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Karyology, nuclear genome quantification and characterization of the carrageenophytes *Eucheuma* and *Kappaphycus* (Gigartinales)

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Abstract

DNA reassociation kinetics were used to determine nuclear genome organization and complexity in the carrageenophyte *Kappaphycus alvarezii*. Results indicate the presence of three second order components corresponding to fast (12%), intermediate (38%) and slow (50%) fractions. Microspectrophotometry with the DNA-localizing fluorochrome DAPI confirmed ploidy level differences in the gametophytic and tetrasporophytic phases. Comparison of mean nuclear DNA (I_f) values to chicken erythrocytes (RBC) resulted in pg/2C genome estimates: *Eucheuma denticulatum* = 0.35, *E. isiforme* = 0.44, *Kappaphycus alvarezii* = 0.32 and *K. striatum* = 0.42. Karyological studies of tetraspore mother cells during diakinesis using aceto-orcein revealed a chromosome complement of 10 for *Eucheuma denticulatum* and *Kappaphycus alvarezii*.

Introduction

Eucheuma denticulatum (N. L. Burman) Collins et Hervey and *Kappaphycus alvarezii* (Doty) Doty are arguably two of the most commercially important seaweeds in the world. Together they account for over 90% of all seaweed exports from the Philippines, and at least 70% of the world's supply of semirefined carrageenan (Trono, 1992). During the late 1980's, harvests of both farmed weed and natural populations began a decline which has continued to the present. A variety of economic and biological factors appear to be involved with this decline in Philippine production, including competition from mariculture initiatives in East Africa (Mollion & Braud, 1993; Lirasan & Twide, 1993) and Indonesia (Luxton, 1993), increasingly poor quality and variability of carrageenan (Trono & Lluisma, 1992) and utilization of severely reduced natural populations as seed stock for successive growing seasons (Dawes & Koch, 1991).

Recently, Dawes and co-workers (Dawes & Koch, 1991; Dawes et al., 1993; Dawes et al., 1994) developed micropropagation techniques to maintain and select seed stock through laboratory cultivation of both *Eucheuma* and *Kappaphycus*. Strain improvement of these carrageenophytes today relies on phenotypic selection of vigorous clones as it did in the early 1970's (Doty & Alvarez, 1975). Before biotechnology techniques developed for seaweeds (Cheney, 1990) can be meaningfully applied to the genetic improvement of these carrageenophytes, some basic information is required on their nuclear genomes. For example, the feasibility of specific biotechnology approaches can be best evaluated with knowledge of the physical structure (chromosome number), size, molecular organization and complexity of the nuclear genomes in these seaweeds (Kapraun et al., 1992). It is remarkable, given the economic importance of *Eucheuma* and *Kappaphycus*; and the reported decrease in vigor of farmed stocks, that such basic information for their nuclear genomes remains unknown.

Table 1. Source of specimens

Species	Isolate	Source
Eucheuma denticulatum	♀	Danajan Reef (Bohol)
(N.L. Burman) Collins et Hervey	♀	Mactan Island (Cebu)
commercial name: 'spinosum'	M-11⊕	Mactan Island (cebu)
	M-12⊕	Mactan Island (Cebu)
	M-17⊕	Mactan Island (Cebu)
Eucheuma isiforme	veg	Bahia Honda (Florida, USA)
(C. Agardh) J. Agardh		
Kappaphycus alvarezii	RP-1 veg	Bolinao (Pangasinan)
(Doty) Doty	RP-2⊕	Bolinao (Pangasinan)
commercial names:	RP-12⊕	Bolinao (Pangasinan)
'cottonii, striatum'	RP-16 veg 'green'	Malalison (Panay)
	RP-17 veg 'brown'	Malalison (Panay)
	RP-18⊕	Malalison (Panay)
Kappaphycus striatum	♀	Danajan Reef (Bohol)
(Schmitz) Doty	♀	Mactan Island (Cebu)
commercial name: 'elkhorn'		

Consequently, we have initiated a program to develop genome profiles for the two most important carrageenophytes in the Philippines: *Eucheuma denticulatum* and *Kappaphycus alvarezii*. This paper presents preliminary information on chromosome numbers and genome sizes for both of these species, as well as molecular organization and complexity data for *Kappaphycus alvarezii*.

Materials and methods

Source and preservation of specimens

Collection sites for specimens included in the present study are listed in Table 1. Algal material for cytogenetics (Table 2) and cytophotometry was fixed in Carnoy's solution (Kapraun et al., 1992) and stored in 70% ethanol. Algal samples for DNA isolation were quick-dried in silica gel and then stored frozen (−20 °C).

Karyotype analysis

Cortical areas containing tetrasporangia were identified with a dissecting microscope, removed and rehydrated in water for 30 min. Tissue was softened in 5% w/v EDTA until tetrasporangia were extruded from the cortical layer. Tissue was then rinsed in water to remove EDTA, and the cortical layer shaved from the thallus with a razor blade and smeared on coverslips treated with subing solution and air-dried (Kapraun et al., 1991). Coverslips were stained with 2% w/v aceto-orcein.

Microspectrophotometry

Detailed procedures for microspectrophotometry with the DNA-localizing fluorochrome DAPI and requirements for reproducible staining have been specified previously (Kapraun et al., 1992). Microspectrophotometric data for chicken erythrocytes (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 et al., 1991).

G + C determination

DNA from a variety of sources was used previously to develop a standard equation: $G+C=T_m$ (2.280–153.7) (Dutcher et al., 1990). Algal DNA samples, with an *E. coli* DNA standard, were heated to 100 °C, 1 °C min^{-1}, in closed thermostatically controlled cuvettes of a Gilford model 2600 spectrophotometer equipped with thermoprogrammer. Thermal denaturation temperatures (T_m's) were determined from the hyperchromatic shift for replicates of each sample using the standard equation specified above.

Table 2. Chromosome numbers in isolates of *Eucheuma* and *Kappaphycus*

Species	Isolate	Chromosome number (1 N)
Eucheuma denticulatum	M-11	c. 10
" "	M-12	10
" "	M-17	10
Kappaphycus alvarezii	RP-2	c. 10

DNA isolation and characterization

Detailed procedures for nuclear DNA isolation and purification (Dutcher et al., 1990a; Kapraun et al., 1992) and requirements for efficient, reproducible reassociation kinetics have been specified previously (Marmur et al., 1963; Britten & Kohne, 1970; Dutcher et al., 1990b). In the present study DNA reassociation kinetics were carried out under moderately stringent conditions (T_m-25 °C, 0.18 M Na^+). Reassociation data were analyzed with a computer program generating a best fit non-linear regression representing three second-order components (Kapraun et al., 1992). DNA of *E. coli* (4.2×10^6 bp, C_ot 1/2 pure 2.93) was used as a standard to convert C_ot values into nucleotide base pairs or complexity of each component for algal samples (Lewin, 1990).

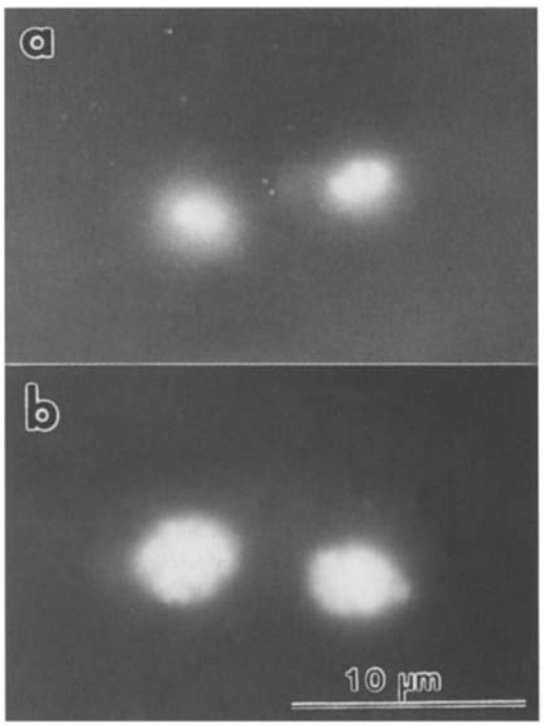

Figure 1. Kappaphycus alvarezii (Isolate RP-2) medulla cells with DAPI-stained nuclei visualized with episcopic (UV) illumination. a. Gametophyte cells showing 1N nuclei with 2C DNA contents. 2. Tetrasporophyte cells showing 2N nuclei with 4C DNA contents.

Results

Microspectrophotometry

DAPI protocols from Kapraun et al. (1992, 1994) resulted in reproducible, stable fluorescence of nuclei (Figure 1). Extrapolated I_f data indicate DNA content mean 2C genome values of 0.30 pg for *Kappaphycus alvarezii*, and 0.35 and 0.42 pg for *Eucheuma denticulatum* and *E. striatum*, respectively (Table 3). Since the *E. isiforme* specimens from Florida were sterile, assignment of the genome size estimate of 0.45 pg to 2C was arbitrary, and requires confirmation.

Karyology

Aceto-orcein staining revealed the presence of 10 bivalents in late prophase-early metaphase (diakinesis) meiotic nuclei of *Eucheuma denticulatum* (Figure 2). The karyotype is characterized by four larger chromosomes and six smaller ones. *Kappaphycus alvarezii* meiotic nuclei have about 10 bivalents as well, but lack any conspicuous size difference (Figure 3). Although numerous tetraspore mother cells were examined in *K. striatum* specimens, no meiotic nuclei were found.

Nuclear DNA base pair composition

Calculations based on UV absorption data indicate yields of approximately 1–2 ml of 100µg.ml DNA from 60 g of algal tissue. The 260/234 nm and 260/280 nm ratios averaged 2.0 and 1.7, respectively. Nuclear DNA base pair composition estimates from thermal denaturation temperatures (T_m) of replicate samples indicate a guanine + cytosine (G + C) content of 46.5 mol % for *Kappaphycus alvarezii*. Absence of double melt profiles in these whole cell DNA preparations excludes the possibility of significant organelle contamination (Kapraun et al., 1993a).

Table 3. Nuclear genome size (pg) in species of *Eucheuma* and *Kappaphycus*

Species	Number of slides	Number of nuclei	Nuclear genome size (pg) 2 C	4 C
Eucheuma denticulatum				
Mactan Is., medulla ♀	11	387	0.35 ± 0.05	
cystocarp	3	79		0.66 ± 0.09
Danajan Reef, medulla♀	5	188	0.35 ± 0.04	
cystocarp	2	73		0.68 ± 0.01
Eucheuma isiforme				
Bahia Honda, medulla veg	2	96	0.45 ± 0.07	
cortex veg	3	129	0.44 ± 0.01	
Kappaphycus alvarezii				
RP-1, medulla ♀	6	136	0.31 ± 0.01	
RP-1, cystocarp	1	47		0.52 ± 0.15
RP-2, medulla ⊕	2	52		0.54 ± 0.01
RP-12, medulla ⊕	7	247	0.28 ± 0.02	
RP-16, medulla veg	4	159	0.30 ± 0.02	
RP-17, medulla veg	4	168	0.32 ± 0.02	
RP-18, medulla ⊕	4	130	0.32 ± 0.01	
RP-18, medulla ⊕	4	181		0.55 ± 0.04
Kappaphycus striatum				
Danajan Reef:				
Cultivar A, medulla veg	3	75	0.41 ± 0.02	
Cultivar A, medulla veg	9	314		0.82 ± 0.03
Cultivar B, medulla veg	12	447	0.43 ± 0.02	

Reassociation kinetics

Repeated attempts to extract and purify whole cell DNA from *Eucheuma denticulatum* and *E. isiforme* were frustrated by the presumed presence of polysaccharides which complexed with the DNA. In contrast, whole cell DNA was routinely extracted and purified from *Kappaphycus alvarezii*. Computer analysis of DNA reassociation kinetics for this species revealed the presence of three second order components (correlation coefficient >0.995): a fast fraction representing highly repetitive sequences (12%), an intermediate fraction of mid-repetitive sequences (38%) and a slow fraction of unique or single copy sequences (50%) (Figure 8). Complexities and copy number for each fraction are summarized in Table 4.

Experimental parameters precluded determination of slow component C_0t 1/2 so genome size estimates from cytophotometry were used to extrapolate a complexity of 0.30×10^9 bp for the genome of *Kappaphycus alvarezii* using 0.965×10^9 bp/pg (Britten & Davidson, 1971).

Table 4. Kinetic analysis of *Kappaphycus alvarezii* DNA reassociation

Kinetic component	Fraction (%)	Complexity (base pairs)	Repetition frequency	Genome size (pg)
Fast	12	–	–	
Intermediate	38	0.63×10^6	180	0.31
Slow	50	1.49×10^8	1	

Discussion

The genus *Kappaphycus* was segregated from *Eucheuma* primarily on the basis of carraageenan type (Doty, 1985). Unfortunately, the binomials formed as a result of the transfer to the new genus (e.g. *Kappaphycus alvarezii* (Doty) Doty) do not fully satisfy the requirements of the *International Code of Botanical Nomenclature* (Greuter et al., 1988). Specifically, Doty's (1985) proposal omitted pagination in the bibliographic citation (Liao, pers. comm.). Despite this taxonomic oversight, the genus *Kappaphycus* has gained wide acceptance for species producing *kappa* carrageenan, while species producing iota car-

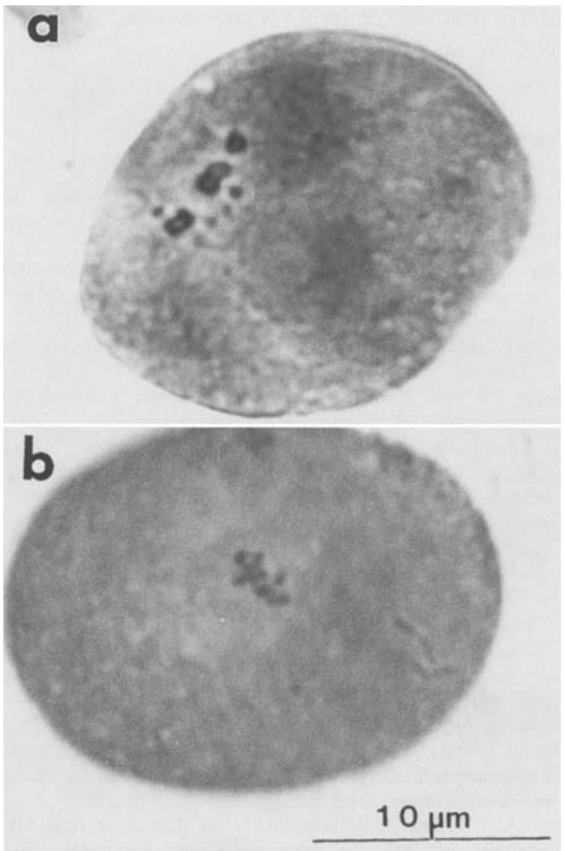

Figure 2. Eucheuma denticulatum (1N = 10) meiotic chromosomes in tetraspore mother cells following aceto-orcein staining. a. Isolate M-11 late prophase (diakinesis) showing 3 larger bivalents. b. Isolate M-12 with maximum chromosome contraction characteristic of metaphase.

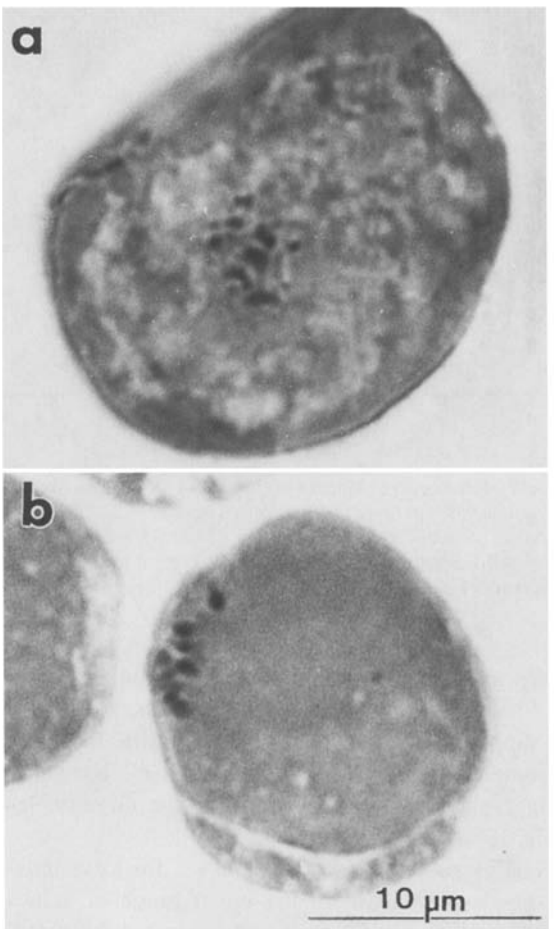

Figure 3. Kappaphycus alvarezii (Isolate RP-2) with 1N = 10. a. Tetraspore mother cell in late prophase showing a size gradation from largest to smallest chromosomes. b. Maximum chromosome contraction in metaphase.

raageenan have been retained in the genus *Eucheuma* (Doty, 1985).

The chromosome numbers and photomicrographs in the present study appear to be unique for *Eucheuma* and *Kappaphycus* (Cole, 1990). The chromosome complements of N = 10 for both *Eucheuma denticulatum* and *Kappaphycus alvarezii* are somewhat lower than for most Gigartinales (Cole, 1990; Kapraun et al., 1992; Kapraun et al., 1993a, 1994). The estimated mean 2C genome sizes of 0.30–0.44 pg for the species of *Eucheuma* and *Kappaphycus* investigated are comparable to those reported for other red algal species in the orders Gracilariales, Gigartinales and Gelidiales (Kapraun et al., 1993b). It is noteworthy that the previous estimates of 2C = 0.48 pg for *Kappaphycus alvarezii* (Le Gall et al., 1993) closely approximate present estimates for the 4C DNA level (0.53 pg). *K. alvarezii* and *K. striatum* were treated as growth forms of a single entity referred to as *Eucheuma* 'cottonii' in the early days of the Philippine carrageenan trade (Doty & Norris, 1985). Nuclear genome size estimates for these taxa of $\bar{x} = 0.30$ and $\bar{x} = 0.42$, respectively, provide substantial support for their current taxonomic status as separate species.

Rarity of reproductive structures (Abbott, 1988) and development of a distinctive physiology (Glenn & Doty, 1981) and morphology (Doty, 1985) in domesticated *Kappaphycus alvarezii* make it tempting to speculate that the cultivated plants are polyploid apomicts (Kapraun et al., 1988; Kapraun, 1989) which arose spontaneously from the wild type. Results of the present study indicating DNA contents for wild (RP-1,2) and cultivated (RP-16,17 & 18) isolates of *Kap-*

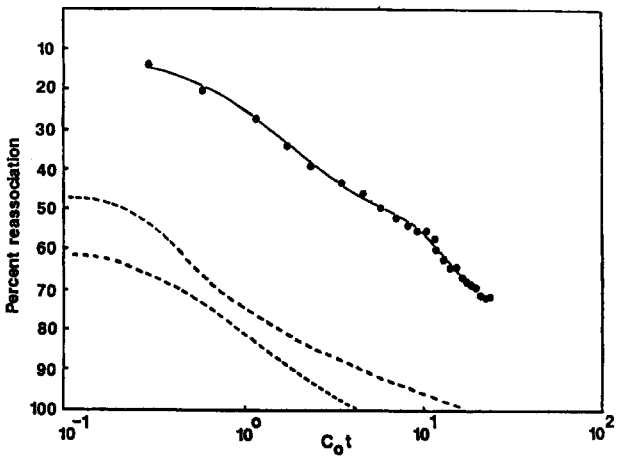

Figure 4. Reassociation kinetics of whole cell DNA from *Kappaphycus alvarezii*. c_0t (moles of nucleotides per liter × S), DNA concentrations were 50–60 μg DNA ml^{-1} in 18 M Na^+ at 60 °C. Solid line: best linear regression analysis of data. Dashed line: predicted second order kinetic components, correlation coefficient >0.995.

paphycus alvarezii to be identical, and similar to values for other *Kappaphycus* and *Eucheuma* species do not support this interpretation. In addition, the chromosome complement of about 1 N = 10 is too small for a typical polyploid number in the Gigartinales (Cole, 1990).

The estimate of 46.5 mol % G+C for *Kappaphycus alvarezii* is close to the upper range of values (46–47 mol % G+C) previously reported for representatives of the Gigartinales (Kapraun et al., 1993c). It should be noted that recent estimates of 51 mol % G+C for *Eucheuma* and *Kappaphycus* refer to nuclear genes encoding small-subunit ribosomal RNAs only and not to the entire nuclear genome (Lluisma & Ragan, 1995). Reassociation kinetics data for *K. alvarezii* indicate a repetitive (fast + intermediate) component of 50%. Repetitive components reported for other carageenophytes range from 11–90% (Kapraun et al., 1992; 1993b). The significance of reassociation kinetics data and nuclear genome profiles for developing commercial red algal resources has been discussed previously.

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