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The use of flow cytometry for species identification and life-cycle studies in dinoflagellates

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ABSTRACT

The difficulties encountered in attempts to differentiate between dinoflagellate species of the genera *Alexandrium* and *Karlodinium* using morphological characteristics are well-known. For this reason, species of these genera were analyzed by flow cytometry to determine whether haploid DNA content served as a valid criterion for species identification. The DNA content of species often confused with each other due to their overlapping size and geographical occurrence, such as *Alexandrium ostenfeldii* and the complexes *Alexandrium catenella*, *Alexandrium tamarense*, *Alexandrium minutum* and *Alexandrium tamutum*, and *Karlodinium veneficum* and *Karlodinium armiger* were analyzed. These species differed greatly in DNA content, which provided a means of distinguishing among them. The only cases of DNA overlap involved *A. ostenfeldii* with *Alexandrium peruvianum*, and *A. catenella* with *A. tamarense*, two groups not yet clearly established either morphologically or genetically. Variability in intraspecies DNA content was observed only in the species *K. veneficum*. Significant differences between the two *A. tamarense* strains analyzed were not detected, and the haploid DNA content (63 pg cell^{-1}) was very different from the one reported previously for this species (103.5 pg cell^{-1}), suggesting cryptic speciation within this group. Flow-cytometric analysis of field samples identified *K. veneficum* as the causative species of a bloom, suggesting this method as a tool to readily identify species responsible for natural blooms. Additionally, after clonal cultures had been established, cytometric analyses corroborated the variability in the haploid DNA content of this species.

1. Introduction

The use of flow cytometry in biological research has expanded dramatically both in scope and in frequency since the initial applications of this technology in medical science (Robinson and Gregori, 2006). Flow cytometry was originally devised to identify and characterize cancerous cells through their DNA content, but it has now become increasingly useful in the fields of ecology, evolutionary biology and systematics. However, the use of this technique to study the dinoflagellate life cycle is relatively new.

Flow cytometry is a high-throughput analytical tool that simultaneously detects and quantifies multiple optical properties (fluorescence and light scatter) of single particles—usually cells whose nuclei have been labeled with fluorescent probes—as they pass in a narrow liquid stream through a powerful beam of light. The technique can therefore be employed to discriminate and quantify cells on the basis of their nuclear DNA content, and thus to distinguish between species and life-cycle stages of dinoflagellates.

Measurement of genome size, i.e, the DNA content of the unreplicated haploid genome (“holoploid” genome size), by flow cytometry is an indirect and relative method since the results are compared to those obtained with a standard of known DNA content (Greilhuber et al., 2005, Leitch and Bennet, 2004). The nuclei of dinoflagellates contain vast amounts of DNA compared to other eukaryotes. Estimates range from 3 to 250 pg · cell^{-1}, or approximately 3,000 to 215,000 megabases (MB). Human nuclei, in comparison, contain 3.2 pg · cell^{-1} (3,180 MB) (Hacket et al., 2004). The evolutionary forces accounting for genome-size variation can be reduced to two main arguments: (1) the difference is due to the accumulation of non-coding DNA (Doolittle and Sapienza, 1980; Ohno, 1972) with no selective value or (2) the difference in DNA content has phenotypic and fitness consequences and, thus, is adaptively significant. While it is clear that genome-size affects phenotype through the physical consequences of increased nuclear size and volume; whether it also accounts for variations in gene expression remains to be shown. The phenotypic effect of a large genome is no doubt important for dinoflagellates, which have large amounts of

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repetitive non-coding DNA in their huge genome (Hacket et al., 2004). In a study by LaJeunesse et al. (2005), relative genomes sizes were measured in dinoflagellates, using 18 cultured "free-living" species and 29 *Symbiodinium* spp. isolates. Genome size was found to directly correlate with cell size, as confirmed in several other reports (Rizzo, 1987; Veldhuis et al., 1997; Parrow and Burkholder, 2002) and which has been documented previously for most eukaryotes.

The question posed in this study was of a more precise and practical nature: If there are interspecific differences in the DNA content of dinoflagellates, can they be quantified in species of overlapping cell size and very similar morphology? This should indeed be possible, since the DNA content within individuals and species is constant (Swift, 1950), apart from chromosomal differences (aneuploidy, polyploidy, B-chromosomes and sex chromosomes), the presence of cryptic species (Greilhuberm, 1998), variations in chromosome size (Gregory, 2005) or a life-cycle stage with a different ploidy level, e.g., in a zygote (Figueroa et al., 2007). Furthermore, polyploidy has been suggested as a speciation mechanism in dinoflagellates and may well be the process behind the evolution of *Gymnodinium catenatum* from *Gymnodinium nolleri* (Bolch, 1999). Although *G. nolleri* is generally smaller than *G. catenatum*; there is some overlap in size and their morphologies are so similar that they have often been confused with each other (Ellegaard et al., 1998). These two species cannot be distinguished by standard flow cytometry, as it is not sensitive enough to discriminate among cells in this size range (under 70 μm) or to recognize cells that have formed chains. However, the speciation mechanism of *Gymnodinium* may be commonplace, and may have occurred also in *A. minutum* and *A. tamutum* (Figueroa et al., 2007), which are, respectively, toxic and non-toxic blooming species with an overlapping size range. The morphologically similar to heterotrophic dinoflagellate species *Pfiesteria piscicida*, *P. shumwayae* and *Cryptoperidiniopsis sp*. have been distinguished from one another based on the DNA content measured with flow cytometry (Parrow and Burkholder, 2003).

We chose to test the hypothesis that species can be distinguished based on DNA content using the genus *Alexandrium*. This genus consists of 29 described species, 8 of which are toxic and produce potent neurotoxins that cause outbreaks of paralytic shellfish poisoning (PSP) in many coastal waters, and thus represent a public-health risk in addition to having an economic impact on aquaculture. Previous attempts to discriminate between species of *Alexandrium* by routine monitoring of cell counts have been inconclusive because of similarities in cell size and general morphology. Moreover, in some cases, even the resting cysts are morphologically identical.

Proper identification of different life-cycle stages is essential in understanding both the ecology and the biology of dinoflagellates. These organisms are haploid, but facultative sex has been described in an increasing number of species. Therefore, in addition to a mixed vegetative population, the water column may contain diploid mobile zygotes as well as diploid vegetative stages resulting from DNA replication prior to mitosis. Life-cycle stage transitions may imply a shift to a different space-time environment (niche), whereas sexual processes and the associated genetic changes may support algal survival strategies (Steidinger and Garcés, 2006). However, the difficulty in differentiating sexual stages from vegetative ones is well-known. Morphological identification is difficult, time-consuming and practically impossible in fixed samples, thus necessitating the development of different tools to identify and quantify zygotes. Recently, a method to quantify culture-formed zygotes of *Alexandrium* by flow cytometry was described by Figueroa et al. (2007).

Dinoflagellate species have traditionally been classified into two mating-type groups: species whose clones are capable of self-cyst production (homothallism), such as *Alexandrium taylori* (Giacobbe and Yang, 1999), and species whose sexuality requires two compatible clonal strains, one plus (+) type and one minus (−) type (heterothallism), as is the case for *A. catenella* (Yoshimatsu, 1984) and *Lingulodinium polyedrum* (Figueroa and Bravo, 2005). The complex sexuality showed by some dinoflagellates (for ex. Blackburn et al., 2001; Figueroa et al., 2007), led us to examine whether flow cytometry could discriminate between homothallic and non-homothallic species. Accordingly, in the present work, we examined the ability of flow cytometry to identify the mating-type group of a given species.

To summarize, the main objective of the present study was to establish valid criteria to differentiate between dinoflagellate species, with the genus *Alexandrium* serving as a model system, by using flow cytometry to measure the haploid DNA content. We then studied the potential of this technique to discriminate between morphologically similar species in a natural bloom as well as among dinoflagellate life-cycle stages.

2. Methods

2.1. Culture conditions, strains and species tested

The strains employed in this study are listed in Table 1. Cultures were grown at 20°C under an illumination of approx. 90 μmol photons $m^{-2}\,s^{-1}$ with a photoperiod of 12:12 h L:D

Table 1
Isolation and history of the strains.

Species	Strain name	Clonal history	Origin	Year of isolation
Alexandrium andersoni	NG1 CSIC	Yes	Catalan Coast (Spain)	2003
Alexandrium margalefi	VGO 763	No	Harbour of Vilanova (Spain)	2003
	VGO 661	No	Alfacs (Spain)	2003
Alexandrium minutum	VG0 A7	Yes	Vigo (Spain)	2005
	VGO B12	Yes		2005
Alexandrium ostenfeldii	FAL 50	Yes	Falmouth (UK)	2004
Alexandrium peruvianum	VGO 10C	Yes	Spain	2002
	VGO D12	Yes		
Alexandrium tamarense	ATA C2	Yes	Tyrrhenian Coast of Sicily (Italy)	1999
	CCMP 1493	No	Da Ya Bay (China)	1991
Alexandrium tamutum	VGO 615	Yes	Tarragona (Spain)	2002
	VGO A8	Yes	Tarragona (Spain)	1997
Alexandrium taylori	CNRAT4	Yes	Syracuse (Italy)	1997
Karlodinium veneficum	GC4 IRTA	Yes	Alfacs Bay (Spain)	2000
	GC5 IRTA	Yes	Alfacs Bay (Spain)	2000
Karlodinium armiger	GC3 IRTA	Yes	Alfacs Bay (Spain)	2000
	GC7 IRTA	Yes	Alfacs Bay (Spain)	2000