in our local context, in the Mediterranean area and elsewhere. It is of interest to know if these new microsatellite markers can amplify other strains of each of the two considered species. Cross-priming has been successfully tested on a wider range of *A. catenella* (IV) strains isolated from sediments of Algeria and Tunisia (unpublished data). Concerning *A. tamarense* (III), no large scale experimentation has yet been performed. However, three microsatellite markers showed cross-priming between both species tested in this study and should therefore amplify on other strains of *A. tamarense*. Furthermore, cross-priming often works inside the *A. tamarense* complex. For example, microsatellite markers developed on *A. tamarense* amplify correctly for *A. fundyense* strains (Erdner et al., 2011) and several markers developed from

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