

Kryptoperidinium foliaceum blooms in South Carolina: a multi-analytical approach to identification

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Abstract

Observations following the discovery of *Kryptoperidinium foliaceum* blooms in South Carolina (SC), USA, suggest that a multi-analytical approach, using a standard, minimal set of criteria, should be adopted for determining dinoflagellate species identity and taxonomic placement. A combination of morphological, molecular, and biochemical analyses were used to determine the identity of this “red tide” dinoflagellate, first documented in SC waters in the spring of 1998. Results from thecal plate tabulations (based on scanning electron and epifluorescence microscopy), gene sequence data, species-specific PCR probe assays, and microalgal pigment profiles were analyzed and compared to reference cultures of *K. foliaceum*. Comparative data showed marked inconsistencies among the *K. foliaceum* reference culture isolates. In addition, the SC bloom isolate was shown to be mononucleate, contrary to previous reports for *K. foliaceum*, suggesting a more transient endosymbiotic association than previously considered.

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1. Introduction

In Spring 1998, dense blooms of a small lightly armored dinoflagellate were observed in Bulls Bay, South Carolina, the first documentation of a “red

tide” localized to SC marine or estuarine waters (Lewitus et al., 2001). Blooms have since been observed with increased frequency and geographic range, and generally occur from early spring to mid-summer. Cell densities have reached as high as 3.5×10^5 cells ml⁻¹ and many blooms appear to be monospecific. Exposure to blooms can cause physiological stress in the eastern oyster, *Crassostrea virginica*, as indicated by elevated measurements of

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percent lysosomal destabilization (Lewitus et al., in press).

Wolny et al. (unpublished data) used a multi-analytical approach, evaluating multiple morphological characteristics and biochemical markers, and a molecular approach targeting multiple gene regions, to confirm the identity of a bloom isolate as *Kryptoperidinium foliaceum* (Stein) Lindemann (Syn. *Peridinium foliaceum* Biecheler, *Glenodinium foliaceum* Stein, *Phyllocladon scutellaris* Conrad). However, the authors noted inconsistencies between morphological and molecular taxonomic criteria when the SC isolate was compared to *K. foliaceum* strains from various culture collections. Although SSU rDNA sequence data for the SC isolate showed 100% similarity to a known strain of *P. foliaceum* (UTEX LB 1688), the latter had an endosymbiotic nucleus, whereas the SC bloom isolate did not. These apparent discrepancies prompted re-evaluation of species identification within the genus *Kryptoperidinium*, with emphasis on the complement of thecal plate tabulation and DNA sequence data as corroborative taxonomic criteria (Montresor et al., 1999; Daugberg et al., 2000).

In this study, morphological, molecular, and biochemical assays were used to compare the SC *K. foliaceum* isolate with four cultures of *K. foliaceum* maintained in culture collections worldwide. Widespread inconsistencies in taxonomic features were noted between culture isolates. This information draws concern over the proper identification of *K. foliaceum* cultures used for reference purposes. The morphological discrepancy between the binucleate *K. foliaceum* reported in the literature and the mononucleate SC *K. foliaceum* isolate raises questions about the association of *K. foliaceum* with its diatom endosymbiont.

2. Materials and methods

2.1. Sample collection and culturing

Four cultures of *K. foliaceum* were used as reference strains for morphological, molecular, and biochemical analyses. Culture UTEX LB 1688 (*P. foliaceum*) was provided by Dr. Peter Rizzo, Texas A&M (originally isolated from Puerto Rico). Culture CCMP 1326 (*P.*

foliaceum) was purchased from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (originally isolated from Scripps Pier, California, USA). Culture CCAP 1116/3 (*G. foliaceum*) was purchased from the Culture Collection of Algae and Protozoa, UK (originally isolated from York River, Virginia, USA). Culture CS 291 (*K. foliaceum*) was purchased from the Australian Commonwealth Scientific and Industrial Research Organization (CSIRO; originally isolated from Port Phillip Bay, Australia). All environmental samples were collected as surface water samples in 1-l acid washed sample bottles. Through the monitoring efforts of the South Carolina Harmful Algal Bloom Program, >10³ whole water samples were collected from January 2001 through April 2002, and screened for harmful algal species based on light microscopy.

2.2. Epifluorescence and scanning electron microscopy

Culture material of *K. foliaceum* strains and field bloom material were stained with Calcofluor White to observe thecal plate structure and patterns, using a modified method of Fritz and Triemer (1985) and Andersen and Kristensen (1995). Observations and digital photographs were made on an Olympus B-Max 50 microscope equipped with a Nikon DXM 1200 digital camera and ACT-1 software. Additional observations and digital photography were done using a Zeiss Axiovert 100 microscope equipped with the Zeiss AxioCam and AxioVision software.

Scanning electron microscopy on *K. foliaceum* cells was performed in addition to light and epifluorescence microscopy. Cells were fixed overnight at 4 °C in a 1% OsO₄ solution made with filtered site water (FSW). Cells were then washed with FSW, sent through a graded ethanol dehydration series, and stored overnight at 4 °C in 70% ethanol. The cells were then rinsed with 100% ethanol three times, followed by a freon TF 2:1, 1:1, 1:2 series and 100% freon rinse two times. Cells were critical point dried after being rinsed with CO₂, sputter coated with gold-palladium, and observed on a Cambridge Stereoscan 240 scanning electron microscope. Comparisons were made with published reports and taxonomies for *P. foliaceum*, *K. foliaceum*, and *G. foliaceum*.

2.3. Dinokaryon and endosymbiont nuclei visualization

K. foliaceum cells were centrifuged down and re-suspended in a small volume of fresh culture medium. Depolymerized paraformaldehyde was added dropwise to a final concentration of 2% and put on a rocker table for 2 h. Cells were concentrated by centrifugation and re-suspended twice in 30:70 EtOH:DW to remove residual seawater salts and formaldehyde. Re-suspended cells were stored at -20°C . Once the cells were fixed, they were concentrated and re-suspended in 30:70 EtOH:DW containing 0.1% Triton-X 100 to wash out lipids, chlorophyll, and other pigments. Cells were again concentrated and re-suspended in 30:70 EtOH:DW. The fluorochrome DAPI was added to a final concentration of approximately $1\ \mu\text{g ml}^{-1}$, and cells were stained for 10 min. Cells were then concentrated and re-suspended in the 30% ethanol rinse solution to rinse out excess DAPI. Cells were rinsed and concentrated in $100\ \mu\text{l}$ of rinse solution plus $5\ \mu\text{l}$ of Molecular Probe's Slow Lightfade 2X reagent (Molecular Probes, Eugene, OR) without glycerol to get a maximum number of cells for viewing. Mounted slides were visualized with a Zeiss Axiomat III epillumination microscope with an Atoarc lamp system using a $60\times$ fluorite oil immersion objective for the maximum resolution and fluorescent brightness. Images were taken using an Olympus BX 50 microscope equipped with a Nikon DXM 1200 digital camera and ACT-1 software.

2.4. Primer selection—*K. foliaceum*-specific assay

K. foliaceum-specific primers were selected from variable regions of the *K. foliaceum* SSU rDNA sequence, accession no. AF231804 (Inagaki et al., 2000; culture UTEX LB 1688). Primer sequences were selected by aligning the 18S rDNA sequence for *K. foliaceum* with other SSU rDNA dinoflagellate sequences to select candidate primers for PCR based on sequence variability. Candidate primers were then screened against all known sequences in GenBank using the BLAST program. Primer specificity was evaluated by testing candidate primers with UTEX LB 1688 as a positive control, and an array of dinoflagellate negative controls and environmental water samples. All PCR amplicons were sequenced for confirmation.

Table 1

Oligonucleotide primers for DNA sequencing and the *K. foliaceum*-specific PCR assay

| Primer name | Sequence 5'–3' |
|---------------------------------------|--------------------------|
| Sequencing primers | |
| 18S F | AACCTGGTTGATCCTGCCAGT |
| 18S R | TGATCCTTCTGCAGGTTACCTAC |
| Dino F | CGATTGAGTGATCCGGTGAATAA |
| LSU380 R | TTTCATCTTTCCCTCACGGTACTT |
| LSU1 R | ATATGCTTAAATTCAGCGGGT |
| <i>K. foliaceum</i> -specific primers | |
| <i>K. foli</i> 236 F | TGATGCTTTGGTGAGTGATAGT |
| <i>K. foli</i> 1693 R | CCTTCCAGGAAGTGGTAAATG |

2.5. DNA isolation and PCR amplification—*K. foliaceum*

All DNA extractions were carried out using either a rapid cetyltrimethylammonium bromide (CTAB) buffer DNA isolation technique (Schaefer, 1997) or by filtering samples onto $5\ \mu\text{m}$ glass microfibers and using a DNeasy plant mini kit (QIAGEN). After testing candidate primers for the *K. foliaceum*-specific PCR assay, the primer pair *K. foli* 236 F and *K. foli* 1693 R were selected (Table 1). A MJ Research PTC-100 Programmable Thermocycler was used under the following PCR reaction conditions: 94°C for 2 min; then 39 cycles (94°C for 1 min, 60°C for 1.5 min, 72°C for 2 min); followed by a 72°C extension for 5 min. Reactions were carried out using a final concentration of 2.5 mM MgCl_2 , 0.2 μM of each primer, and 10 μM bovine serum albumin in 50 μl reactions.

2.6. PCR and sequencing—*K. foliaceum*

PCR amplicons from the *K. foliaceum*-specific PCR assay were sequenced for confirmation at the Biotechnology Resources Laboratory, Medical University of South Carolina (MUSC). SSU rDNA sequence data for *K. foliaceum* were derived using the universal primers 18S F and 18S R (Medlin et al., 1988; Sogin, 1990) coupled with the species-specific internal primers (Table 1). Internal transcribed spacer (ITS) 1, 5.8S, and ITS2 rDNA sequence data were generated using the dinoflagellate-specific forward primer Dino F (Oldach et al., 2000) coupled with either the general reverse primer LSU380 R or the reverse primer

LSU1 R. The reaction conditions were the same conditions used for the *K. foliaceum*-specific PCR assay. All sequences were aligned using DNASIS v. 3.6.

2.7. High performance liquid chromatography (HPLC)

The protocol used for HPLC analyses followed Van Heukelem and Thomas (2001). Water samples from cultured isolates and field samples were filtered onto 25 mm glass fiber filters (GF/F), flash frozen in liquid nitrogen and extracted in 1 ml of 100% HPLC-grade acetone for 1 h. Pigments were separated on an Agilent Technologies™ 1100 HPLC system using a reverse-phase C8 column, and quantified with a UV-VIS diode array detector. Individual pigment spectra were identified (HPCHEMstation software, Agilent Technologies Inc.), and calibrated pigments were quantified (ng ml⁻¹ filtered water).

3. Results

3.1. Morphological comparisons—*K. foliaceum*

P. foliaceum cultures UTEX LB 1688 and CCMP 1326, *K. foliaceum* culture CS 291, and *G. foliaceum*

culture CCAP 1116/3 were compared to the SC *K. foliaceum* isolate using light, epifluorescence, and scanning electron microscopy. The combination of morphological analyses allowed us to determine cell shape and size, orientation, and plate configurations. Initial investigations with light microscopy indicated that all *K. foliaceum* strains had the same cell shape; rounded-to-pointed epithecas with rounded hypothecas and dorsoventral compression (Table 2). Strains CCMP 1326, CCAP 1116/3, and the SC isolate all had extremely convex dorsal surfaces and concave ventral surfaces. Because of the concavity and convexity of the cells, along with the dorsoventral compression, strains CCMP 1326 and CS 291 appeared to have lobed hypothecas.

The eyespot, which is often made reference to in *K. foliaceum* literature, was conspicuous and ‘comma’ shaped in both the CCAP 1116/3 and SC isolates (Table 2). The bright red eyespots in these two strains were located in the hypotheca of the cell and ‘hooked’ inward. CS 291 has a round, reddish-orange eyespot located in the epitheca/hypotheca junction. Eyespots were absent from the cultures of UTEX LB 1688 and CCMP 1326.

Using epifluorescence and scanning electron microscopy, we determined the plate configurations for the *K. foliaceum* strains. Data for the following key

Table 2

Comparison of morphological characteristics for *P. foliaceum*, *K. foliaceum*, and *G. foliaceum* cultures, and the SC *K. foliaceum* isolate

| Isolate | UTEX LB 1688 | CCMP 1326 | CCAP 1116/3 | CS 291 | SC isolate |
|-----------------------|---------------------------|--|--|---|--|
| Diameter (μm) | | | | | |
| Median | 37.0 | 37.2 | 38.8 | 36.4 | 26.6 |
| Range | 17–53 | 17–54 | 18–60 | 15–47 | 10–50 |
| Number | 44 | 39 | 26 | 25 | 100 |
| Cell shape | Dorsoventrally compressed | Dorsoventrally compressed with extreme concavity and convexity | Dorsoventrally compressed with extreme concavity and convexity | Dorsoventrally compressed | Dorsoventrally compressed with extreme concavity and convexity |
| Eye spot | Absent | Absent | Comma shaped, located in hypotheca | Round, located at epitheca/hypotheca junction | Comma shaped, located in hypotheca |
| Cingular displacement | Descending | Descending | Ascending | Ascending | Descending |
| Thecal plates | | | | | |
| 1' | Ortho, wide, asymmetrical | Ortho, narrow, symmetrical | Ortho, wide, asymmetrical | Ortho, wide, asymmetrical | Ortho, wide, asymmetrical |
| 1a | Long, six-sided | Short, five-sided | Long, six-sided | Long, six-sided | Long, six-sided |
| 2a | Large, six-sided | Large, six-sided | Large, six-sided | Large, six-sided | Large, six-sided |
| c | 4 | 4 | 5 | 4 | 4 |

diagnostic plates are presented in Table 2: first apical ($1'$), anterior intercalaries (a), and cingular (c). More work needs to be done on the dinoflagellates used in this study with respect to the apical pore complex (apc) and sulcal plate configurations. *P. foliaceum* strain CCMP 1326 exhibited a high degree of variability in culture at several salinities (24, 31 and 36 psu) and may not provide the most reliable data for plate patterns.

Our observations indicated a similar plate formula for all strains tested (Po X $4'$ 2a $7''$ 4 or 5c 5s $5'''$ $2''''$). The *K. foliaceum* strains all had a canal plate (X) and a small inset “D” shaped pore that lacked a collar. All strains had ortho arranged $1'$ plates (Fig. 1). The $1'$ plates of CCMP 1326 was narrow and symmetrical (Fig. 1B), while all other strains had wide and asymmetrical $1'$ plates (Fig. 1A, C–E). All strains con-

tained two anterior intercalary plates. The 1a plates were long and six-sided in UTEX LB 1688, CCAP 1116/3, CS 291, and the SC isolate. The 1a plate in CCMP 1326 was short and five-sided. All 2a plates were large six-sided plates that bisected the $3'$ and $4'$ plates. The cingular series of plates for all strains contained four plates, except CCAP 1116/3 which contained 5c plates. The cingulum in all strains but CCAP 1116/3 and CS 291 were displaced 0.5–1 times and descending. CCAP 1116/3 and CS 291 had 0.5–1 displaced, ascending cingulum.

Biecheler (1952) described only five series in her plate formula for *P. foliaceum*. Based on her formula of $4'$ 2a 6– $7''$ $5'''$ $2''''$, all four cultures and the SC isolate would be grouped together. Wolny et al. (unpublished data) described the SC strain with a more complete formula: Po X $4'$ 2a $7''$ 4c 4–5s $5'''$ $2''''$. This same

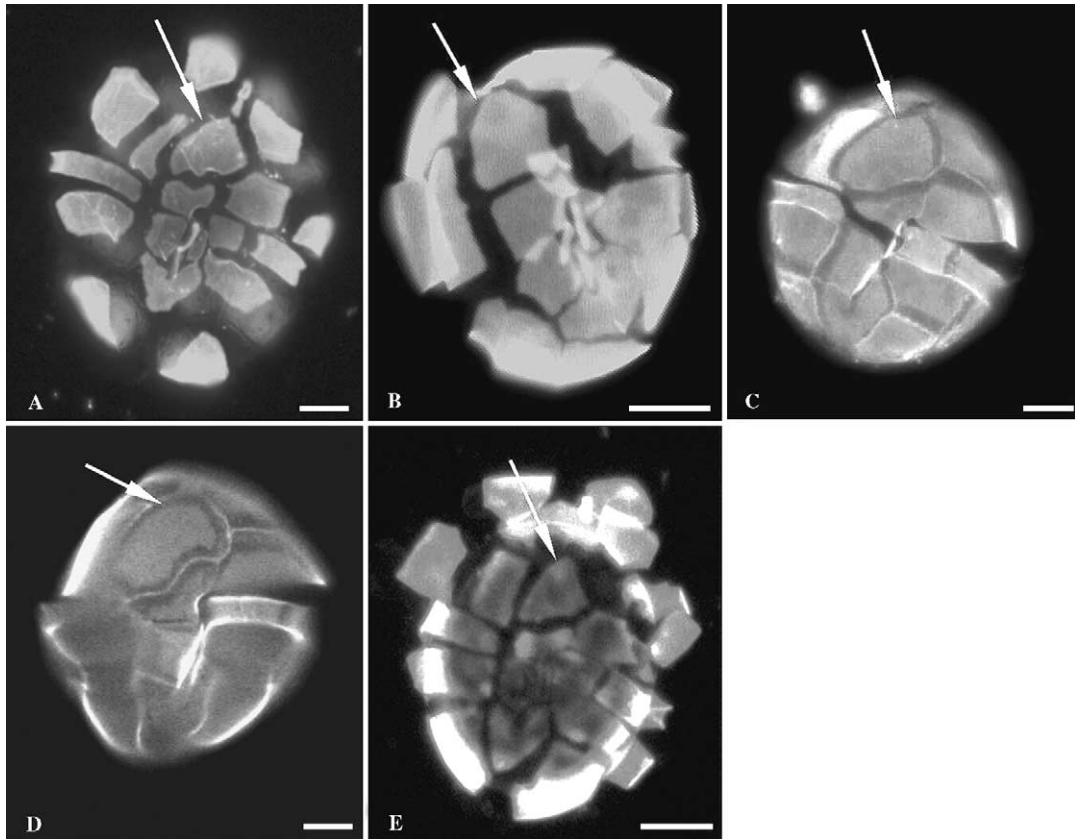


Fig. 1. Micrographs of Calcofluor White-stained cells highlighting the first apical plate (arrows): (A) UTEX LB 1688 *K. foliaceum*; (B) CCMP 1326 *P. foliaceum*; (C) CCAP 1116/3 *G. foliaceum*; (D) CS 291 *K. foliaceum*; (E) SC bloom isolate (*K. foliaceum*). Dimension bar represents 10 μm .

series was found in all strains but CCAP 1116/3, which had an additional cingular plate. When we examined the sizes, shapes, and orientations of the 1', anterior intercalaries, and 7'' plates, only the UTEX LB 1688 strain and SC isolate exhibited the descriptions similar to what Biecheler (1952) proposed for *P. foliaceum*.

3.2. Morphological comparisons—*K. foliaceum* endosymbiont

Dinokaryon and endosymbiont nuclei were stained with DAPI for testing the presence of the endosymbiont nucleus and for general morphological comparisons. DAPI stained nuclei from the four *K. foliaceum* cultures and the SC *K. foliaceum* isolate were compared. The *P. foliaceum* cultures UTEX LB 1688, CCMP 1326, *K. foliaceum* CS 291, and *G. foliaceum* CCAP 1116/3 had the endosymbiont nucleus in addition to the dinokaryon nucleus (Fig. 2A, Table 3). However, the endosymbiont nucleus was absent from the SC *K. foliaceum* isolate (Fig. 2B). In addition, morphologies of the endosymbiont nuclei were evaluated for the four culture isolates. The endosymbiont nuclei were all highly lobed in each of the cultures, however, the dinokaryon nucleus in CCAP 1116/3 appeared larger and stained brighter than the other culture isolates. The endosymbiont nucleus appeared to be highly lobed or fragmented because of the pigment extraction method used to aid visualization of the nuclei. The dinokaryon nucleus was spherical in each of the culture isolates, but was larger and 'bean-shaped' in the SC *K. foliaceum* isolate. Although the thecal plate arrangements for culture UTEX LB 1688 and the SC *K. foliaceum* isolate support their placement in *K. foliaceum*, the mononucleated morphology of the SC

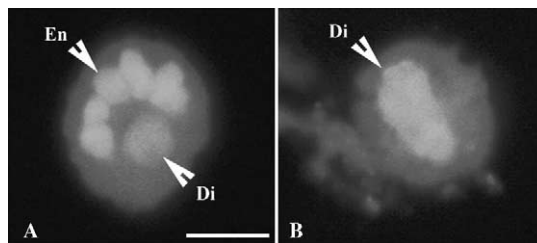


Fig. 2. (A) UTEX LB 1688 strain of *P. foliaceum* with DAPI stain showing the dinokaryon (Di) and endosymbiotic (En) nuclei. (B) South Carolina *K. foliaceum* with DAPI stain showing the dinokaryon (Di) nucleus. Dimension bar represents 10 μ m.

K. foliaceum isolate is inconsistent with descriptions for *K. foliaceum*.

3.3. Molecular comparisons—*K. foliaceum*

The PCR assay was used to confirm *K. foliaceum* culture designations and screen environmental water samples (Fig. 3). Primers *K. foli* 236 F and *K. foli* 1693 R amplified target DNA from *P. foliaceum* cultures UTEX LB 1688 and CCMP 1326 (lanes 2 and 3, respectively), but did not amplify DNA from *G. foliaceum* culture CCAP 1116/3 or *K. foliaceum* culture CS 291 (lanes 7 and 8, respectively). Isolated DNA from a *K. foliaceum* bloom in Bulls Bay, McClellanville, SC (SC isolate) during the spring of 2001 was amplified with the *K. foliaceum*-specific primers (lane 4), but DNA isolated from bloom events on the Cooper River (Charleston, SC) and a brackish retention pond on Hilton Head Island, SC did not amplify (lanes 5 and 6, respectively). Both bloom isolates appeared similar to *K. foliaceum* under light microscopy,

Table 3
Comparative summary for morphological, biochemical, and molecular data

| Isolate | UTEX LB 1688 | CCMP 1326 | CCAP 1116/3 | CS 291 | SC isolate |
|----------------------|--------------|-----------|-------------|---------|------------|
| Morphology | | | | | |
| Plate configuration | Same | Unique | Unique | Unique | Same |
| Endosymbiont nucleus | Present | Present | Present | Present | Absent |
| Biochemical | | | | | |
| Pigment profiles | Same | Same | Unique | Unique | Same |
| Molecular | | | | | |
| SSU rDNA | Same | Same | Unique | Unique | Same |
| ITS | Same | Same | Unique | Unique | Same |



Fig. 3. Gel electrophoresis of PCR products using *K. foliaceum*-specific primers (236F-1693R): (lane 1) Hi Lo™ DNA marker, 1400 bp indicated at left side of figure; (lane 2) UTEX LB 1688; (lane 3) CCMP 1326; (lane 4) SC bloom isolate; (lane 5) Cooper River, SC bloom; (lane 6) Hilton Head, SC isolate; (lane 7) CS 291; (lane 8) CCAP 1116-3; (lane 9) *Peridinium balticum*; (lane 10) *P. cinctum*; (lane 11) *Amphidinium carterae*; (lane 12) *Gymnodinium dorsum*; (lane 13) *Karenia mikimotoi*; (lane 14) *Linguldinium polyedrum*; (lane 15) *Cryptoperidiniopsis* sp.; (lane 16) *Pfiesteria piscicida*; (lane 17) *P. shumwayae*; (lane 18) *Oxyrrhis marina*; (lane 19) *Prorocentrum minimum*; (lane 20) *Scrippsiella trochoidea*; (lane 21) no DNA control; (lane 22) Hi Lo™ DNA marker.

however, after evaluating plate configurations for the Cooper River bloom isolate, the isolate was excluded as *K. foliaceum* (Wolny, unpublished data). Species designations of the Cooper River and Hilton Head Island bloom isolates are still pending further morphological and molecular examination.

The *K. foliaceum*-specific PCR assay was used to screen a subset of environmental samples collected through the monitoring efforts of the South Carolina Harmful Algal Bloom Program. A total of 1070 samples were collected over a 16-month period during the 2001–2002 sampling season. Of these, 311 samples were identified to have *Kryptoperidinium* spp. present based on light microscopy. The *Kryptoperidinium* spp. identifications were spread over 26 sites in the coastal counties of South Carolina. The *K. foliaceum* primers were used to assay for the presence of *K. foliaceum* at 17 of those sites for a total of 27 samples. A subset of the sites was screened more than once with the PCR assay due to additional observations of *K. foliaceum* at the sampling site. Of the 17 sites assayed, 6 sampling locations were positive for *K. foliaceum* based on the PCR assay results. A subset of the PCR positives was sequenced for confirmation.

In each case, the target sequence was amplified and confirmed.

In order to confirm results from the *K. foliaceum*-specific PCR assay with the culture strains and the SC isolate, gene sequence data were derived for further comparisons. SSU and ITS rDNA sequence data were generated for the SC *K. foliaceum* isolate, and *P. foliaceum* cultures UTEX LB 1688 and CCMP 1326. Comparisons of SSU rDNA sequence data revealed that sequence data for the SC *K. foliaceum* isolate were identical to the SSU rDNA sequence data generated for *P. foliaceum* cultures UTEX LB 1688 and CCMP 1326, and identical to SSU rDNA sequence data deposited in GenBank by Saldarriaga et al. (2001) for *P. foliaceum* (accession no. AF274268, strain UTEX LB 1688). A four base pair difference was noted in the sequence data generated for the SC isolate compared to the sequence data deposited by Inagaki et al. (2000) for *P. foliaceum* (accession no. AF231804, UTEX LB 1688). Comparisons of the ITS region for the SC *K. foliaceum* isolate, and *P. foliaceum* cultures UTEX LB 1688 and CCMP 1326 also indicated identical sequences. Cultures CCAP 1116/3 and CS 291 did not amplify with the SSU rDNA targeted probes specific

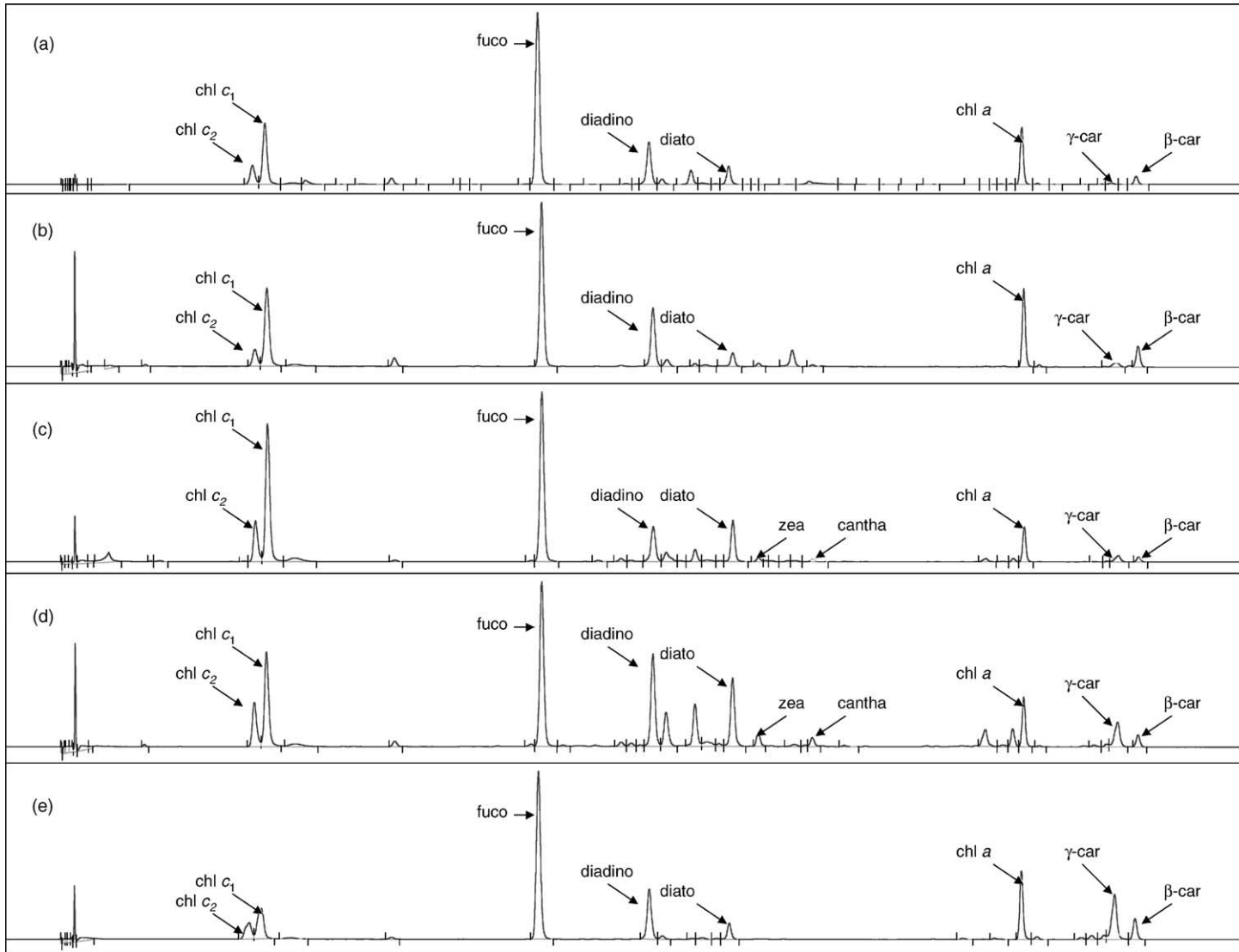


Fig. 4. HPLC chromatograms displaying pigment profiles of: (a) UTEX LB 1688 *P. foliaceum*; (b) CCMP 1326 *P. foliaceum*; (c) CCAP 1116/3 *G. foliaceum*; (d) CS 291 *K. foliaceum*; (e) SC bloom isolate (*K. foliaceum*). Abbreviations: chl c_1 = chlorophyll c_1 , chl c_2 = chlorophyll c_2 , chl a = chlorophyll a , fuco = fucoxanthin, diadino = diadinoxanthin, diato = diatoxanthin, zea = zeaxanthin, cantha = canthaxanthin, γ -car = γ -carotene, β -car = β -carotene.

to *K. foliaceum* (Fig. 3), and the ITS sequences were divergent from that of *P. foliaceum* strain UTEX LB 1688. These results question the taxonomic designation of *G. foliaceum* CCAP 1116/3 and *K. foliaceum* CS 291, implying that they are different species from *P. foliaceum*.

3.4. Microalgal pigment comparisons—*K. foliaceum*

Pigment profiles for all four *K. foliaceum* cultures and SC bloom material were examined for comparisons in pigment composition (Fig. 4a–e). *P. foliaceum* cultures UTEX LB 1688 and CCMP 1326 each contained chlorophylls *a*, *c*₁ and *c*₂, fucoxanthin, diadinoxanthin, diatoxanthin, and β -carotene (β -carotene). The pigment composition of the SC isolate (Fig. 4e) was similar to that of UTEX LB 1688 and CCMP 1326. Isolates CCAP 1116/3 (*G. foliaceum*; Fig. 4c) and CS 291 (*K. foliaceum*; Fig. 4d) differed from the others due to the presence of zeaxanthin and canthaxanthin in these isolates. These results were consistent with the DNA sequencing evidence placing the SC isolate, UTEX LB 1688, and CCMP 1326 together and CCAP 1116/3 and CS 291 as an outgroup. The carotenoid β , ψ -carotene (γ -carotene), eluting just prior to β -carotene, was found in all cultures but CCAP 1116/3.

4. Discussion

A multi-analytical, broad-based approach was used to determine the identity of the SC red tide dinoflagellate as *K. foliaceum*. Plate configurations based on epifluorescence and scanning electron microscopy for the SC *K. foliaceum* were consistent with those previously reported for *K. foliaceum* by Lebour (1925), Biecheler (1952, as *P. foliaceum*), and Sournia (1986) (Wolny et al., unpublished data). Here, we also presented plate configuration characteristics for the cingulum. However, more work needs to be done to clearly define the APC and sulcal regions. SSU and ITS rDNA sequence data were identical between the SC isolate and *P. foliaceum* cultures UTEX LB 1688 and CCMP 1326, providing further confirmation of the SC isolates' designation as *K. foliaceum*. Although the plasticity of microalgal pigment composition warrants caution in taxonomic applications, HPLC pigment profiles sup-

ported the taxonomic linkages between the SC isolate, UTEX LB 1688, and CCMP 1326.

A number of inconsistencies were noted among the four reference cultures selected for comparison purposes. One of the more notable inconsistencies was observed when comparing cultures UTEX LB 1688 and CCMP 1326. These cultures were identical in terms of their SSU and ITS gene sequence data; however, they differed with respect to the size and shape of the first apical (Table 2 and Fig. 1). The morphological differences observed in culture CCMP 1326 may be attributed to culturing artifacts. Culture CCMP 1326 has been maintained in culture since 1984, and its morphology has been shown to be highly variable when cultured at varying salinities (unpublished observations). The shape and orientation of the first apical plate was the same for cultures UTEX LB 1688, CCAP 1116/3, and CS 291, but the molecular data were not consistent among these *K. foliaceum* cultures, illustrating the need to use both morphological and molecular analyses to determine taxonomic placement below the genus level. This also suggests that *Kryptoperidinium* may not be a monospecific genus as previously reported.

It is well known that *K. foliaceum* has a fucoxanthin-containing diatom as a cytoplasmic endosymbiont (Chesnick et al., 1997). The endosymbiont has lost its cell wall, suggesting that the endosymbiont's relationship with its *K. foliaceum* host is an obligate association (Inagaki et al., 2000). However, a notable aspect of the data presented here was the absence of the endosymbiont nucleus from the SC *K. foliaceum* isolate. A bloom sample from Lassing Park in St. Petersburg, Florida was also determined to be *K. foliaceum* based on the *K. foliaceum*-specific PCR assay (Steidinger and Kempton, unpublished data). Like the SC isolate, the Florida *K. foliaceum* isolate did not contain an endosymbiont nucleus. The presence of the endosymbiont nucleus in *K. foliaceum* cultures, but not field isolates, suggests that the association of the *K. foliaceum* endosymbiont with its host is more transient than previously reported.

5. Conclusions

Morphological and molecular analyses were used to confirm the South Carolina bloom dinoflagellate

isolate as *K. foliaceum*. These results illustrate the value of multi-analytical taxonomic assessment, and the importance of confirming reference culture identity. Of the four putative cultures of *K. foliaceum* selected from culture collections maintained worldwide, only two of these were identified as *K. foliaceum* based on our criteria. Although “sorting” of reference cultures and bloom species is ongoing, the present findings are a significant step toward developing molecular tools for species detection and quantification necessary for understanding bloom ecology, and evaluating the taxonomic and evolutionary relationships of the *Kryptoperidinium* clade.

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References

- Andersen, P., Kristensein, H., 1995. Rapid and precise identification and counting of thecate dinoflagellates using epifluorescence microscopy. In: Lassus, P., Arzul, G., Gentlen, P., Marcaillou, C. (Eds.), *Harmful Marine Algal Blooms*. Lavoisier Publishers, New York.
- Biecheler, B., 1952. Recherches sur les Péridiniens. *Bull. Biol. France Belg.* 36 (Suppl.), 1–149.
- Chesnick, J.M., Kooistra, W.H.C.F., Wellbrock, U., Medlin, L.K., 1997. Ribosomal RNA analysis indicates a benthic pennate diatom ancestry for the endosymbionts of dinoflagellates *Peridinium foliaceum* and *Peridinium balticum* (Pyrrhophyta). *J. Eukaryot. Microbiol.* 44, 314–320.
- Daugberg, N., Hansen, G., Larsen, J., Moestrup, Ø., 2000. Phylogeny of some of the major genera of dinoflagellates based on ultrastructure and partial LSU rDNA sequence data, including the erection of three new genera of unarmoured dinoflagellates. *Phycologia* 39, 302–317.
- Fritz, L., Triemer, R., 1985. A rapid simple technique utilizing Calcofluor White M2R for the visualization of dinoflagellate thecal plates. *J. Phycol.* 21, 662–664.
- Inagaki, Y., Dacks, J.B., Doolittle, W.F., Watanabe, K.I., Ohama, T., 2000. Evolutionary relationship between dinoflagellates bearing obligate diatom endosymbionts: insight into tertiary endosymbiosis. *Int. J. Syst. Evol. Microbiol.* 50, 2075–2081.
- Lebour, M., 1925. *The Dinoflagellates of Northern Seas*. Marine Biological Association of the United Kingdom, Plymouth, UK, 250 pp.
- Lewitus, A.J., Hayes, K.C., Gransden, S.G., Glasgow Jr., H.B., Burkholder, J.M., Glibert, P.M., Morton, S.L., 2001. Ecological characterization of a widespread *Scrippsiella* red tide in South Carolina estuaries: a newly observed phenomenon. In: Hallegraef, G.M., Blackburn, S., Bolch, C., Lewis, R. (Eds.), *Harmful Algal Blooms 2000*. IOC UNESCO 2001, Paris, pp. 129–132.
- Lewitus, A.J., Schmidt, L.B., Mason, L.J., Kempton, J.W., Wilde, S.B., Wolny, J.L., Williams, B.J., Hayes, K.C., Hymel, S.N., Keppeler, C.J., Ringwood, A.H. *Harmful algal blooms in South Carolina residential and golf course ponds*. *Popul. Environ.*, in press.
- Medlin, L.H., Elwood, J., Stickel, S., Sogin, M.L., 1988. The characterization of enzymatically amplified eukaryotic 16S-like rRNA-coding regions. *Gene* 71, 491–499.
- Montresor, M., Procaccini, G., Stoecker, D.K., 1999. *Polarella glacialis*, Gen. Nov., Sp. Nov. (Dinophyceae): Suessiaceae are still alive!. *J. Phycol.* 35, 186–197.
- Oldach, D.W., Delwiche, C.F., Jakobsen, K., Tengs, T., Brown, E., Kempton, J., Schaefer, E., Bowers, H., Glasgow Jr., H., Burkholder, J., Steidinger, K., Rublee, P., 2000. Heteroduplex mobility assay-guided sequence discovery: elucidation of the small subunit (18S) rDNA sequences of *Pfiesteria piscicida* and related dinoflagellates from complex algal culture and environmental sample DNA pools. *Proc. Natl. Acad. Sci. U.S.A.* 97, 4303–4308.
- Saldarriaga, J.F., Taylor, F.J.R., Keeling, P.J., Cavalier-Smith, T., 2001. Dinoflagellate nuclear SSU rRNA phylogeny suggests multiple plastid losses and replacement. *J. Mol. Evol.* 53, 204–213.
- Schaefer, E.F., 1997. A DNA assay to detect the toxic dinoflagellate *Pfiesteria piscicida*, and the application of a PCR based probe. MS thesis, UNC, Greensboro.
- Sogin, M.L., 1990. Amplification of ribosomal RNA genes for molecular evolution studies. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J., White (Eds.), *PCR Protocols: A Guide to Methods and Applications*. Academic Press, San Diego, pp. 307–314.
- Sournia, A., 1986. Introduction, Cyanophycées, Dictyochophycées, Dinophycées et Raphidophycées. In: Sournia, A. (Ed.), *Atlas du Phytoplancton Marin*. Editions du Centre National de la Recherche Scientifique, Paris, France, pp. 91–92.
- Van Heukelem, L., Thomas, C.S., 2001. Chromatography modeling software in pigment method development: an evaluation of eleven HPLC columns. *J. Chromatogr. A* 910, 31–49.