

Development of Real-Time PCR Assays for the Detection of *Chattonella* Species in Culture and Environmental Samples

Holly Bowers¹, Torstein Tengs², Sayaka Goto¹, Carmelo Tomas³, Chitari Ono⁴, Sadaaki Yoshimatsu⁴, and David Oldach¹

¹University of Maryland, Institute of Human Virology, 725 W. Lombard St. Baltimore MD 21201, USA;

²University of Oslo, P.O. Box 1031 Blindern 0315, Oslo NORWAY; ³University of North Carolina at Wilmington, 5600 Marvin K. Moss Ln., Wilmington, NC 28409, USA; ⁴Akashiwo Research Inst. of Kagawa Prefecture, 75-5 Higasi-machi, Takamatsu Kagawa 761-0111 JAPAN

Abstract

Raphidophytes have been associated with fish kill events in Japanese, European and U.S. coastal waters, and many produce toxins that can pose a threat to human health. The recognition of raphidophytes as HAB species in mid-Atlantic estuarine waters led us to initiate molecular phylogenetic analyses of these taxa and to develop real-time PCR assays for rapid detection of important species. The molecular studies and PCR detection methods will enhance ongoing taxonomic, toxicologic and ecological assessment of these organisms and will be a useful tool in HAB monitoring programs.

Introduction

Chattonella subsalsa (Raphidophyceae) was first described by Biechler (1936) and since that description, several other *Chattonella* species have been identified: *C. antiqua* (Ono *et al.*, 1980), *C. globosa* (Hara *et al.*, 1994), *C. ovata* (Hara *et al.*, 1994), *C. marina* (Hara and Chihara, 1982) and *C. verruculosa* (Hara *et al.*, 1994). Other notable species in the family Raphidophyceae include *Fibrocapsa japonica* (Toriumi and Takano, 1973) and *Heterosigma akashiwo* (Hada, 1967). Effects of raphidophyte species on fish health vary (Schimada *et al.*, 1983, Onoue *et al.*, 1989, Chang *et al.*, 1990, Tanaka *et al.*, 1994, Ahmed *et al.*, 1995). Fish kills attributed to *Chattonella* spp. have been reported from inland seas of Japan (Okaichi, 1983), and in 1990, fish kills in New Zealand, Chile and British Columbia were associated with *Heterosigma* spp. (Chang *et al.*, 1990). Blooms of raphidophyte species have also occurred in the coastal waters of Florida (Tomas, 1998) and along the eastern seaboard. Microscopic methods have been traditionally utilized for identifying raphidophyte species in environmental water samples, however, these cells do not preserve well and may alter their morphology when disturbed by discharging ejectosomes, trichocysts and mucocysts (Hallegraeff *et al.*, 1995). Vrieling *et al.* (1995) developed antibodies against Japanese strains of *Chattonella*, while Murayama-Kayano *et al.* (1998) detected differences between species and strains of cultured *Chattonella* spp. using the random amplified polymorphic DNA technique. More recently, Tyrrell *et al.* (2001) adapted a sandwich hybridization assay for detecting *H. akashiwo* and *F. japonica*, and Connell (2000, 2002) designed primers targeting the ITS region of the raphidophyte genome.

We utilized Taqman[®] PCR technology (Wittwer *et al.*, 1997, Bowers *et al.*, 2001) which involves labeling a probe complementary to the PCR-amplified target sequences. Previous assays developed by our laboratory were based on this technology and have proven successful in detecting other harmful algal species in culture and environmental samples (Bowers *et al.*, 2000, Tengs *et al.*, 2001, Jakobsen *et al.*, 2002). PCR-based assays, coupled with morpholog-

ical descriptions, are strong tools for identifying organisms in culture and assessing diversity in environmental samples. Furthermore, they can aid in determining whether closely related species are actually the same organism or if strain variation is apparent at the genetic level.

Materials and Methods

Samples and DNA Extraction A commercially available kit (Puregene, DNA Isolation Kit; Gentra Systems) was used for extraction of total DNA from an environmental sample collected from a *Chattonella* bloom in Arnell Creek, and other raphidophyte cultures (Table 1).

PCR-Primer Design, Cloning and Sequencing A matrix was generated (MacClade 4.04; Sinauer Associates, Inc.) from raphidophyte 18S sequence data downloaded from GenBank: *Chattonella subsalsa* (U41649), *Heterosigma carterae* (L42529), *Heterosigma akashiwo* (AB001287) and Heterokont/Stramenopile 18S sequence data downloaded from an SSU rRNA database (www.rrna.uia.ac.be). Raphidophyte group-specific primers were designed and used with eukaryotic-specific primers designed by others (Medlin *et al.*, 1988) and our laboratory (unpublished) to obtain sequence data from four CCMP cultures (*C. marina* 2049, *C. antiqua* 2050, *C. sp.* 218, *F. japonica* 1661), five *C. subsalsa* cultures, a *C. marina* culture, three *C. verruculosa* cultures, and the environmental sample. 50 µL PCR reactions were performed, gel purification was utilized on target bands using a commercially available kit (MinElute[™] Qiagen), and the TOPO TA Cloning[®] kit (Invitrogen) was used for ligation and transformation. PCR products were further purified using a Performa[™] DTR gel filtration column (Edge Biosystems) and sequenced using the DYEnamic[™] ET Terminator Cycle Sequencing kit (Amersham Pharmacia Biotech, Inc.).

Phylogenetic Analysis An alignment was made using ClustalX (Thompson *et al.*, 1997), and phylogenetic analyses were performed using PAUP* (Swofford, 2002; Figure 1). A minimum evolution (ME) tree was made using Kimura 2-parameter (K2P) distances and random addition of the

Table 1 Specificity testing of various raphidophyte real-time PCR assays. Species-specific real-time PCR assays were developed for *C. “Delaware”* (based on a novel raphidophyte sequence derived from an environmental sample), *C. cf. verruculosa* (*C. Tomas* isolate), *Chattonella verruculosa* (strain KA-GAWA 111), and *C. subsalsa* (CCMP 217). The assay developed for *C. subsalsa* does not differentiate between *C. subsalsa*, *C. marina*, *C. antiqua* and *C. sp.*, and has been designated as a “*Chattonella* species” (*C. sp.*) assay. *18S sequence data from these cultures used to design real-time PCR assays.

Culture/Source	Morphological ID	Location	Real-Time PCR Results			
			<i>C. “Delaware”</i>	<i>C. cf. verr.</i>	<i>C. sp.</i>	<i>C. verr.</i>
CCMP 2049	<i>C. marina</i>	North Pacific, Japan	NEG	NEG	POS	NEG
S. Yoshimatsu	<i>C. marina</i>	Japan	NEG	NEG	POS	NEG
CCMP 2050	<i>C. antiqua</i>	North Pacific, Japan	NEG	NEG	POS	NEG
CCMP 2052	<i>C. antiqua</i>	Mikawa Bay, Japan	NEG	NEG	POS	NEG
S. Yoshimatsu	<i>C. antiqua</i>	Japan	NEG	NEG	POS	NEG
CCMP 216	<i>C. cf. ovata</i>	North Pacific, Japan	NEG	NEG	POS	NEG
CCMP 218	<i>C. sp.</i>	North Pacific, Japan	NEG	NEG	POS	NEG
CCMP 1596	<i>H. akashiwo</i>	Narragansett Bay, RI	NEG	NEG	NEG	NEG
CCMP 1680	<i>H. cf. akashiwo</i>	Sandy Hook Bay, NJ	NEG	NEG	NEG	NEG
CCMP 1870	<i>H. akashiwo</i>	Los Angeles River, CA	NEG	NEG	NEG	NEG
CCMP 1912	<i>H. akashiwo</i>	Kalalich, WA	NEG	NEG	NEG	NEG
CCMP 302	<i>H. akashiwo</i>	Milford Sound, New Zealand	NEG	NEG	NEG	NEG
CCMP 452	<i>H. carterae</i>	Long Island Sound	NEG	NEG	NEG	NEG
CCMP 1661	<i>F. japonica</i>	Australia	NEG	NEG	NEG	NEG
C. Ono	<i>C. verruculosa</i>	Japan	NEG	NEG	NEG	POS
J. Goebel	<i>C. verruculosa</i>	Japan	NEG	NEG	NEG	POS
C. Ono/S. Yoshimatsu	* <i>C. verruculosa</i>	Japan/KAGAWA 111	NEG	NEG	NEG	POS
C. Tomas	* <i>C. cf. verruculosa</i>	Delaware	NEG	POS	NEG	NEG
CCMP 217	* <i>C. subsalsa</i>	Gulf of Mexico	NEG	NEG	POS	NEG
C. Tomas	<i>C. subsalsa</i>	Rehoboth Bay, DE	NEG	NEG	POS	NEG
C. Tomas	<i>C. subsalsa</i>	Salton Sea, CA	NEG	NEG	POS	NEG
C. Tomas	<i>C. subsalsa</i>	Corpus Christi, TX	NEG	NEG	POS	NEG
C. Ono	<i>C. subsalsa</i>	Japan (NIES culture)	NEG	NEG	POS	NEG
M. Holms	<i>C. subsalsa</i>	Singapore	NEG	NEG	POS	NEG
Environmental sample	N/A	Arnell Creek, Delaware	POS	N/A	N/A	N/A

sequences using tree-bisection-reconnection branch swapping. Ten random additions were done, and the same topology was found every time. Maximum likelihood values for a general-time reversible substitution matrix, gamma distribution (4 rate categories), base frequencies and proportion of invariable sites were estimated simultaneously using the K2P topology, and a new ME tree was made using maximum likelihood distances and these parameters. Ten random additions of sequences were done using TBR branch swapping, and the same tree was found in all searches. Bootstrapping was done (100 pseudo replicates) using the same model.

Real-Time PCR Design and Validation Based on the sequence matrix, group-specific primers were designed to be used in conjunction with species-specific Taqman® probes for *C. “Delaware”* (based on the novel sequence derived from the environmental sample), *C. cf. verruculosa* (*C. Tomas* isolate), *C. subsalsa* (CCMP 217) and *C. verruculosa* (strain KAGAWA 111).

Results and Discussion

Raphidophyte blooms in US coastal waters with associated fish kills prompted our laboratory to design PCR-based assays to rapidly and specifically detect these species, avoiding problems associated with distortion of cells by fixatives. Our strategy involved developing common primers and unique probes for each species based on real-time Taqman® PCR technology (see Bowers *et al.*, 2000 and Tengs *et al.*, 2001). Using all available raphidophyte cultures for validation, we found the *C. verruculosa* and *C. cf. verruculosa* assays to be

highly specific, and validation will be ongoing as more isolates become available. Since there were no morphological data or culture isolates derived from the environmental sample collected from the Arnell Creek bloom, we will need to continue to screen cultures with the *C. “Delaware”* assay to identify the source organism. Based on the cross-reactivity of the assay designed for *C. subsalsa* and subsequent sequence data, 18S SSU rRNA is not a feasible target for differentiating *C. subsalsa* (not known to produce toxins) and *C. marina* (a toxin producer), which differ by only 0.01% across the 18S SSU sequence and interestingly are morphologically difficult to distinguish. Preliminary data suggest that ITS1 and ITS2 may be variable enough between these two species to permit using this region for assay development (data not shown). This approach was utilized by Connell (2002) to identify raphidophyte species in fresh and archived material. Additionally, we have generated plastid 16S sequence data from the majority of these cultures and believe that this locus has sufficient heterogeneity to allow development of species-specific assays (see Tengs *et al.*, 2001). Our assay, designed to be specific for *C. subsalsa*, also cross-reacted with three cultures of *C. antiqua* and an isolate identified as *C. sp.* deposited at CCMP. Sequencing of the 18S region revealed that these sequences are 100% identical to *C. marina*, and further supports the need for targeting the ITS region for assay development. The genetic observations presented here clearly illustrate the need for coupling molecular assays with morphological descriptions to verify whether organisms that are difficult to distinguish are in fact two separate species or are demonstrating strain variation. Once

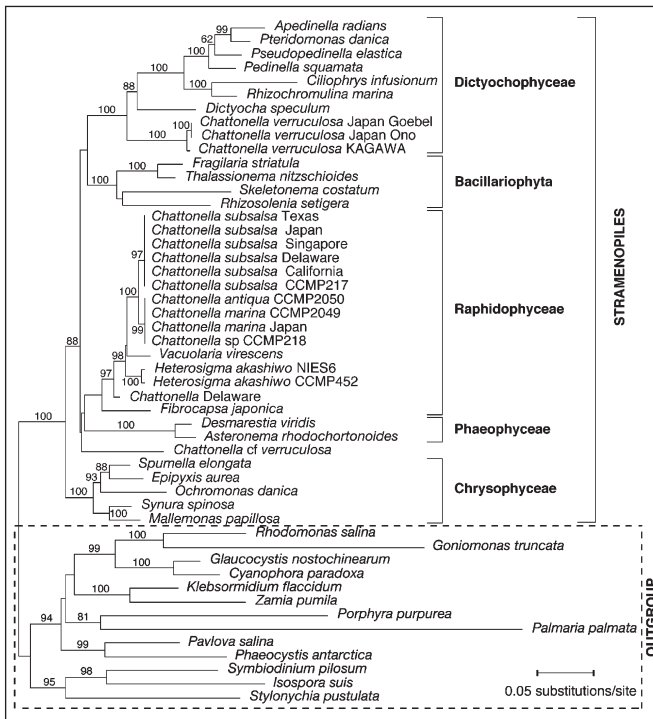


Figure 1 Phylogenetic distance analyses of SSU rRNA sequences from raphidophytes and other protists. Bootstrap values above 75% are indicated.

validated, these assays can be utilized on archived samples to better understand temporal and spatial distributions (*i.e.*, bloom dynamics and consequences) of these species. Interestingly, the phylogenetic analyses suggest that *C. verruculosa* is more likely a member of the Dictyochophyceae. We acknowledge Dr. Daisuke Honda for first bringing the Dictyochophyceae character of *C. verruculosa* to our attention, based on his own detailed morphologic and sequencing studies (Honda, pers. comm.) and await his publication of this work with great interest. Finally, we note that the novel brevetoxin-producing *Chattonella*-like organism isolated by C. Tomas (labeled *C. cf. verruculosa* in Fig. 1) remains phylogenetically ambiguous. In BLAST searches, the 18S sequence of this organism is most closely related to the freshwater raphidophyte *Vacuolaria virescens*. However, placement of the sequence in phylogenetic analysis is unstable, pointing to the need for further morphologic analysis and greater taxon sampling.

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