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Single-copy DNA-DNA hybridizations among five species of *Laminaria* (Phaeophyceae): phylogenetic and biogeographic implications*

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ABSTRACT: DNA-DNA hybridizations between single-copy nuclear DNA from *Laminaria digitata* and total DNA from *L. saccharina, L. hyperborea, L. rodriguezii, L. ochroleuca* and *Chorda filum*, respectively, show that these species of *Laminaria* are genotypically closely related. *Chorda filum* is only distantly related with *L. digitata*. Based on the thermal elution patterns of the DNA hybrids, as quantified by $\Delta T_{m(e)}$ values, it is hypothesized that all five species of *Laminaria* evolved at about the same time from their most recent common ancestor some 15–19 Ma ago. This phylogenetic hypothesis is discussed in relation to the history of modern laminariaiean distribution patterns.

INTRODUCTION

Laminaria is a dominant genus along the rocky subtidal domains of most northern-hemisphere, temperate shores. Within the North Atlantic Ocean, the present northern and southern boundaries of a number of *Laminaria* species are apparently determined by various critical temperature effects (lethal temperatures, temperatures inhibiting the onset of life history stages) (for reviews see van den Hoek, 1982; Breeman, 1988 this volume); it is likely that latitudinal boundaries of *Laminaria* species in the N Pacific have comparable causes.

So far, the possible significance of historical events in the geological past for the present day distributions of *Laminaria* species has not been investigated. In the present study we will formulate hypotheses on these historical aspects on the basis of DNA-DNA hybridization among five *Laminaria* species.

In this study, the following questions were of interest: (1) what are the phylogenetic relationships among species of *Laminaria* common to the North Atlantic; (2) how long ago did these species diverge from their most recent common ancestor; and (3) in the absence of a fossil record, do the divergence times help to elucidate modern distribution patterns in the context of climate conditions and paleogeographic configurations in the historical past?

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Data are presented from single-copy nuclear DNA-DNA hybridization experiments on five species of *Laminaria*. By using the scDNA-DNA hybridization technique both genotypic relationships between organisms and an estimate of elapsed time since the species evolved from their most recent common ancestor were determined. The *Laminaria* species are among the major North Atlantic representatives of the genus and were chosen in order to provide a mix of distributional limits. They are: *Laminaria digitata* (Huds.) Lamouroux, occurring on both sides of the North Atlantic; *L. saccharina* (L.) Lamouroux, circumpolar distribution; *L. hyperborea* (Gunn.) Foslie, northern Northeast Atlantic only; *L. ochroleuca* La Pylaie, more southern Northeast Atlantic and Mediterranean; and *L. rodriguezii* Bornet, Mediterranean endemic. *Chorda filum* (L.) Stackh. was used as an outgroup.

MATERIALS AND METHODS

Organisms

Laminaria digitata, L. saccharina, L. hyperborea and *Chorda filum* were collected from the Helgoländer Nordostwatt (Helgoland, W Germany). *Laminaria rodriguezii* and *L. ochroleuca* were collected in the Straits of Messina (Italy) and near Roscoff (Brittany, France), respectively. Young non-sporogeneous plant tissue was selected and thoroughly cleaned in seawater followed by a freshwater rinse and blotting between sheets of filter paper. The specimens were then lyophilized on-site, sent to our laboratory and stored at −20 °C. Prior to use, small tissue samples were rehydrated from each species and screened for epiphytic contamination by staining with the fluorescent DNA specific dye, DAPI. The samples were examined under a fluorescent microscope and only tissue with less than ca 3% contaminating cells, mostly diatoms and bacteria, were considered acceptable for DNA extraction.

Materials

The following chemicals were purchased from Boehringer Mannheim (W Germany): 4',6-diamidine-2-phenylindol (DAPI), RNase, pronase, DNA molecular weight marker III, deoxy-adenosine-triphosphate (dATP), dCTP, dTTP, polymerase I and bovine serum albumin (BSA). CsCl p.a. was obtained from Janssen Chimica (Belgium), agarose from Sigma (USA), Chelex 100 and hydroxyapatite (HAP) DNA-grade from Biorad (USA), [^{3}H]dCTP from Amersham (England), Sephadex G-50 from Pharmacia (Sweden) and the scintillation fluid (Pico-Fluor) + counter from Packard (USA).

DNA extraction

Routinely 6 g (lyophilized weight) of tissue were pulverized in a mortar with liquid N_2. The pulverized tissue was added to 40 ml lysis buffer A (100mM TRIS/20mM $CaCl_2$) at room-temperature. The mixture was gently homogenized and transferred to a cold (−20 °C) X-press (Biotec, Sweden). Following overnight incubation at −20 °C, the mixture was extruded through the X-press at $8–10 \cdot 10^3$ kg · cm^{-2} and pulverized a second time in a mortar with liquid N_2. The pulverized tissue was added to 80 ml of warm (50 °C) lysis buffer B (100mM TRIS with 1% Na-dodecylsulphate, 1M $NaClO_4$, 200mM $CaCl_2$ and an

additional 1 g $CaCl_2$ in order to adjust the $CaCl_2$ concentration in lysis buffer A to ca 200mM), mixed and incubated at 50 °C for 2 h. The aqueous lysate was collected by centrifugation (10 000 g, 30 min). Deproteinization of the lysate was achieved with two phenol-extractions [phenol saturated with TE 10:1 buffer (10mM TRIS, 1mM EDTA), 20 min incubation, after 10 min addition of 0.5 volume chloroform] followed by 2–4 treatments with CIA (chloroform-isoamylalcohol 24:1 v/v) until no protein precipitate was observed at the interphase. Separation of the aqueous phase and the phenol or CIA layer was achieved by centrifugation (2000 g, 20 min). Finally, crude DNA was precipitated from the aqueous layer by addition of 0.075 volume 4M Na-acetate and 2.3 volumes 100 % ethanol p.a. at −20 °C overnight.

DNA purification

The ethanol precipitated DNA pellet was collected by centrifugation (2000 g, 30 min), washed with a small volume of 70 % ethanol in TE 10:1, recentrifuged, dried in a vacuum desiccator (ca 30 min) and dissolved in TE 10:1 (4 ml per 1 g lyophilized tissue). The redissolved crude DNA extract was then subjected to CsCl ($1\,g \cdot ml^{-1}$ solution) density gradient centrifugation (Beckman L8-M ultracentrifuge with Ti 75 fixed angle rotor, 40 000 rpm, 44 h) in the presence of ethidium bromide ($300\,\mu g \cdot ml^{-1}$ solution). The fluorescent DNA band was removed from the centrifuge tube and ethidium bromide and CsCl removed by extractions with 2-propanol (saturated with both CsCl and TE 10:1), followed by dialysis against TE 10:1 (40 h, two TE changes). After dialysis the solution was incubated with RNase ($70\,\mu g \cdot ml^{-1}$, 37 °C, 1 h; preincubated for 10 min at 80 °C) and then pronase ($100\,\mu g \cdot ml^{-1}$, 37 °C, 2 h; predigested for 1 h at 37 °C). A second ultracentrifugation step (including 2-propanol extractions and dialysis) was performed as above. The concentration and purity of the DNA was determined in a spectrophotometer (PYE Unicam PU8600). The purity was considered satisfactory when both the ratios A_{260}–A_{320}/A_{230}–A_{320} (polysaccharides) and A_{260}–A_{320}/A_{280}–A_{320} (proteins) were 1.9 or more. The DNA concentration was adjusted to $200\,\mu g \cdot ml^{-1}$ either by diluting with TE 10:1 or concentrating with 2-butanol.

Determination of DNA-base composition

The thermal midpoint of dissociation (T_m) of DNA from *Laminaria digitata, L. hyperborea* and *L. saccharina* was determined from U.V. absorbance-temperature profiles according to de Bont et al. (1981). The DNA-base composition (% GC) was calculated according to Gillis et al. (1970):

$$T_m = 0.41 \times \%\ GC + 78.7 + 13.3 \log[Na^+]$$

Preparation of sheared DNA

DNA ($200\,\mu g \cdot ml^{-1}$ TE 10:1, 0.5-ml aliquots) was sheared by sonication in an ultrasonic disintegrator (MSE, 2 bursts of 25 s at an amplitude of 10 μm with an interval of 1 min). DNA fragments with an average length of 450 bp were obtained as confirmed by electrophoresis on 1 % agarose gels, in the presence of the DNA molecular weight

marker III. Bivalent cations were removed from the sheared DNA solution with Chelex 100. The DNA was then precipitated with 2.3 volume 100% ethanol (−20 °C) in the presence of 0.3M Na-acetate and stored at −20 °C until ready for use.

Gap-labeling of DNA

Reassociated DNA in 50mM Na-phosphate buffer (pH = 7.6) was used as the DNA template. The labeling mixture (100 µl) contained the following: 50mM Na-phosphate buffer (pH = 7.6), 10mM $MgCl_2$, 1mM EDTA, 10mM β-mercaptoethanol, 50 $\mu g \cdot ml^{-1}$ BSA, 40µM each of dATP, dTTP and dGTP, 15µM [^{3}H]dCTP (1.8–3.15 $TBq \cdot mM^{-1}$) and 20 $\mu g \cdot ml^{-1}$ DNA. After addition of 6 units polymerase I per µg DNA, the mixture was incubated for 40–42 h at 11 °C. The reaction was stopped by addition of EDTA to a final concentration of 100mM (Galau et al., 1976b). The bulk of unincorporated [^{3}H]dCTP and other reagents were removed with Sephadex G-50 column exchange according to the spin-column procedure of Maniatis et al. (1982). The labeled DNA was then denatured (5 min, 100 °C), incubated to Cot 10^{-3} at 60 °C, rapidly cooled on ice and loaded onto a 0.03M NaPB/0.135M NaCl (= 0.03M Na-phosphate buffer, i.e. an equimolar buffer of Na_2HPO_4 and NaH_2PO_4, pH = 6.8, with 0.135M NaCl) pre-equilibrated HAP column. The loaded column was washed first at 50 °C with 0.03M NaPB/0.135M NaCl and then at 60 °C with 0.03M NaPB. Single-stranded (ss) radioactive DNA was removed from the HAP with 0.12M NaPB and double-stranded (ds) radioactive foldback DNA with 0.3M NaPB. Radioactivity of each fraction was determined by liquid scintillation. In general 50% of the DNA was recovered in the ss-fractions with about 4×10^6 cpm $\cdot \mu g^{-1}$ DNA. Labeled DNA fragment length was determined by electrophoresis on 1% alkaline (20mM NaOH) agarose gels, in the presence of DNA molecular weight marker III. Unlabeled, marker DNA was stained with ethidium bromide, whereas [^{3}H] labeled fragments were cut into 0.5 cm cubes, incubated in 0.2M HCl for 3 h and counted in Pico-Fluor.

Determination of Cot-curve

The reassociation kinetics of the genome (Cot-curve, Cot = *co*ncentration × *t*ime in Moles $\cdot$ sec^{-1}) were determined from *Laminaria digitata* since DNA from this species was used as the tracer in subsequent hybridization experiments.

Mixtures of total [^{3}H] labeled DNA (tracer, average fragment size = 220 bp) and homologous unlabeled total DNA (driver, 450 bp) were prepared over a broad range of concentrations (25–4000 $\mu g \cdot ml^{-1}$) and NaPB (0.03–0.36M). A 2000-fold excess of driver DNA was used. Aliquots of these dilution series were sealed in capillaries, denatured (5 min, 100 °C) and incubated at the appropriate reassociation temperature (= T_m−25), as calculated from the formula according to Gillis et al. (1970) given above. A time series of 10 sampling points ranged from 0.5–96 h. Reassociation was stopped by immersion of the capillaries into −20 °C ethanol. The capillaries were emptied into 250 µl 0.03M NaPB containing 0.1% SDS and then loaded onto 0.03M NaPB pre-equilibrated HAP columns at room-temperature. Following a 0.03M NaPB wash at 60 °C, ss-DNA was removed with 0.12M NaPB at the same temperature and ds-DNA with the same buffer at 95 °C. Radioactivity in each of the eluted fractions was counted. The fraction of ds-DNA present in each reassociation mixture and the coordinate equivalent Cot-value (in M.s.) was

calculated according to the standard method of Britten et al. (1974). The kinetic reassociation profile for total DNA from *L. digitata* was calculated and plotted with an independent second-order kinetics program according to the least square method.

Preparation of single-copy DNA

Single-copy DNA of *Laminaria digitata* was prepared in a two-step reassociation cycle as follows: in the first cycle, total DNA (in 0.48M NaPB) was sealed in capillaries, denatured (5 min, 100 °C) and incubated at the appropriate reassociation temperature (= T_m−25 = 68 °C). Incubation time was predetermined by selecting the equivalent Cot-value at which 97 % reassociation of the repetitive DNA sequences was known. This is referred to as Cot_1 (= 160 M.s. for *L. digitata*) as calculated from the Cot-curve determined (Fig. 1). HAP chromatography was used following Cot_1 incubation at 60 °C and DNA concentrations of the eluted ss-fractions were determined spectrophotometrically. The fraction containing the most ss-DNA was chosen and concentrated with 2-butanol. NaPB molarity was also adjusted and the first-cut scDNA resealed in capillaries. In this reassociation cycle, an equivalent Cot-value of 64 M.s. was chosen and referred to as Cot_2 (Cot_2 = Cot_1 × the single-copy fraction as given by the total DNA Cot-curve = 160 × 0.4012). HAP chromatography was used following Cot_2 incubation and, as before, DNA concentration of the eluted ss-DNA fractions were determined. The fraction containing the most ss-DNA was further concentrated with 2-butanol, desalted with Sephadex G-50 according to the spincolumn procedure, and the DNA was precipitated with 2.5 volumes 100 % ethanol (−20 °C) in the presence of 0.3M Na-acetate. After overnight precipitation at −20 °C, the DNA was collected by microfuge centrifugation, dissolved in 0.48M NaPB to a concentration of ca 4000 $\mu g \cdot ml^{-1}$, sealed in capillaries and incubated at T_m−25 (= 68.8 °C) to Cot 20 000 M.s. The reassociated single-copy DNA was diluted in 50mM NaPB (pH = 7.6) to a concentration of 40 $\mu g \cdot ml^{-1}$ and gap-labeled.

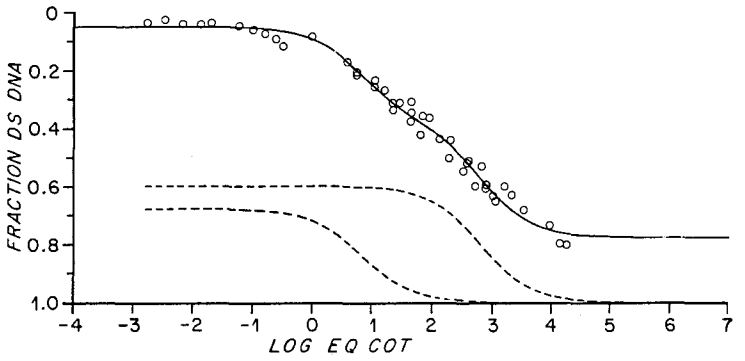

Fig. 1. Reassociation kinetics of total DNA from *Laminaria digitata*. The solid line represents the best least squares fit based on two second-order components (dashed line insets). Highly repetitive sequences (5.17 %) reassociated before Cot = 0.002 M.s. A repetitive fraction of 33.11 % reassociated with a reaction constant of 0.14 $M^{-1} \cdot s^{-1}$. From this fraction 97 % is reassociated at Cot = 160 M.s. The single-copy fraction of 39.23 % reassociates with a reaction constant of 0.0014 $M^{-1} \cdot s^{-1}$, while 22.49 % of the DNA failed to reassociate. The root mean square (RMS) error of the best least squares solution was 0.029

Monitoring single-copy DNA

Purity of the single-copy DNA preparation, i.e. absence of repetitive sequences, was double-checked by determining a Cot-curve of the scDNA. The procedure is the same as discussed above with the following alterations: the reassociation mixture contains single-copy [^{3}H] labeled DNA as tracer instead of total [^{3}H] labeled DNA and the DNA dilution series were 200 and 2000 $\mu g \cdot ml^{-1}$ in 0.12 and 0.48M NaPB, respectively.

Hybridization conditions

One part single-copy tracer DNA from *Laminaria digitata* (150 bp) and 2000 parts driver DNA (450 bp) from all species tested (final concentration ca 4500 $\mu g \cdot ml^{-1}$ in 0.36M NaPB) were sealed in capillaries, denatured (5 min, 100 °C) and incubated at $T_m - 25$ (= 67 °C) to an equivalent Cot-value of 20000 M.s. (*L. digitata* DNA was used as homologous driver). Two sets of three replicates of each hybridization were run. Reassociation was stopped by immersing the capillaries in −20 °C ethanol. Capillaries were emptied into 0.03M NaPB/0.1 % SDS and loaded onto a 0.03M NaPB pre-equilibrated HAP columns. Following a 0.03M NaPB wash at 60 °C, ss-DNA was eluted with 0.12M NaPB at the same temperature. The column temperature was then raised to 95 °C in 5 °C increments. At each temperature step, "melted" DNA was eluted from the column with 0.12M NaPB and counted.

Reassociation calculations

The renaturation rate (% RR) was calculated according to:

$$\% \text{ RR} = \frac{\text{total cpm in 65–95 °C 0.12M NaPB eluates}}{\text{total cpm in 60–95 °C 0.12M NaPB eluates}} \cdot 100\,\%$$

The relative binding (% RB) of the heterologous hybridizations was calculated according to:

$$\% \text{ RB} = \frac{\% \text{ RR of the heteroduplex}}{\% \text{ RR of the homoduplex}} \cdot 100\,\%$$

The thermal midpoint of eluation ($T_{m(e)}$) is defined as the temperature at which 50 % of the total cpm's (in the 65–95 °C, 0.12M NaPB eluates) are eluted from the HAP. $T_{m(e)homoduplex} - T_{m(e)heteroduplex}$ yields the $\Delta T_{m(e)}$ value in °C for the heterologous hybridization.

Estimation of divergence times

Single-copy DNA-DNA hybridization data permit a molecular level estimate of divergence time among closely related taxa, in the absence of fossil evidence, under the following assumptions and considerations: (1) DNA sequences evolve in a clocklike manner (Wilson et al., 1977); (2) 1 °C $\Delta T_{m(e)}$ = 1 % mismatch (Bonner et al., 1973) = 0.5 % average sequence change per genome; (3) $\Delta T_{m(e)}/2$ = the average sequence change per genome = rate of sequence change × divergence time; (4) Rate of sequence change has been or can be calculated from results of scDNA-DNA hybridizations done with sea-

urchins (Angerer et al., 1978; Harpold & Craig, 1978), *Xenopus* (Galau et al., 1976a), primates (Kohne et al., 1972), teleost fishes (Schmidtke & Kandt, 1981) and *Osmunda* (Stein et al., 1979). In all these studies, the results were obtained by using the hydroxyapatite/phosphatebuffer method as described above; the $\Delta T_{m(e)}$ values were determined exclusively with single-copy DNA and, most important, a well delineated fossil record was available for paleocalibration. An average rate of sequence change of 0.09 % · Ma^{-1} was calculated. Sibley & Ahlquist (1984, using a modified protocol) calculate an average rate of 0.11–0.125 % · Ma^{-1}. The following formula is suggested:

$$\text{divergence time} = \Delta T_{m(e)}/2 \times 1/0.09 \text{ M.a}$$

See "Discussion" for accuracy and applicability of these estimates for plant systems, in general, and the algae, in particular.

RESULTS

DNA extraction and purification

Extraction and subsequent purification of DNA from brown algae require special methods because of the vast amount of cell-wall-associated polysaccharides. Standard methods used for bacteria were unsuitable because it was found that the phaeophycean alginate co-precipitated and subsequently redissolved with the DNA during the extraction process. Elimination of alginate contamination is essential because failure to do so results in gelling of the highly concentrated DNA solutions needed in the experimental protocols.

To overcome this problem a new method was developed based on a procedure used in our laboratory for siphonocladous green algae (Bot et al., in press). The principal adaptations are the use of an X-press for mechanical disruption of the tough, rubbery cell walls, and the addition of 200mM $CaCl_2$ to the lysis mixture, which results in bulk precipitation of the alginate during initial lysis. Further traces of alginate are removed by the two ultracentrifugation steps. The main point is that excellent DNA-yields can be obtained, both with respect to quantity (ca 700 µg from 6 g lyophilized tissue = ca 40 g wet weight in *Laminaria*) and quality (U.V. absorption patterns) from marine algae, whose cell-wall-associated polysaccharides have heretofore presented insurmountable problems. This seemingly small but critical breakthrough will now make it possible for a wide variety of nucleic acid-level studies to be undertaken in the algae.

Reassociation kinetics of total and single-copy DNA

The reassociation kinetics (Cot-curve) of total DNA from *Laminaria digitata* are shown in Figure 1. The repetitive fraction sizes 43.40 % (corrected for the fraction unreassociated DNA sequences; see Table 1). This fraction is also assumed to contain chloroplast DNA since whole-cell DNA was used. Chloroplast DNA has a kinetic behaviour like nuclear repetitive DNA because, for each single-copy nuclear DNA sequence, there are numerous chloroplast DNA sequences. The single-copy DNA fraction accounts for 51.34 % of the sequences. The relatively high amount of DNA (22.49 %) that failed to reassociate possibly represents a class of small and/or damaged fragments

Table 1. Kinetic analysis of total and single-copy DNA from *Laminaria digitata*

Component	Fraction* size	K_{obs}**	$Cot_{½obs}$**	K_{corr}***	$Cot_{½pure}$†	Rep.†† freq.	Kin.††† compl.
Repetitive	0.4340	0.14	7.15	0.2128	2.0	100	1.87×10^6
Single-copy	0.5143	0.0014	725.15	0.002128	241.7	1	2.26×10^8
Single-copy††††	0.9240	0.0019	523.86	0.004248	–	–	–

* Fraction size. Unreassociated DNA sequences accounted for 22.49 % in the total DNA Cot-curve (Fig. 1) and for 22.63 % in the single-copy DNA Cot-curve (Fig. 2). In this table, fraction sizes are normalized with the assumption that these unreassociated sequences are randomly distributed among the components involved.

** Observed reaction constant (K in $M^{-1} \cdot s^{-1}$) and the Cot value (in $M \cdot s$) at which 50 % of the fraction reassociated ($Cot_{½} = 1/K$) as given by the second order component calculations.

*** Reaction constant after correction of fragment size to 250 bp according to Okamura & Goldberg (1985): $K_{corr} = (L_d/L_t)^{½} \cdot (250/L_t)^{½} \cdot K_{obs}$, where L_d and L_t are the fragment sizes of the driver and tracer respectively (driver = 450 bp, tracer = 220 bp in total DNA Cot-curve and 150 in single-copy DNA Cot-curve).

† $Cot_{½pure}$ = fraction size. $1/K_{corr}$ = the $Cot_{½}$ in the reassociation of the homogeneous kinetic component (Zimmerman & Goldberg, 1977).

†† Repetitive frequency = K_{corr} repetitive fraction/K_{corr} single-copy fraction.

††† Kinetic complexity of each fraction is expressed in nucleotide pairs relative to the complexity of the *Escherichia coli* genome standard (= 4.2×10^6 with a $Cot_{½}$ of 4.5 $M \cdot s$ for 250 bp fragments, Okamura & Goldberg, 1985), as follows: kin. compl. = $Cot_{½pure}$ fraction. $4.2 \times 10^6/4.5$. Note that the Cot-curves are extrapolated, based on the assumption that under ideal conditions the actual reassociation would proceed to completion.

†††† See Fig. 2.

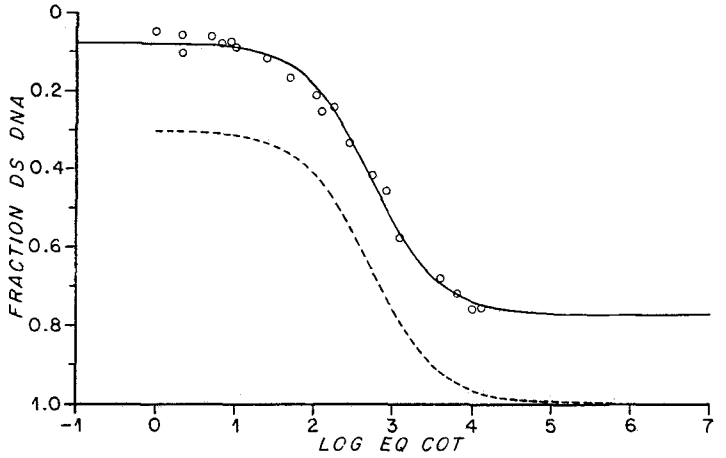

Fig. 2. Reassociation kinetics of single-copy DNA from *Laminaria digitata*. The solid line represents the best least squares solution based on a single second order component (dashed line inset). A fraction of 7.60% reassociated before Cot = 0.0088 M.s. The single-copy fraction of 69.77% reassociated with a reaction constant of 0.0019 $M^{-1} \cdot s^{-1}$ and 22.63% failed to reassociate. The RMS was 0.023

possibly created by the X-press treatment. Highly repetitive DNA sequences (fold-back, palindromic) reassociating before Cot = 0.001 are not measured as a consequence of the procedure used for the purification of labeled tracer DNA.

The reassociation kinetics of the isolated single-copy fraction of DNA are shown in Figure 2. The single-copy fraction accounts for 92.40 % of the sequences when corrected for the 22.63 % of the sequences which failed to reassociate (Table 1).

Kinetic analyses from both Cot-curves are summarized in Table 1. Our analyses show that for the haploid genome of *L. digitata*, the repetitive DNA sequences (including chloroplast DNA) are about 100 times more frequent than single-copy sequences, and that its complexity is 0.434×10^9 nucleotide pairs or approximately 0.48 pg DNA as calculated from the K_{corr} from the single-copy fraction in the total DNA Cot-curve when related to the K_{corr} ($= 0.22\ M^{-1} \cdot s^{-1}$) and the complexity ($= 4.2 \times 10^6$ bp) of the genome of *Escherichia coli* (Okamura & Goldberg, 1985). This calculation is an overestimate because chloroplast DNA is included. Ersland & Cattolico (1981) report that whole-cell DNA of *Olisthodiscus luteus* (Heterokontophyta, Chrysophyceae) contains 4.85 % chloroplast DNA. Using their value for *L. digitata*, a more realistic estimate is 0.413×10^9 bp or approximately 0.45 pg DNA is suggested. Finally it is noted that the rate constant of the single-copy DNA is, though not identical, quite similar (0.002128 and 0.004248 $M^{-1} \cdot s^{-1}$, respectively), whether determined from total DNA or from a separate single-copy fraction, demonstrating that this fraction is highly enriched for unique DNA sequences.

DNA-DNA hybridizations

Results of the scDNA-DNA hybridizations in which *Laminaria digitata* was used as the tracer are shown in Table 2. All species of *Laminaria* tested are genotypically closely related to *L. digitata* since the RB values are high (84.7–97.5 %) and the $\Delta T_{m(e)}$ values low

Table 2. Percentages of relative binding (% RB) and differences in thermal midpoint of elution (°C $\Delta T_{m(e)}$) of single-copy DNA-DNA hybridizations. Tracer: 150 bp fragments of single-copy DNA from *Laminaria digitata*. Driver: 450 bp fragments of DNA from *L. digitata*, *L. saccharina*, *L. hyperborea*, *L. rodriguezii*, *L. ochroleuca* and *Chorda filum* (outgroup). RB and $\Delta T_{m(e)}$ values are related to the homologous *L. digitata* hybridization. DNA base compositions (% GC) and estimated divergence times (in Ma) are also shown

Tracer	*Laminaria digitata*		% GC	Divergence time (Ma)
Driver	RB (%)	$\Delta T_{m(e)}$(°C)		
L. digitata	100*	0**	41.6	–
L. saccharina	97.5 (±0.1)	2.7 (±0.3)	39.9	15
L. hyperborea	88.4 (±1.6)	3.2 (±0.2)	42.6	18
L. rodriguezii	86.4 (±0.8)	3.2 (±0.7)	n.t.	18
L. ochroleuca	84.7 (±1.1)	3.5 (±0.2)	n.t.	19
Chorda filum	12.8 (±0.5)	12.0 (±1.2)	n.t.	(67)

* Set at 100 % (see 'Materials and Methods'). Measured renaturation rates averaged 77.5 %
** Set at 0 °C (see 'Materials and Methods'). Measured $\Delta T_{m(e)}$ values averaged 82.9 °C.
n.t. = not tested

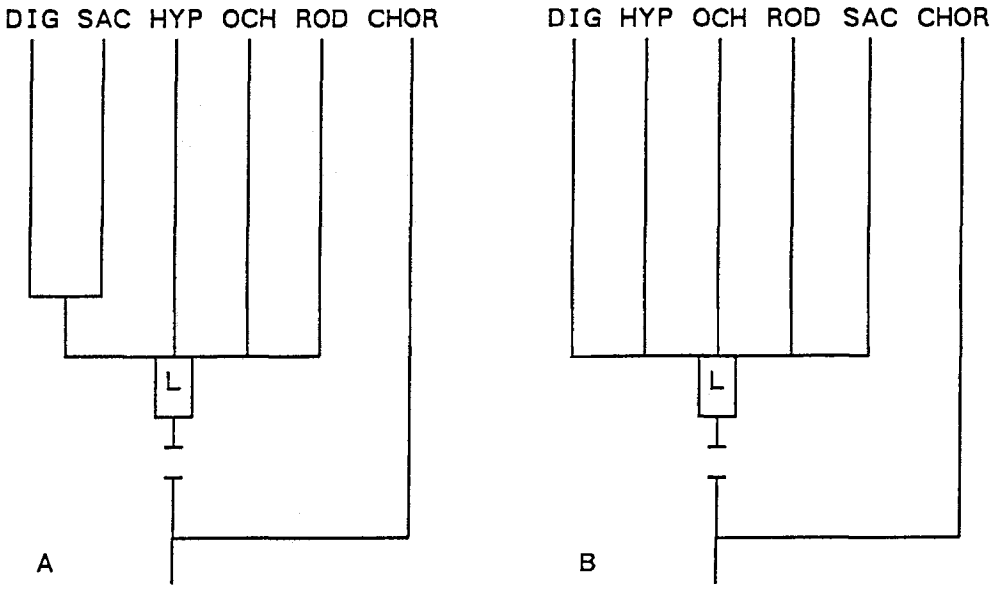

Fig. 3. Phylogenetic hypotheses among five species of *Laminaria*. A. Hypotheses based on % RB values; B. Hypotheses most consistent with $\Delta T_{m(e)}$ data in which all five taxa arise from a hypothetical ancestor. See text for discussions. L = most recent common ancestor. DIG = *Laminaria digitata*, SAC = *L. saccharina*, HYP = *L. hyperborea*, OCH = *L. ochroleuca*, ROD = *L. rodriguezii* and CHOR = *Chorda filum* (outgroup)

(3.5–2.7 °C). *L. digitata* shows a significantly higher RB value to *L. saccharina* than to *L. hyperborea*, *L. rodriguezii* and *L. ochroleuca* on the individual experimental data as determined by Wilcoxon's signed-ranks test (Sokal & Rohlf, 1973). No significant differences were found in the RB percents among *L. hyperborea*, *L. rodriguezii* and *L. ochroleuca* with respect to *L. digitata*. This might initially suggest that *L. saccharina* is most closely related to *L. digitata* and the others are equidistant to this species (Fig. 3 A). When the same statistical tests are applied to the $\Delta T_{m(e)}$ data, no significant differences were found among *L. saccharina*, *L. hyperborea*, *L. rodriguezii* and *L. ochroleuca* with regard to *L. digitata*. This suggests that all four species are phylogenetically equidistant to *L. digitata* (Fig. 3 B). Considering the RB values, *L. digitata* does in fact share more sequences with *L. saccharina* than with *L. hyperborea*, *L. rodriguezii* and *L. ochroleuca*. This could be the result of lost single-copy sequences by deletions in *L. hyperborea*, *L. rodriguezii* and *L. ochroleuca* following divergence from an ancestral sequence they had in common with *L. digitata*. The problem with this interpretation lies in the fact that %RB is strictly a measure of reassociation, whereas thermal stability of heteroduplexes, as quantified by the $\Delta T_{m(e)}$ values, provides a measure of sequence mismatch, which is more indicative of actual divergence. Among the four *Laminaria* species, all sequences matched equally well with *L. digitata*. Thus, the most conservative phylogenetic interpretation of the hybridization data, without any additional a priori assumptions, is that all five species diverged at about the same time from a common ancestor (Fig. 3 B).

The close genotypic relationship of *L. digitata* with *L. saccharina* and *L. hyperborea*

is confirmed by the small differences in % GC. These DNA base composition determinations have an experimental error of ca 1% (Stulp & Stam, 1984).

Chorda filum was used as an outgroup. Its putative taxonomic position is confirmed by its low genotypic relationship with *L. digitata* (RB = 12.8% and $\Delta T_{m(e)}$ = 12.0°C). Hybridization with calf thymus DNA (RB = 6.3% and $\Delta T_{m(e)}$ beyond the sensitivity of the method) and self-reassociation of the tracer DNA (RB = 4.5%) were also included as a standard control on the method.

Estimate of divergence time

From the $\Delta T_{m(e)}$ data and application of the formula for real divergence time, we estimate that these five species of *Laminaria* diverged from a common ancestor between 15–19 Ma ago. Although the $\Delta T_{m(e)}$ for *Chorda filum* lies near the limits of the resolving power of this technique, we estimate a rough divergence time of about 67 Ma.

DISCUSSION

Haploid genome size

The 0.48 pg DNA that characterize the haploid genome of *Laminaria digitata* can only be compared with very distant organisms because, to our knowledge, no other benthic algal macrophyte has been investigated. The closest relative is *Olisthodiscus luteus* (Chrysophyceae) with 2.06 pg DNA (Ersland & Cattolico, 1981). Despite this large difference, both organisms have similar single-copy fraction sizes and a two component Cot-curve. Two species of green, giant-celled, cladophoralean algae have also been measured in our laboratory. *Cladophora albida* has a haploid genome of 0.82 pg DNA and *Dictyosphaeria cavernosa* 1.79 pg DNA. These sizes are of the same order of magnitude when compared with *L. digitata*. However, both taxa show an additional repetitive fraction and hence a three-component Cot-curve. Most higher plants have a larger haploid nuclear genome size ranging from 0.2–127 pg DNA (Ohri & Khoshoo, 1986). Until more data are accumulated within and across many algal groups, it is difficult to draw conclusions about the significance of any particular size and complexity.

Phylogenetic hypotheses and divergence time

Recent papers on the reliability of the molecular clock and the constancy of evolutionary rates of DNA sequences make it clear that the interpretation of phylogenetic patterns and actual divergence times must be handled judiciously, as working hypotheses and not as fixed conclusions (Thorpe, 1982; Ayala, 1986; Britten, 1986; Li & Tanimura, 1987; Nei, 1987). Still we are confident that our phylogenetic interpretations reflect nature for the following reasons: (1) Only single-copy DNA sequences were compared. This makes it possible to measure the relative number of nucleotide substitutions which have occurred since divergence from the most recent common ancestral sequence. (2) Despite the fact that the final sequence homology is summarized as a distance measure, it is based on an enormous sample size, not just a single gene or protein. It is, therefore, a phylogenetic measurement rather than a phenetic one. (3) All comparisons were made within a single genus except for the intentionally chosen

outgroup, so that mutually exclusive differences in rates of DNA sequence evolution are highly unlikely.

A radiational burst of the five *Laminaria* species from their most recent common ancestor, i.e. all five species evolving at or about the same time (Fig. 3 B), is consistent with the statistically identical $\Delta T_{m(e)}$ and provides the most conservative phylogenetic hypothesis. This result is somewhat disappointing in the context of taxonomic classification, but similar bursts have been observed in other groups of organisms such as birds (Sibley & Ahlquist, 1983, 1986) and some ulvophycean green algae (Cladophorales, cf. Olsen-Stojkovich et al., 1986). Since only *L. digitata* was used as a tracer, the results provide information about the amount of sequence homology between *L. digitata* and each of the other species, but not among the other species in relation to *L. digitata*. Although good reciprocity is generally obtained with this type of data, there is still the possibility that these species are not equidistant from each other.

The assignment of divergence times to phylogenetic hypotheses is more controversial. Rates of DNA sequence change are not necessarily identical among different groups and, virtually all of the rate measurements available are for animal systems only. It is, therefore, difficult to know which rates should be applied. Are DNA sequences in particular plant groups evolving slowly and in others rapidly? Finally, small differences in hybridization protocols as applied in different laboratories make rate comparisons in different data sets difficult. These three factors do not invalidate divergence estimates, rather they serve as a reminder that these numbers are the best estimates currently available and subject to change as new and better evidence comes along.

The calculated divergence times for these *Laminaria* species at 15–19 Ma ago are consistent with other current scientific data that suggest a relatively short evolutionary history of the Phaeophyceae and its groups. The divergence time of approximately 67 Ma between *L. digitata* and *Chorda filum* does not exceed the divergence time of ca 200 Ma estimated for the brown algae as a whole (Lim et al., 1986), and an estimated Miocenic (7–26 Ma ago) origin for the species of *Laminaria* agrees with the limited fossil evidence (Parker & Dawson, 1965).

In contrast, species and genera in some green algal lineages, notably the order Cladophorales (class Ulvophyceae) appear to have a much older evolutionary history (Olsen-Stojkovich et al., 1986, immunological distance data and, Olsen et al., 1987, scDNA-DNA hybridizations; Bot, in progress, scDNA-DNA hybridizations). In both *Dictyosphaeria* and *Cladophora*, for example, interspecific distances are of the same order of magnitude as the intergeneric distance between *Laminaria* and *Chorda* reported here. If we assume that evolutionary genomic change takes place at a comparable pace in these unrelated algal classes (Phaeophyceae, Ulvophyceae), then the evolutionary morphological change in the Laminariales is apparently much faster than in the Cladophorales (however, cf. Britten, 1986).

Speciation, fossils and biogeographic scenarios

Based on the data from 5S rRNA (Lim et al., 1986), the Phaeophyceae are at least 200 Ma old, having diversified from a common ancestor to the diatoms. The Laminariales are believed to be an evolutionarily advanced order of recent origin based on such criteria as oogamy, level of tissue differentiation, certain ultrastructural features, and

their overall taxonomic uniqueness (see Kain, 1979 for overview). Unfortunately, the fossil record for the order is most uncertain. Extant phaeophytes cannot reliably be compared with fossil deposits older than Tertiary, 65 Ma ago (Clayton, 1984) and the oldest well-documented laminarialean fossil, *Julescraneia*, is from the "Upper Mohnian Monterey Formation" in California (site 1267, cf. Parker & Dawson, 1965), between 10 and 7 Ma ago.

Other evidence that suggests close genetic relatedness in *Laminaria*, specifically, are a number of interfertility studies between species (overview in Mathieson et al., 1981; Cosson & Olivari, 1982; Bolton et al., 1983).

The high diversity of Laminariales in general and of *Laminaria* in particular in the N Pacific, as compared to the N Atlantic and the southern hemisphere oceans, suggest that Laminariales originated and radiated in the N Pacific Ocean. Only comparatively recently, after the late Cainozoic opening of the Beringian Seaway, could a few *Laminaria* species possibly invade the N Atlantic Ocean through the Arctic (Lüning, 1985).

The above hypothesis explaining the origin of the N Atlantic *Laminaria* species is consistent with a general scenario implying the immigration, after the opening of the Beringian Seaway, of a much greater number of N Pacific biota into the N Atlantic, than of N Atlantic biota into the N Pacific. Two possible reasons are mentioned: (1) the pool of species was much bigger in the Pacific; (2) the eastward circumpolar current in the Arctic Ocean would facilitate gradual dispersal through the relatively short way of the Canadian Arctic (Pielou, 1979). There is fossil evidence for the passage of many N Pacific mollusc species into the N Atlantic (McKenna, 1983), and this suggests that the numerous cool temperate to arctic algal species and red algal genera shared by the N Pacific and N Atlantic are predominantly of N. Pacific origin (van den Hoek, 1975, 1984; Joosten & van den Hoek, 1986).

According to McKenna (1983), the first inundation of the Bering Land Bridge took place in Mid-Pliocene, at about 3.5 Ma ago, when the climate was somewhat milder than it is now. He presents evidence in support of this idea and he rather emphatically rejects possible earlier (e.g. late Miocene, ca 10 Ma) inundations. Later, the Bering Land Bridge reemerged several times during the Pleistocene sea level drops. Free exchange of biota between the Arctic and N Atlantic Oceans was possible already since the Middle Miocene (ca 15 Ma) subsidence of the Greenland-Scotland Ridge below sea level (Kennett, 1982; Thiede, 1979).

As *Laminaria* could invade the N Atlantic much later (at 3.5 Ma ago) than the time of radiation in the genus as here estimated for 5 N Atlantic species (15–19 Ma), this radiation apparently took place in the N. Pacific and resulted in the origin of the N Pacific predecessors of these 5 N Atlantic species.

The implication is that all five species can be expected to have close relatives in the N Pacific from which they have diverged in less than 3.5 Ma (unless these relatives have become extinct in the meantime). Actually it is possible to indicate five groups of N Pacific species which, on morphological grounds, can be considered as being related to the five N Atlantic *Laminaria* species here investigated.

L. rodriguezii, with its single blades and branched rhizomes, is morphologically close to the NE Pacific species *L. sinclairii* and *L. longipes* (these three species are united by Petrov in the subgenus *Rhizomaria*) (Petrov, 1974). The restricted distribution of *L.*

rodriguezii in deep (~60–100 m), cool (temp. range 12–19 °C) water of the Mediterranean (van den Hoek, 1982) is possibly the result of this species' extinction along the N Atlantic shores in the course of the Pliocene/Pleistocene glaciations. *L. rodriguezii* would then be a Pliocene relic. An alternative hypothesis is that *L. rodriguezii* evolved from a locally isolated population of *L. saccharina* from which it differs by having branched rhizomes (van den Hoek, 1982). These two alternative hypotheses could in principle be tested by DNA-DNA hybridization between *L. rodriguezii, L. sinclairii, L. longipes* and *L. saccharina*. On the basis of morphology, and against the background of the general scenario, the first hypothesis is the more likely one.

L. hyperborea and *L. ochroleuca* are very distinct species with digitate blades in the N Atlantic Ocean where they are restricted to the NE Atlantic shores (cf. van den Hoek, 1982); *L. ochroleuca* has a somewhat more southerly distribution than *L. hyperborea*. Both species have rigid stipes able to bear the blade's weight (the stipe is rugose in *L. hyperborea*, smooth in *L. ochroleuca*). They share this trait with the NE Pacific digitate species *L. dentigera* and *L. setchellii* (Kain, 1979; Druehl, 1968; Druehl, 1979). The S Atlantic species *L. pallida* probably also belongs in this group (Kain, 1979). Van den Hoek (1982) suggests that *L. pallida* crossed the equator during a glacial temperature lowering. The restriction of *L. hyperborea* to the NE Atlantic shores cannot be explained by its known temperature responses which suggest that it is capable of surviving and growing under Arctic and NW Atlantic conditions (Breeman, 1988). Possibly, this species became extinct along Arctic and NW Atlantic shores during the glaciations and did not succeed in reinvading these shores after the last glaciation. However, most species of *Laminaria* are lacking in the Arctic and this suggests that there are either unknown adverse temperature effects (e.g. too low temperatures when the days are short enough for sorus formation [Lüning, in preparation]) or other adverse effects (e.g. too long ice-covering).

L. digitata, which has a NE and NW Atlantic distribution, is characterized by a digitate blade and a flexible stipe which is not capable of bearing the blade's weight. These characters are shared by the NE Pacific species *L. "groenlandica"* (sensu Druehl, 1968).

L. saccharina, with a N Pacific–Arctic–N Atlantic distribution, belongs to a complex of closely related (sub)species in the N Atlantic as well as in the N Pacific (Kain, 1979; Lüning, 1985). The flexible stipes and the undivided fronds are characteristic. Successful crosses (resulting in F2-sporophytes) were obtained between *L. saccharina* from the NE Atlantic, *L. saccharina* from the NE Pacific, *L. longicruris* from the NW Atlantic and *L. ochotensis* from Japan, indicating that these species are closely related (Lüning et al., 1978; Bolton et al., 1983). Incipient genetic barriers, however, were observed between *L. ochotensis* and *L. longicruris*.

In the present study, no species were included with discoid holdfasts: the digitate *L. yezoensis* (NW Pacific), the single bladed *L. ephemera* (NE Pacific) and the single bladed *L. solidungula* (NW Atlantic–Arctic) (Kain, 1979). These three species are considered closely related by Petrov (1974; subgenus *Solearia*). This implies that the structure of the blade (digitate or simple) is not considered fundamental to the subdivision of the genus (into the sections Digitatae and Simplices). This idea is supported by results of our DNA-DNA hybridization experiments. *L. digitata* is just as closely related to *L. saccharina* (with single blades) as to other species with digitate blades *(L. hyperborea, L. ochroleuca)*.

An alternative to the above theory implying a N Pacific origin of the N Atlantic *Laminaria*-species, would be a Miocene (15–19 Ma) radiation of a primaeval N Atlantic–Arctic *Laminaria*-species into the five N Atlantic *Laminaria*-species. It is difficult, however, to explain how this primaeval species could reach the N Atlantic from the Pacific. Another possible passage would be the then open tropical Panamic Seaway. This is, however, extremely unlikely as three of the five species *(L. saccharina, L. digitata, L. hyperborea)* have upper lethal temperatures between 21–23 °C, and it is likely that *L. rodriguezii* and *L. ochroleuca* have at most somewhat higher lethal temperatures (van den Hoek, 1982; Breeman, 1988). Lethal temperatures are apparently genetically highly fixed traits (Breeman, 1988). It is therefore unlikely that an ancestral *Laminaria* species could pass the tropical Panamic Seaway (with temperatures of 25–30 °C).

The Miocene radiation (15–20 Ma) of the N Pacific *Laminaria* species (and probably also of other N Pacific Laminariales; cf. Parker & Dawson, 1965) coincided with the Middle Miocene steepening of the temperature gradient between high and low latitudes (Kennett, 1982). Possibly, this cooling of the higher latitudes is the driving force of speciation in the Laminariales, a then rather recent lineage (~65 Ma ago) with innate capacities to occupy the new cold water niches. The N Pacific with, on both sides, archipelagos with continuously changing configurations due to tectonic events and sea-level fluctuations, could provide the scenario for alternating genetic isolation of subspecific populations (demes) and mixing of these populations after speciation.

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