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scDNA-DNA hybridization studies in Pacific and Caribbean isolates of *Dictyosphaeria cavernosa* (Chlorophyta) indicate a long divergence

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ABSTRACT: Genomic size and complexity were calculated for the pantropical chlorophyte, *Dictyosphaeria cavernosa* (Cladophorales). Genome characterization of the Hawaiian material by means of DNA renaturation studies showed highly repetitive (31.3 %), middle repetitive (42.7 %), and single-copy (25.8 %) fractions; and an estimated haploid genome size of 1.79 pg DNA. A G:C content of 43 % was calculated from a melting curve of DNA. Pacific and Caribbean isolates of this species were compared using single-copy nuclear DNA-DNA hybridization. Results show a relatively low thermal stability of the hybridized DNA (Δ Tm(e) = 10 °C) which suggests that these Pacific and Caribbean lineages may have been separated for up to 55 Ma.

INTRODUCTION

Levels of genetic divergence among widely disjunct isolates of morphologically indistinguishable species is a central area of interest in understanding species evolution and distribution patterns in the context of historical biogeography. The use of single-copy (sc) nuclear DNA-DNA hybridization measurements on isolates of putatively the same species and "closely" related species, collected from widely differing geographic localities, provides a distance measure of DNA sequence homology that can help to elucidate some of these puzzles. In this research note we present data on size and complexity of the genome, and provide a preliminary estimate of divergence time between Pacific and Caribbean isolates of the tropical chlorophyte, *Dictyosphaeria cavernosa* (Forssk.) Børgesen.

MATERIALS AND METHODS

Extensive theoretical discussions of single-copy DNA-DNA hybridization principles and methods can be found in Bonner et al. (1973) and Britten et al. (1974). In brief, by measuring the renaturation rate of complementary sequences, the total DNA content of the haploid genome, as well as the proportion of repetitive and single-copy DNA can be calculated. Subsequent comparisons of homologous and heterologous reassociations

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within and among species provides a measure of genetic divergence reflected by two parameters. These are percent relative binding (RB %) and thermal stability of the hybrids as measured by the thermal midpoint of elution [Tm(e) °C].

Protocols used in this research are briefly summarized here following Bot (personal communication) and Stam et al. (1988, in preparation).

Specimens. Specimens of *Dictyosphaeria cavernosa* were collected from Coconut Island, Oahu, Hawaii and Boiler Bay, St. Croix, U.S.V.I. *Dictyosphaeria versluysii* was collected from Waikiki, Oahu, Hawaii. Only young, clean thalli were selected. Further cleaning consisted of epidermal peels and removal of chunks of unexposed thallus. Tissue was further examined with the DNA fluorescent stain, DAPI, to screen for bacterial and cyanophytic contaminants. Only material passing this test was finally considered acceptable for DNA extraction. Samples were frozen at −20 °C. Most of the material was subsequently lyophilized for transport prior to DNA extraction. We caution that, while axenic material is always preferred, field-collected material can be used if care is taken. Comparative experiments have been conducted in our laboratory with cleaned, field-collected material and cultured species of *Cladophora*. Although cultured material gave a homologous hybridization of up to 10 % higher, overall results were favorable. Field-collected *Laminaria* species have also been successfully used.

DNA extraction and purification. Fresh or lyophilized algal tissue was pulverized in liquid nitrogen and lysed at 50 °C for 2 h. The lysis buffer consisted of TE (100:50) plus 1 % SDS and 1M sodium perchlorate, pH = 8.0. From the lysate, DNA was purified by deproteinization with phenol-chloroform followed by chloroform-isoamyl alcohol treatments and then, two ultracentrifugation runs on CsCl gradients in the presence of ethidium bromide. Incubation with RNase and pronase was performed between ultracentrifugations. DNA concentration and purity were monitored spectrophotometrically. DNA was sheared to ca 450 bp fragments by sonication. The length of the sheared fragments was measured electrophoretically against markers of known length.

Genome characterization. A Cot [DNA concentration (mol nucleotides) × time] curve was determined for *D. cavernosa* (Hawaii) from which genetic complexity, an estimate of genome size and conditions necessary for single-copy DNA isolation could be calculated (Stam et al., 1988 in prep). G:C content was calculated from a melting curve of total unsheared DNA in 0.1 SSC buffer in order to determine incubation temperature for hybridization (Mandel & Marmur, 1968).

Hybridizations. The Hawaiian *D. cavernosa* was used as the tracer. One part of ^{3}H-gap-labeled, single-copy tracer DNA was mixed with 2000 parts of total driver DNA. The mixture was denatured, allowed to reassociate to Cot 20 000 at 25 °C below the melting temperature of native DNA. The reaction was stopped in −20 °C EtOH.

Chromatography. Reassociated DNA was subjected to hydroxylapatite chromatography. Single stranded (ss) DNA was removed at 60 °C, whereas double stranded (ds) DNA was subjected to stepwise thermal elution (65–95 °C).

Relative binding and thermal stability. Radioactivity in the ds fraction relative to the total (dsDNA + ssDNA) gives the renaturation for each hybrid. Renaturation rate of a heterologous comparison relative to the homologous one gives relative binding (RB %), a measure of sequence homology. The extent to which this parameter reflects genotypic difference is a function of true sequence mismatch and/or deletions in

the genome. The difference in thermal stability between homoduplexes and heteroduplexes was determined from the thermal elution profiles. The temperature at which 50 % of the hybrids were eluted is also a measure of genotypic relationship.

Divergence time. It is estimated that 1 °C △ Tm(e) equals an average of 5.5 Ma of elapsed time. This is based on the assumption that DNA sequences evolve in a clocklike manner (cf. Wilson et al., 1977; Ayala, 1986 for thoughtful discussions); that 1 °C △ Tm(e) = 1 % mismatch (Bonner et al., 1973) = 0.5 % average sequence change per genome; that △ Tm(e)/2 = the average sequence change per genome = rate of sequence change × divergence time; and that an average rate of sequence change of 0.09 %/Ma is accepted (Stam et al., 1988, in prep.)

RESULTS

Reassociation kinetics analysis of *Dictyosphaeria cavernosa* shows that 31.27 % of the DNA is highly repetitive, 42.47 % is middle repetitive and 25.8 % behaved as a kinetic class consistent with that predicted for single copy sequences (Fig. 1). The haploid

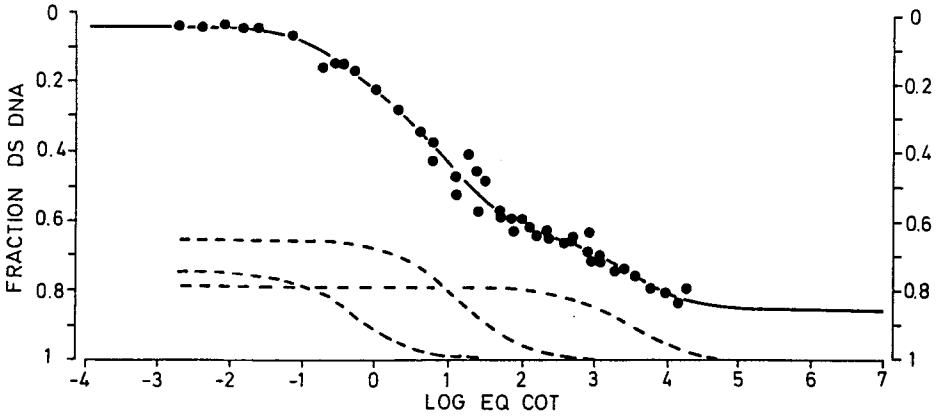

Fig. 1. Reassociation of *Dictyosphaeria cavernosa* (Coconut I., Oahu, Hawaii). The solid line represents the best least squares fit of the data to three second-order reassociation components. Dashed lines represent the highly repetitive, middle repetitive and single-copy fractions, respectively. Root mean square of the fit was 0.026. Reassociation was carried out over a concentration range of 25–4000 μg/ml, in phosphate buffer molarities ranging from 0.03–0.36, incubation times 0.5–96 h, and a temperature of 60 °C (25, 100, 1000 and 2000 μg/ml series) and 67 °C (4000 μ/ml series)

genome size is estimated at 1.79 pg DNA. A melting temperature of 72 °C was determined on total unsheared DNA from which the G:C content was calculated to be 43 %. Other technical parameters are summarized in Table 1.

D. cavernosa from Coconut I, Oahu, Hawaii was used as the radioactive tracer to which the Caribbean isolate and other species were hybridized. Results are given in Table 2 and the relationships summarized in Figure 2. These data show that disjunct isolates of *D. cavernosa* from Hawaii and St. Croix are genetically very divergent, a finding that supports earlier protein immunological distance measurements on isolates from the same localities (Olsen-Stojkovich et al., 1986). *D. versluysii* is even more distant,

Table 1. Kinetic analysis of total DNA from *Dictyosphaeria cavernosa* (Coconut Island, Oahu, Hawaii) from Figure 1

DNA component	Fraction* size (%)	K_{obs}**	$Cot½_{obs}$**	K_{corr}***	$Cot½_{pure}$****	Repet.† freq.	Complexity†† (bp)
Highly repetitive	31.27	2.078	0.48	2.68	0.116	5709	10.82×10^4
Middle repetitive	42.74	0.0745	13.42	0.0960	4.452	205	4.16×10^6
Single copy	25.81	0.000364	2744.56	0.0004691	550.20	1	5.15×10^8
Total†††	100.00	–	–	–	–	–	1.97×10^9 (1.79 pg)

* Unreassociated DNA accounted for 18.81 % of the total (see Fig. 1). In this table, fraction sizes are normalized with respect to unreassociated DNA under the assumption that these sequences are randomly distributed among the components

** Observed reaction constant (K in $M^{-1} \times s^{-1}$) and Cot value (Concentration [M] × time [s]) at which 50 % of the fraction reassociated from Fig. 1

*** Corrected reaction constant for fragment size to 250 bp. In this experiment the tracer and driver were 260 bp and 450 bp, respectively

**** $Cot½_{pure}$ = fraction size/K_{corr}

† Repetitive frequency = $K_{corr\ (repetitive\ component)}/K_{corr\ (single\ copy\ component)}$ where single copy is taken as 1

†† Fraction complexity is expressed as nucleotide pairs relative to *E. coli* (= 4.2×10^6 bp, $Cot½_{pure}$ = 4.5 M.s, 250 bp fragments). $Cot½_{pure}$ fraction × $4.2 \times 10^6/4.5$. Note that the Cot curve is extrapolated, based on the assumption that under ideal conditions the actual reassociation would proceed to completion

††† Total complexity is expressed as $K_{(E.\ coli\ =\ 0.22)} \times G_{(E.\ coli\ =\ 4.2 \times 10^6)}/K_{corr}$ single copy fraction

Table 2. Summary of hybridization results, based on relative binding (RB %) and on differences in thermal stability, i. e. thermal midpoint of elution [ΔTm(e) °C], from 150-bp fragments of *D. cavernosa* scDNA tracer and 450-bp fragments of total DNA drivers

Species	Status	ΔTm(e) (°C)	RB (%)	Hypothesized divergence (Ma)
Dictyosphaeria cavernosa (Coconut I., Oahu, Hawaii)	tracer	0*	100**	
Dictyosphaeria cavernosa (Boiler Bay, St. Croix, U.S.V.I)	driver	10.0 (+/− 0.6)	13.9 (+/− 0.8)	55
Dictyosphaeria versluysii (Waikiki, Oahu, Hawaii)	driver	12.3 (+/− 0.3)	9.8 (+/− 0.5)	68***
Struvea sp. (outgroup)	driver		2.7 (+/− 1.2)	

* Measured thermal midpoint of elution = 77.3 °C

** Measured renaturation = 51 %

*** Estimate at the outer limit of sensitivity for the method

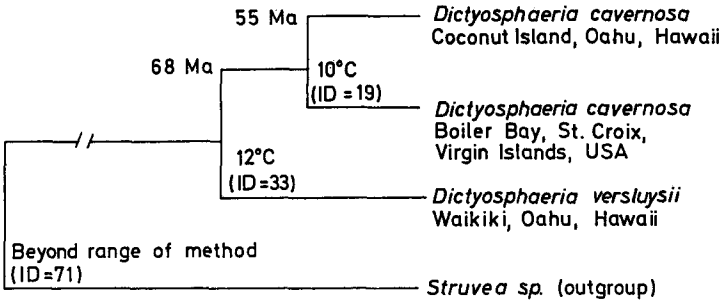

Fig. 2. Phylogenetic hypothesis based on single-copy DNA-DNA hybridization data from Table 2. Topology is identical for both RB % and ΔTm(e) values. This topology and estimate of divergence also confirms a parallel study based on immunological distance measurements (values in parentheses) (Olsen-Stojkovich et al., 1986)

at the limits of sensitivity of the method. Assignment of real divergence time under a molecular clock model suggests a lineage separation of up to 55 Ma based on a ΔTm(e) value of 10 ° C.

DISCUSSION

The only other benthic macrophytes that have had their nuclear genomes characterized are *Cladophora albida* [0.48 pg DNA, 3-component complexity, 32 % single-copy (Bot pers. communication)] and *Laminaria digitata* [0.51 pg DNA, 2-component complexity, 51.43 % single-copy (Stam et al., 1987). Until a data base is generated for many algal groups the significance of any particular genome size and complexity remains obscure.

Assignment of real geological divergence time to phylogenetic topologies is dependent on either a fossil record or knowledge of vicariant events that are hypothesized to have resulted in phyletic radiations. For tropical marine algae, these vicariant events include plate tectonic movements that have opened or closed various bodies of water, especially the Tethys Sea. The Tethys Sea is thought to have extended back at least into the Jurassic (180 Ma ago), during which time a continuous marine flora is believed to have existed (Melville, 1967; Sylvester-Bradley, 1967). With the gradual formation of contemporary continental configurations during the Miocene (ca 20 Ma ago), the Tethys Sea closed and fragmented this primordial marine flora. It is hypothesized that Tethyan remnants have been best preserved in the tropical oceans and that some lineages have continued to evolve, essentially morphologically unchanged, for millions of years. Support for these hypotheses is partly based on limited fossil remains from the Jurassic and Lower Cretaceous (Schopf, 1970; Wray, 1978; Tappan, 1980) and on the living fossil concept (Stanley, 1979; Schopf, 1984) in which extant taxa are morphologically conservative, show little taxonomic diversity, and little provincial endemism. Cladophoralean species fit these criteria.

Other evidence that suggests ancient origins has come from recent protein immunological distance studies among cladophoralean genera and species (Olsen-Stojkovich, 1986; Olsen-Stojkovich et al., 1986). Interspecific distances among five species of *Dictyosphaeria* were nearly twice as large as those observed among species in the majority of

other genera in that study; and intraspecific distances among widely separated biogeographic isolates of *Dictyosphaeria cavernosa* were also inexpectedly large. These results suggested that there were two lineages, morphologically indistinguishable but genetically divergent. This result is further supported by the DNA-DNA hybridization studies reported here in which isolates were collected from the same localities as used in the immunological work.

Most recently, similarly large intraspecific distances have been found in our laboratory in another cladophoralean species, *C. albida*, using single-copy DNA-DNA hybridizations (Bot et al., unpublished). Isolates from the North Atlantic French coast were compared with isolates from Japan and Australia. All reciprocal comparisons were made. The Australian and Japanese isolates had a ΔTm(E) of 1.5–2.0 °C between themselves, whereas each had a ΔTm(e) between 5–6 °C from the European isolate, suggesting a 33-Ma divergence.

Single-copy DNA-DNA hybridization studies among five North Atlantic species of *Laminaria* (Phaeophyceae) show somewhat smaller interspecific distances (ΔTm(e) ca 3.0 °C) indicating that a species radiation occurred between 15–19 Ma ago (Stam et al., 1987, 1988, in prep.) That study, however, did not investigate intraspecific divergence.

The picture emerging thus far suggests that while *Laminaria* is a tightly circumscribed genus, whose species are of comparatively more recent origin (15–19 Ma ago), at least some cladophoralean taxa are much older, having evolved as long ago as 55–60 Ma. It is also interesting to ponder how such great genetic divergence could have resulted in so little morphological dissimilarity.

The *D. cavernosa* divergence estimate is, however, offered cautiously for two reasons, one theoretical and one technical. First, this estimate is based on an average rate of sequence change of ca 5.5 Ma/°C ΔTm(e) calculated from a variety of fossil records (Stam et al., 1988, in prep.). A similar divergence estimate based on protein immunological distance data measured by the same techniques and calibrated from fossil pinacean genera (Prager et al., 1976; Price et al., 1987) suggests a divergence time of about 60 Ma for *D. cavernosa*. These estimates are in good agreement. However, since rates have been recently shown to be different for different animal groups (Britten, 1986), it has to be assumed that rates may also differ among plant groups. Unfortunately, similar comparative rate studies for plant groups have not been made. Therefore, these estimates are based on nested assumptions and are crude at best. Second, the data presented here are based on only three isolates and the relative binding of the homologous hybridization was low. Normally, 70–80 % homologous reassociation can be expected. In these experiments, runs that utilized lyophilized material showed good homologous reassociation (80 %), whereas experiments that utilized frozen material gave a lower percentage (51 %). This material may have been partially thawed during transport and thus damaged. Despite this, reproducibility of the 10 °C ΔTm(e) value was good (10 % sequence difference), and corresponds well with a 13.9 % RB value. Moreover, hybridization with another Hawaiian *D. cavernosa* isolate from Waikiki gave a ΔTm(e) of 0.6 °C (= 0.6 % difference), indicating virtual identity between the two Hawaiian isolates, which provides an additional check on the hybridization.

In conclusion, although the actual divergence time is subject to some error, the overall result stands that these *Dictyosphaeria* lineages have been separated for as long as 55–60 Ma as estimated by DNA hybridization data. DNA hybridization studies thus

provide strong support to the Tethyan vicariance model and raise the question of the species concept in these otherwise morphologically identical taxa.

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