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## Nuclear DNA content estimates in the Trentepohliales (Chlorophyta): Phylogenetic considerations

By JUAN M. LÓPEZ-BAUTISTA<sup>1</sup>

Department of Biological Sciences, The University of Alabama, Tuscaloosa, AL

DONALD F. KAPRAUN

Department of Biological Sciences, The University of North Carolina Wilmington,  
Wilmington, NC

RUSSELL L. CHAPMAN

Center for Marine Biodiversity and Conservation, Scripps Institution of Oceanography,  
University of California, San Diego, CA

With 3 figures and 1 table in the text

**Abstract:** Static microspectrophotometry and the DNA-localizing fluorochrome DAPI (4',6'-diamidodino-2-phenylindole) and RBC (chicken erythrocytes) standard were used to estimate nuclear DNA contents for eight species representing three genera of the sub-aerial green algal order Trentepohliales (Chlorophyta). Estimated nuclear DNA content values for the Trentepohliales (2C = 1.1–4.1 pg) closely approximate published values for sister groups of Ulvophyceae. Both estimated genome sizes and published chromosome numbers suggest a discontinuous distribution which can be explained in terms of ancestral polyploidy events. In *Cephaleuros parasiticus* KARSTEN, I<sub>f</sub> (fluorescence) levels in 2C nuclei in the gametophyte phase closely approximated 50% of the 4C values in the sporophytic phase, consistent with an alternation of ploidy levels in a sexual life history.

**Key words:** Trentepohliales, DAPI, Phylogeny, Microspectrophotometry, Nuclear DNA Content, *Cephaleuros*, Phylogeny, Chlorophyta

### Introduction

Phylogenetic analyses of molecular markers including 18S rDNA and *rbcL* (BHATTACHARYA et al. 1994, MCCOURT et al. 1996, HUSS et al. 1999, KANTANA et al. 2001) and morphological data (MATTOX & STEWART 1984) present a compelling case that an ancient divergence separates green algae into two major monophyletic lineages: the Chlorophyta and the Streptophyta (MCCOURT 1995, KAROL et al. 2001). The Streptophyta includes the charophycean lineage, along with bryophytes and tracheophytes (MISHLER et al. 1994; TURMEL et al. 2002a, b, c;

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DELWICHE et al. 2002). The Chlorophyta contain the classical "green algae", primarily the Chlorophyceae, Trebouxiophyceae (= Pleurostrophyceae) and Ulvophyceae (MISHLER et al. 1994, WATANABE et al. 2001). Conflicting hypotheses have been offered about the systematic position of the Trentepohliales. Phragmoplast type cytokinesis suggests an affinity with the Streptophytes (WATERS et al. 1998; CHAPMAN & HENK 1986; CHAPMAN et al. 2001), while flagellar apparatus components indicate a relationship with the Ulvophyceae in the Chlorophyta (ROBERTS 1984). Recent phylogenetic analyses of the nuclear encoded small-subunit rDNA sequences from taxa representing both Streptophytes and Chlorophytes consistently indicated that the subaerial Trentepohliales are closely related to the marine green algae or ulvophycean lineage in the Chlorophyta (LÓPEZ-BAUTISTA & CHAPMAN 2003). The Ulvophyceae are primarily marine species, most with larger and more complex morphologies than typically found in the Chlorophyceae. Molecular data support a model for the Ulvophyceae (*sensu* MATTOX & STEWART 1984) with two separate lineages: a clade including the Ulotrichales und Ulvales (HAYDEN & WAALAND 2002, O'KELLY et al. 2004) and a clade with the Caulerpales (Codiales), Cladophorales/Siphonocladales complex, Dasycladales and the Trentepohliales (ZECHMAN et al. 1990, HANYUDA et al. 2002, LÓPEZ-BAUTISTA & CHAPMAN, 2003). Thus, the Trentepohliales appear to be sister group to the siphonous and hemisiphonous ulvophycean algae with an implied divergence from an Ulvales/Ulotrichales macroscopic filamentous marine ancestor (LÓPEZ-BAUTISTA & CHAPMAN 2003).

New availability of these consensus higher-level molecular phylogenies provides a framework for viewing C-value data for the Chlorophyta in a phylogenetic context. The present investigation was initiated to describe in detail the trentepohlialean nucleotype and to suggest patterns of nuclear genome transformation that may have accompanied their evolution. In related multinucleate coenocytic green algae, very large nuclear genomes (2C DNA contents = 2.6–4.9 pg) are reported to have a role in maintaining nucleus/cytoplasm 'domains' (KAPRAUN & NGUYEN 1994). In the Dasycladales (e.g. *Acetabularia*), nuclear genome content data superimposed on a phylogeny of the group suggest that ancient polyploidy events accompanied major radiations in extant families (KAPRAUN & BURATTI 1998). In the Cladophorales/Siphonocladales complex, a combination of karyotype pattern and nuclear genome size appear to be unique and diagnostic, distinguishing a core clade of cladophoralean algae (KAPRAUN 2005).

The present investigation was initiated to determine the extent of nuclear DNA content variation in the Trentepohliales and to identify any correlation between genome size and phylogeny.

### Methods

Sources of isolates included in this study are indicated in Table 1. Specific identifications are based on previously published descriptions and diagnostic criteria (THOMPSON & WUJEK 1992, 1997, KARSTEN 1891, PRINTZ 1921, 1939). Specimens of Trentepohliales were

Table 1. Trentepohliales. Source of specimens. Data standardized to the DNA level of chicken

Species	Location	Repro
<i>Cephaleuros parasiticus</i> KARSTEN	LSU BR-51	gam
<i>Cephaleuros parasiticus</i>	LSU BR-52	spo
<i>Cephaleuros virescens</i> KUNZE	LSU BR-50	
<i>Physolinum monile</i> (DE WILDEMAN) PRINTZ	unknown	
<i>Trentepohlia arborum</i> (C. A. AGARDH) HARIOT	Brazil	
<i>Trentepohlia aurea</i> (LINNAEUS) MARTIUS	IRE 293	
<i>Trentepohlia aurea</i>	UTEX LB 429	
<i>Trentepohlia iolithus</i> (LINNAEUS) WALLROTH	IRE 293	
<i>Trentepohlia odorata</i> (WIGGERS) WITTRÖCK	CCAP 483/4	
<i>Trentepohlia umbrina</i> (KÜTZING) BORNET (= <i>Phycopeltis umbrina</i> (KÜTZING) THOMPSON et WUJEK)	IRE 294	

maintained in culture in MBB-medium. Material was fixed in Carnoy's solution, rehydrated in water and soft tissue was rehydrated in water and soft tissue for 30 min–3 h. Algal specimens were then air dried and stained with DAPI (0.5% in water) (KAPRAUN & NGUYEN 1994). Detailed methods and requirements for reproducible staining of DNA (NGUYEN 1994) using a protocol modified from that of CLOWES (1983) were used to determine the algal specimens (KAPRAUN 1994). DNA contents were determined by measuring the thymine rich regions of DNA with a scintillation counter (WARING 1998). Consequently, RBC DNA when the A-T contents of both species were compared (COLEMAN et al. 1981). *Gallus* has a DNA content of 2.6 mol% for the Chlorophyta (SUEON & KOOISTRA et al. 1992, LE GALL et al. 1992). This study is assumed to have a similar relationship between DAPI-DNA binding and algal sample (KAPRAUN 1994).

tain the classical "green algae", primarily (= Pleurastrophyceae) and Ulvales (al. 2001). Conflicting hypotheses exist on the Trentepohliales. Phragmotrichs of the Streptophytes (WATERS et al. 2001), while flagellar apparatus of the Ulvophyceae in the Chlorophyta is of the nuclear encoded small-subunit rDNA. Both Streptophytes and Chlorophyta Trentepohliales are closely related lineages in the Chlorophyta (LÓPEZ-EGUIA et al. 2004). Ulvophyceae are primarily marine species, with ecologies than typically found in the model for the Ulvophyceae (*sensu lato*) lineages: a clade including the Trentepohliales (ANDERSON 2002, O'KELLY et al. 2004) and Siphonocladales/Siphonocladales composites (SCHMIDT et al. 1990, HANYUDA et al. 2003). Thus, the Trentepohliales appear to be synonymous with ulvophycean algae with an algal-like macroscopic filamentous morphology.

Recent level molecular phylogenies provide support for the Chlorophyta in a phylogenetic tree and to describe in detail the trentepohlioid nuclear genome transformation that has occurred in the multinucleate coenocytic green algae. Nuclear genome contents = 2.6–4.9 pg are reported to be in 'domains' (KAPRAUN & NGUYEN 1994), nuclear genome content data suggest that ancient polyploidy events have occurred (KAPRAUN & BURATTI 1998). In a combination of karyotype patterns and diagnostic, distinguishing a Trentepohliales (2005).

To determine the extent of nuclear DNA content and to identify any correlation between

Table 1. Trentepohliales. Source of specimens, reproductive phase, estimated nuclear genome size. Data standardized to the DNA level of chicken erythrocytes (RBC = 2.4 pg).

Species	Location	Reproductive Phase	Number of Slides	Number of Nuclei	Nuclear Genome Size (pg)	
					2C	4C
<i>Cephaleuros parasiticus</i> KARSTEN	LSU BR-51	gametophyte	3	105	3.9 ± 1.4	
<i>Cephaleuros parasiticus</i> <i>Cephaleuros virescens</i> KUNZE	LSU BR-52	sporophyte	4	90		7.2 ± 1.3
<i>Physolinum monile</i> (DE WILDEMAN) PRINTZ	unknown		3	95	4.1 ± 0.9	
<i>Trentepohlia arborum</i> (C. A. AGARDH) HARIOT	Brazil		4	110	3.0 ± 0.6	
<i>Trentepohlia aurea</i> (LINNAEUS) MARTIUS	IRE 293		3	64	2.0 ± 0.4	
<i>Trentepohlia aurea</i> (LINNAEUS) WALLROTH	UTEX LB 429		4	141	1.2 ± 0.3	
<i>Trentepohlia iolithus</i> (LINNAEUS) WALLROTH	IRE 293		3	64	2.0 ± 0.4	
<i>Trentepohlia odorata</i> (WIGGERS) WITTROCK	CCAP 483/4		4	147	1.1 ± 0.2	
<i>Trentepohlia umbrina</i> (KÜTZING) BORNET (= <i>Phycopeltis umbrina</i> (KÜTZING) THOMPSON et WUJEK)	IRE 294		3	95	1.3 ± 0.3	

maintained in culture in MBB-medium (FRIEDL 1989), and illuminated by 75W cool-white fluorescent tubes on a continuous light regimen at room temperature (20 °C). Algal material was fixed in Carnoy's solution and stored in 70% ethanol at 4 °C. Preserved material was rehydrated in water and softened in 5% w/v EDTA (GOFF & COLEMAN 1990) for 30 min–3 h. Algal specimens were transferred to cover slips treated with subbing solution, air dried and stained with DAPI (0.5 µg/mL 4'-6'-diamidodino-2-phenylindole) (Sigma Chemical Co., St. Louis, MO 63178) as previously described (GOFF & COLEMAN 1990, KAPRAUN & NGUYEN 1994). Detailed procedures for microspectrophotometry with DAPI and requirements for reproducible staining have been specified previously (KAPRAUN & NGUYEN 1994) using a protocol modified after GOFF & COLEMAN (1990). Microspectrophotometric data for *Gallus* (chicken erythrocytes or RBC) with a DNA content of 2.4 pg (CLOWES et al. 1983) were used to quantify mean fluorescence intensity ( $I_f$ ) values for algal specimens (KAPRAUN 1994). DAPI binds a non-intercalative mechanism to adenine and thymine rich regions of DNA which contain at least four A-T base pairs (PORTUGAL & WARING 1998). Consequently, RBC are best used as a standard for estimating amounts of DNA when the A-T contents of both standard and experimental DNA are equivalent (COLEMAN et al. 1981). *Gallus* has a nuclear DNA base composition of 42–43 mol% G + C (MARMOR & DOTY, 1962). Limited published data indicate similar mean values of 46 mol% for the Chlorophyta (SUEOKA 1961, OLSEN et al. 1987; FRESHWATER et al. 1990, KOOISTRA et al. 1992, LE GALL et al. 1993, SIMON et al. 1994). Algae investigated in this study are assumed to have a similar range of base pair compositions, and linearity is accepted between DAPI-DNA binding in both RBC and algal samples (LE GALL et al. 1993). Nuclear DNA contents were estimated by comparing the  $I_f$  values of the RBC standard and algal sample (KAPRAUN 1994).

as detailed in Table 1. Specific identifications and diagnostic criteria (THOMPSON & WUJEK 1999). Specimens of Trentepohliales were

## Results and Discussion

DAPI staining with the protocol modified from GOFF & COLEMAN (1990) yielded reproducible, stable nuclear fluorescence with little apparent interference from autofluorescence, nonspecific binding, or other cellular material. Comparison of  $I_f$  values for species of the Trentepohliales to  $I_f$  values for chicken erythrocytes (RBC), which have a DNA content of 2.4 pg (TIERSCH et al. 1989), permitted estimation of nuclear DNA contents for taxa investigated in this study. The source of specimens, number of nuclei for each sample and the estimated nuclear genome size (pg DNA  $\pm$  SD) are given in Table 1. DNA content estimates in 2C nuclei ranged from 1.1–4.1 pg (Table 1). These data superimposed on a phylogeny derived from nuclear-encoded SSU rDNA sequence comparisons (LÓPEZ-BAUTISTA & CHAPMAN 2003) suggest that nuclear DNA contents are highly correlated with phylogenetic advancement in the Trentepohliales such that basal taxa have the smallest genomes while more derived taxa, including *Physolinum* and *Cephaleuros* have substantially larger genomes (Figure 1).

Published chromosome complements for the Trentepohliales range from  $1n = 4-28$  (SARMA 1982) and suggest a pattern of large-scale discontinuous variation. It is tempting to speculate that reported  $1n$  chromosome numbers of 12, 18 and 24 correspond to polyploidy levels of  $2x$ ,  $3x$  and  $4x$ , respectively (Figure 2). Additional values can be explained in terms of fission and/or fusion events that result in aneuploid chromosome complements (KAPRAUN 1993, KAPRAUN & BAILEY 1992). We note that nuclear DNA content data show a similar pattern of large-scale discontinuous variation (Figure 1). When results of the present investigation are examined in the context of comprehensive studies of the Ulvophyceae (Figure 3), it appears that the basal orders Ulvales and Ulotrichales (O'KELLY et al. 2004) are characterized by both small nuclear genome sizes and chromosome complements (KAPRAUN 2005). In contrast, the more derived orders (HANYUDA et al. 2002) can be circumscribed as having both larger nuclear genome sizes and chromosome complements, consistent with one or more polyploidy events in their evolution (Figure 3).

Variations in nuclear DNA contents associated with ploidy level differences in gametophytic and sporophytic phases in green algae have been demonstrated previously with microspectrophotometry (HOPKINS & MCBRIDE 1976, KAPRAUN 1994, KAPRAUN & SHIPLEY 1990). In the present study, *Cephaleuros parasiticus* exhibited DNA levels for 2C nuclei in the gametophytic phase that closely approximate 50% of the 4C values in the sporophytic phase (Table 1). Vegetative growth is the only form of reproduction reported in most trentepohlialean species (RINDI et al. 2005) and the diplobiontic life cycle proposed for the Trentepohliales is not well documented (LÓPEZ-BAUTISTA et al. 2002). THOMPSON'S (1961) observations on life history of *Trentepohlia* and *Phycopeltis*, and an alternation of heteromorphic generations in *Cephaleuros* and *Stomatochroon* involving a dwarf sporophyte. In *Cephaleuros virescens*, a tentative ultrastructural confirmation of THOMPSON'S observations was made by the discovery of syna-

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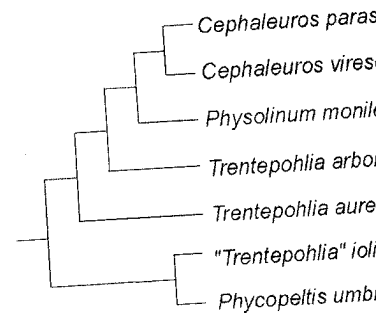


Fig. 1. Cladogram inferred from SSU rDNA sequence comparisons (LÓPEZ-BAUTISTA & CHAPMAN 2003) and nuclear DNA content estimates for Trentepohliales.

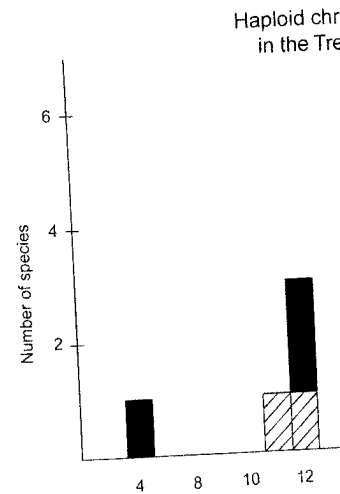


Fig. 2. Histogram of haploid ( $1n$ ) chromosome numbers in the Trentepohliales (SARMA 1982).

tonema complexes in sporangia during meiosis and sexual reproduction. The life cycle found for *Cephaleuros parasiticus* in this study is consistent with the alternation of haploid and diploid

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GOFF & COLEMAN (1990) yielded little apparent interference from cellular material. Comparison of  $C_f$  values for chicken erythrocytes (MERSCH et al. 1989), permitted investigated in this study. The source sample and the estimated nuclear DNA content estimates in 2C are superimposed on a phylogenetic tree (A sequence comparisons (LÓPEZ-BAUTISTA & CHAPMAN 2003) and nuclear DNA contents are highly correlated in Trentepohliales such that basal taxa, including *Physolinum* and *Phycopeltis* (Figure 1).

Trentepohliales range from  $1n = 4x$ , respectively (Figure 2). Addition and/or fusion events that result in chromosome numbers of 12, 18 and 24, respectively (Figure 2). Additional data show a similar pattern of chromosome numbers of 12, 18 and 24, respectively (Figure 2). When results of the present investigation are compared with the results of comprehensive studies of the Ulvales and Ulotrichales (SARMA 1982), the more derived orders are consistent with one or more polyploidization events.

and with ploidy level differences in algae have been demonstrated previously (SARMA & MCBRIDE 1976, KAPRAUN & BAUTISTA 1993). In this study, *Cephaleuros parasiticus* is in a vegetative phase that closely resembles the vegetative phase of *Trentepohlia* in most trentepohlialean species (SARMA 1982). The life cycle proposed for the Trentepohliales (SARMA 1982, THOMPSON & BAUTISTA et al. 2002). THOMPSON & BAUTISTA et al. (2002) proposed that *Trentepohlia* and *Phycopeltis*, and an ultrastructural study of *Cephaleuros* and *Stomatochroom* in *Trentepohlia* made by the discovery of syn-

Molecular phylogeny of the Trentepohliales

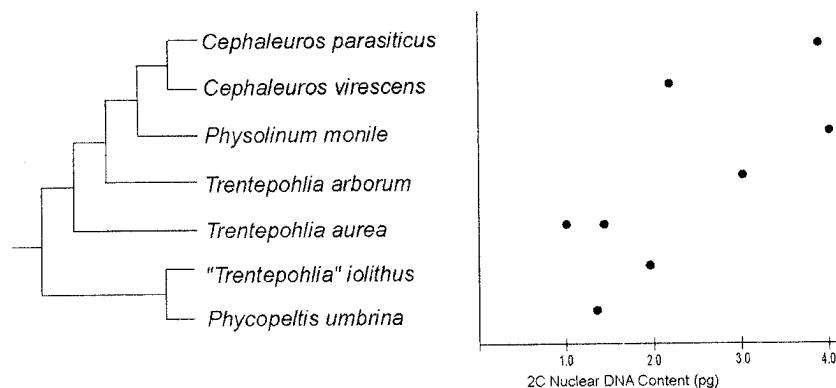


Fig. 1. Cladogram inferred from SSU rDNA (LOPEZ-BAUTISTA et al. 2002, LOPEZ-BAUTISTA & CHAPMAN 2003) and nuclear DNA content estimates for members of the Trentepohliales.

Haploid chromosome numbers reported in the Trentepohliales (SARMA 1982)

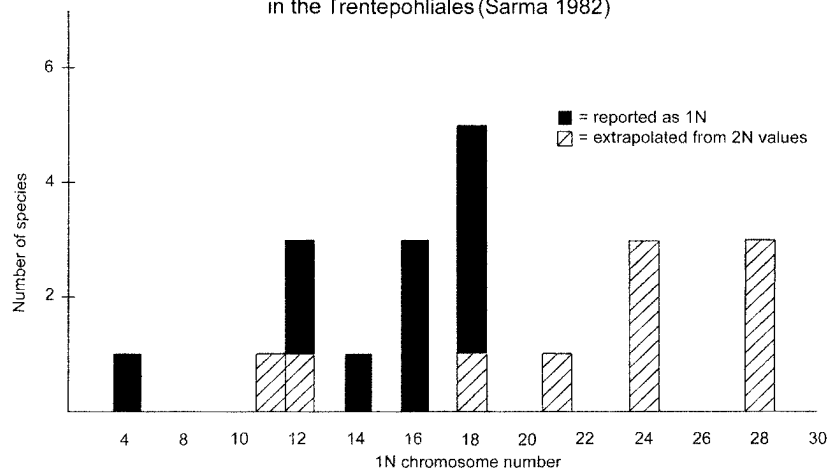


Fig. 2. Histogram of haploid (1n) chromosome numbers reported in the Trentepohliales (SARMA 1982).

tonema complexes in sporangia of this alga (CHAPMAN & HENK 1981), indicative of meiosis and sexual reproduction (LEWIN 1997). The DNA content values found for *Cephaleuros parasiticus* isolates identified as gametophyte and sporophyte in this study is consistent with the presumptive sexual life cycle and an alternation of haploid and diploid phases.

## Nuclear genome size variation in the Ulvophyceae

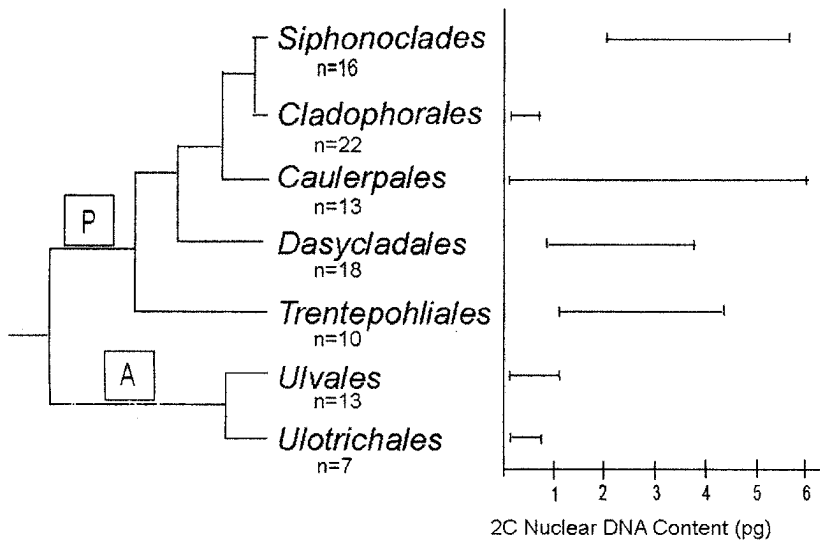


Fig. 3. Cladogram inferred from SSU rRNA sequence data (ZECZMAN et al. 1990) and nuclear DNA content estimates for the Ulvophyceae (KAPRAUN 2005); P = Polyploidy event; A = Aneuploidy event; n = number of species investigated.

## Acknowledgements

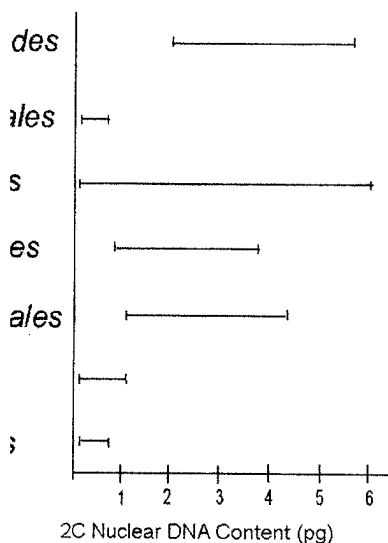
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## Resumen

Microespectrofotometría con el fluorocromo localizador de DNA DAPI (4'-6 diamidodino-2-fenilindole) y estándares de RBC (eritrocitos de *Gallus*) fueron utilizados para estimar el contenido de DNA nuclear en ocho especies representando tres géneros de algas verdes subaéreas del orden Trentepohliales (Clorofita). Los valores del contenido de DNA nuclear estimados para las Trentepohliales ( $2C = 1.1-4.1$  pg) se aproximan estrechamente a los valores publicados para los grupos cercanos en Ulvophyceae. Los tamaños estimados del genoma junto con los números cromosómicos publicados sugieren una distribución continua que puede ser explicada en términos de eventos ancestrales de poliploidia. En *Cephaleuros parasiticus* KARSTEN, los niveles  $I_f$  (fluorescencia) en los núcleos 2C en la fase gametofítica se aproximan cercanamente al 50% de los valores 4C en la fase esporofítica, consistente con una alternancia de niveles de ploidia en un ciclo biológico sexual.

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## variation in the Ulvophyceae



sequence data (ZECZHMANN et al. 1990) and Ulvophyceae (KAPRAUN 2005); P = Polyploidy species investigated.

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un localizador de DNA DAPI (4'-6 diamidino-2-fenilindol) fueron utilizados para analizar los niveles de DNA en células de los tejidos de *Gallus gallus* y *Escherichia coli*. Los valores del contenido de DNA nuclear (2C = 1.1-4.1 pg) se aproximan a los valores reportados para otros grupos cercanos en Ulvophyceae. Los números cromosómicos publicados sugieren una explicación en términos de eventos ancestrales de poliploidización. Los niveles  $I_f$  (fluorescencia) se aproximan cercanamente al 50% de los valores reportados para una alternancia de niveles de ploidia en un

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The authors' addresses:

JUAN M. LOPEZ-BAUTISTA  
The University of Alabama  
Department of Biological Sciences  
500 Hackberry Lane  
Tuscaloosa, AL. 35487-0345, USA

DONALD F. KAPRAUN  
The University of North Carolina  
Department of Biological Sciences  
601 South College Road  
Wilmington, NC 28403-5915, USA

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The authors' addresses:

JUAN M. LOPEZ-BAUTISTA  
The University of Alabama  
Department of Biological Sciences  
500 Hackberry Lane  
Tuscaloosa, AL 35487-0345, USA

DONALD F. KAPRAUN  
The University of North Carolina Wilmington  
Department of Biological Sciences  
601 South College Road  
Wilmington, NC 28403-5915, USA

RUSSELL L. CHAPMAN  
 University of California  
 Scripps Institution of Oceanography  
 Centre for Marine Biodiversity and Conservation  
 San Diego, CA 92093, USA

## Valorization attempt $\beta$ -carotene production by *Dunaliella salina*

By JAMILA RIYAH<sup>1</sup>, YOUSSEF  
 THIERRY GIVERNAUD<sup>2</sup>, YVES

<sup>1</sup> Université Ibn Tofaïl, Faculté de  
 Biotechnologies

<sup>2</sup> Setexar

<sup>3</sup> Laboratoire de phycologie et  
 « ELICO », Vill

With 4 figures

**Abstract:** This study was realized during  
 a commercial salt pond (Société Schérienne)  
 in September for two years. Water sample  
 parameters (temperature, pH, oxygen,  
 chlorophyll *b*,  $\beta$ -carotene) and phytoplankton  
 were prospected at 10 stations.

The unicellular green alga *Dunaliella salina*  
 (Chlorococcales) is the most salt-tolerant eukaryote  
 which can grow at very high salinities.  $\beta$ -  
 carotene which is the most important  
 pigment is currently used as food coloring agent, as  
 pharmaceuticals and in medical treatment for  
 diseases. Synthetic  $\beta$ -carotene is synthetic; the algal  
 $\beta$ -carotene is natural and its value is twice the value  
 of the synthetic product.

The purpose of our study was to determine  
 the optimal conditions for the cultivation of  
 this species in a salt pond without trouble. The  
 results presented by this paper reveal that *Dunaliella salina*  
 in the last two crystallization basins with  
 salinity conditions  $\beta$ -carotene accumulated  
 close to  $88 \mu\text{g l}^{-1}$  without any nutrient.

Key words: *Dunaliella salina*, salt