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Molecular divergence between North Atlantic and Indo-West Pacific *Cladophora albida* (Cladophorales: Chlorophyta) isolates as indicated by DNA-DNA hybridization

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Abstract

Genomic relationships between North Atlantic, Australian and Japanese isolates of the benthic seaweed *Cladophora albida* (Huds.) Kütz. were examined in 1987 by means of DNA–DNA hybridization. The data indicate that *C. albida* can be divided into a North Atlantic and an Indo-West Pacific group with an intergroup hybridization response of 25 to 30% and 5.5° to 6.0 °C for hybridization percentage and ΔT_{me} the melting temperature reduction of hybridized sequences, respectively. This level of genome divergence is considerably higher than that observed in most other eukaryotes. The separation between the two *C. albida* groups presumably dates back to the closure of the Asian part of the Tethys Ocean, about 12 million years ago. The data also indicate that transatlantic *C. albida* populations have a greater genetic inter-relatedness than have Japanese and Australian populations. In *C. albida* there is no clear correlation between molecular evolution and the evolution of morphological traits. *C. albida* and *C. rupestris* (L.) Kütz have hardly any DNA sequences in common.

Introduction

In recent years, significant progress has been made toward understanding the ecological factors that keep seaweeds within their geographic boundaries (van den Hoek 1982a, b, Lüning 1985, Breeman 1988). However, present-day ecological factors alone are insufficient to explain global seaweed distribution patterns which are also the result of evolutionary processes and configurations of the oceans in the geologic past. For example, knowledge about its environmental requirements is of little help to decide whether the disjunct distribution of a seaweed species is caused by fragmentation of a formerly continuous distribution area or by dispersal.

Genetically isolated groups of a species appear to accumulate changes in their single copy DNA as a function of time (Britten 1986), which potentially provides a means of tracing the evolutionary history of the genomes. A direct method to determine overall DNA differences between large portions of genomes is DNA–DNA hybridization. This technique relies on the fact that the temperature at which double stranded DNA becomes single stranded is largely determined by the fidelity of base pairing. The decrease in thermostability in hybrid DNA is proportional to the degree of mismatch between the DNA strands.

We have used DNA–DNA hybridization here to determine genetic relationships between *Cladophora albida* isolates collected worldwide. This benthic seaweed, with multinucleate cells, is distributed in the warm temperate to temperate regions of the northern and southern hemispheres (van den Hoek 1963, 1982c, Sakai 1964, van den Hoek and Womersley 1984). In the strictly tropical regions the species seems to occur rarely (van den Hoek 1982a), at least in the Atlantic Ocean. In the Pacific Ocean the taxonomy and the distribution of tropical *Cladophora* species awaits critical examination. The distribution of this seaweed offers a good opportunity for assessing both the existence of tropical dispersal barriers and the effects of historical geological events on the present day distribution. Measurements were thus designed to determine DNA sequence divergence between *C. albida* isolates from Australia and Japan, from the N. Atlantic and Indo-W. Pacific Ocean and between N. Atlantic isolates (W. Europe and NE. America). *C. rupestris* was included as an outgroup.

Materials and methods

Table 1 lists *Cladophora albida* (Huds.) Kütz. and *C. rupestris* (L.) Kütz. isolates used in this study and includes, if available, details on collection sites.

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Table 1. *Cladophora albida*. Collecting sites for DNA-DNA hybridization

Location	Isolate	Habitat	Time of collection
C. albida			
Roscoff (France)	A83-4	Littoral pool	May 1983
Roscoff (France)	A83-3	Littoral pool	May 1983
Roscoff (France)	A83-6	Littoral pool	May 1983
Connecticut (USA)	A83-c	Lagoon	Mar 1983
Parker Point, Rottnest (West Australia)	A85-12	Littoral pool	Dec 1984
Claremont, Swan River estuary (West Australia)	A85-23	Littoral pool	Dec 1984
West Hokkaido (Japan)	A85-101	Unknown	Apr 1985
West Hokkaido (Japan)	A85-101	Unknown	Apr 1985
C. rupestris			
Roscoff (France)	R83-5	Sublittoral	May 1983

Culturing conditions

Axenic *Cladophora albida* cultures from cloned apical cells were grown in enriched heat-sterilized seawater medium (Provasoli 1966) at 20 °C (*C. rupestris* at 16 °C) and a photon fluence rate of 10 μmol m^{-2} s^{-1}. Cultures were exposed to a daily cycle of 16 h light and 8 h dark. After about 4 wk plants were collected and washed with freshwater. Residual water was removed by squeezing. Samples were stained with 4',6-diamidino-2-phenylindole (DAPI) and examined for bacterial contamination by means of epifluorescence microscopy. When not immediately used, plants were stored at −20 °C.

DNA–DNA hybridization

Details of DNA isolation and purification: DNA shearing, fragment length determination, preparation of radioactive tracers, and analysis of reassociation kinetics have been described previously (Bot et al. 1989). A brief overview of the procedure is given below.

Algal tissue was pulverized in liquid nitrogen and lysed for 2 h at 50 °C in 3 vol. (w/v) of 0.1 *M* Tris-HCl (pH 8.0), 0.05 *M* Ethylenediaminetetraacetate (EDTA), 1 *M* $NaClO_4$ and 1% sodium dodecylsulphate (SDS). DNA was purified by phenol/chloroform extractions and two cycles of CsCl/ethidium bromide centrifugation. DNA fragments with an average length of 450 basepairs (bp) were obtained by sonification. Tracer DNAs were labeled with [^{3}H]dCTP by gap translation (Galau et al. 1976).

To analyze DNA reassociation kinetics, thermally denatured (5 min 100 °C) driver DNA fragments were reassociated for various times with a much smaller quantity of homologous tracer DNA. After incubation, the ratio between single and double stranded DNA was determined by means of hydroxyapatite (HAP) fractionation. The reassociation data were analyzed by a non-linear least-squares computer program, designed to fit theoretical second order components to the observed kinetics. The kinetics are expressed as the fraction of double stranded DNA versus Cot (Cot is the product of DNA concentration in moles of nucleotides per litre and time after initiation of reaction in seconds). All Cot values are given as equivalent Cot (eq. Cot) in 0.18 *M* Na^+ at 60 °C according to Britten et al. (1974).

To isolate single copy sequences, DNA fragments with an average length of 450 bp were thermally denatured and subsequently reassociated to Cot 300. Single stranded fragments were separated by HAP fractionation and reassociated to Cot 100. Sequences remaining single stranded after the second incubation (about 15% of the input mass) were concentrated, reassociated to Cot 25 000 and labeled.

DNA hybridizations were performed by mixing labeled single copy sequences with a 2 000-fold excess of unlabeled DNA fragments. The mixtures were denatured and incubated to Cot 20 000 under two criteria: a standard criterion, at 25 °C below T_m (T_m-25 °C), which is the optimum temperature for reassociation, and a permissive criterion at T_m-35 °C. T_m is the midpoint temperature of the melting transition of native *Cladophora albida* DNA and was corrected for Na^+ concentration (Mandel and Marmur 1968) and fragment length (Chamberlin et al. 1978). The extent of self-reassociating tracer was determined by mixing tracer DNA with calf thymus DNA instead of *C. albida* DNA. After incubation the samples were loaded on HAP columns. Single stranded DNA was eluted with 0.12 *M* PB (an equimolar buffer of NaH_2PO_4 and Na_2HPO_4) at 60° and 50 °C for standard and permissive criteria, respectively. Double stranded DNA fragments were denatured by raising the temperature of the columns in steps of 5 °C up to 95 °C. The normalized percentage of hybridization (NPH) was determined as the ratio between single stranded and double stranded tracer DNA at the initial temperature, corrected for self-reassociating and unreactable tracer DNA. T_{me} was determined as the temperature at which 50% of the hybridized tracer DNA was eluted. ΔT_{me} was defined as the difference between the T_{me} for homologous (the same isolate) and heterologous (different isolates) hybridizations. Under the standard criterion a ΔT_{me} of 1 °C represents about 1% base sequence mismatch (Bonner et al. 1973).

Results

Reassociation kinetics of total cellular DNA

Genome organization and complexity of two *Cladophora albida* isolates (A85-12 and A83-4) were examined by studying the reassociation kinetics of their DNA (Table 2 and Fig. 1). Computer analysis revealed three kinetic classes: a rapidly reassociating fraction made up of highly repetitive sequences, an intermediate fraction (middle repetitive sequences) and a slow reassociating fraction, which is usually identified with single copy sequences. Under our experimental conditions we were unable to follow the initial 3% reassociation. This was probably due to the formation of intramolecular secondary structures in single stranded molecules.

Table 2. Summary of *Cladophora albida* and *Escherichia coli* DNA reassociation kinetics experiments. Reassociation percentages were normalized to 100% after subtracting unreactable tracer DNA. To allow direct comparison, rate constants were normalized to a driver and tracer DNA fragment length of 250 bp (Chamberlin et al. 1978). Isolated single copy DNA was reassociated with total cellular driver DNA of the same isolate

Isolate and kinetic component	Fraction	Rate constant (M^{-1} s^{-1})	Basepairs
A85-12			
Highly repetitive	0.15	4.45	3.0×10^4
Middle repetitive	0.49	0.12	3.4×10^6
Single copy	0.36	0.00115	2.6×10^8
Isolated single copy DNA	1.0	0.00147	
A83-4			
Highly repetitive	0.13	9.85	1.0×10^4
Middle repetitive	0.53	0.10	4.5×10^6
Single copy	0.34	0.00131	2.2×10^8
Isolated single copy DNA	1.0	0.00124	
A85-101			
Isolated single copy DNA	1.0	0.00114	
E. coli	1.0	0.20	4.2×10^6

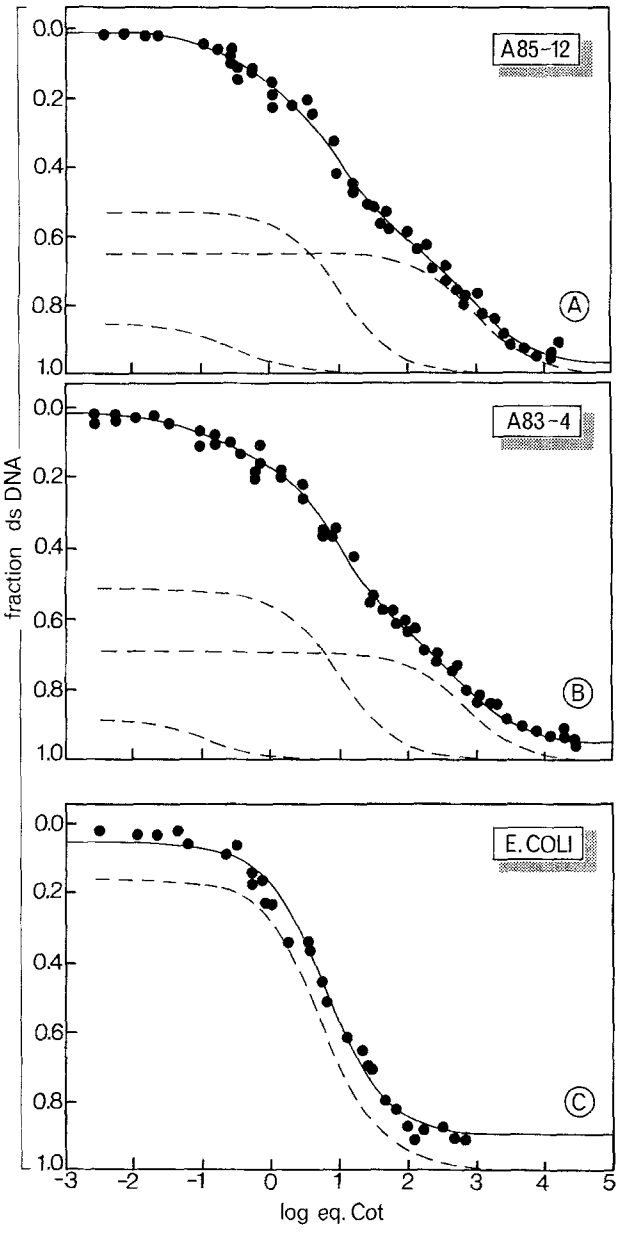

Fig. 1. A and B *Cladophora albida*. Reassociation kinetics of total cellular DNA. A: Isolate A85-12. B: Isolate A83-4. Reassociations were carried out at DNA concentrations of 25 to 4000 µg DNA ml^{-1}. Driver/tracer DNA ratios ranged between 4 000 and 25. Cot value are given as equivalent Cot (eq. Cot) in 0.18 *M* Na^+ at 60 °C (Britten et al. 1974). Continuous lines: best non-linear regression analysis of data; dashed lines: computer generated kinetics components. Root mean square (RMS) for A85-12 and A83-4 were 0.011 and 0.016, respectively. C: Reassociation kinetics of *Escherichia coli* DNA. RMS: 0.023

Genome size is inversely proportional to the reassociation rate of the single copy component (Britten and Kohne 1968). To calculate the genome size of the two *Cladophora albida* isolates we used *Escherichia coli*, which has a genome size of about 4.2×10^6 bp, as a standard. *E. coli* DNA reassociated under identical conditions with a rate constant of 0.2 M^{-1} s^{-1} (Table 2, Fig. 1). This implies a haploid genome size of 7.3×10^8 bp ($0.2/0.00115 \times 4.2 \times 10^6$) or 0.80 pg for A85-12 and 6.4×10^8 bp (0.70 pg) for isolate A83-4.

These reassociation experiments provided the conditions for isolating single copy sequences. We designated DNA that was not reassociated at Cot 300 as single copy DNA and separated this fraction from total cellular DNA of A85-12, A83-4 and A85-101 as described under Material and methods. To verify the kinetic purity of the isolated sequences, their rate constant was independently measured by reassociation in the presence of unlabeled total DNA of the same isolate (Table 2, Fig. 2). The best fit through the data could adequately be described with one kinetical component which had a rate constant comparable to that expected for single copy DNA. This indicated that the isolated tracers were free from detectable repetitive sequences. The data also showed that there is little variation in the complexity of the single copy component in different *Cladophora albida* isolates.

DNA sequence homology

Table 3 shows NPH and ΔT_{me} from DNA hybridizations with single copy sequences of *Cladophora albida* isolates A83-4 (France), A85-12 (Australia) and A85-101 (Japan). NPH and ΔT_{me} can both be considered as indicators of divergent evolution. NPH is a measure of the degree to which DNA being compared is complementary, and ΔT_{me} gives an insight into the extent to which sequences have diverged from each other. On the basis of these parameters, *C. albida* can be divided into two distinct groups with an intergroup NPH of 25 to 30% and ΔT_{me} values between 5.5° and 6.0 °C. One group contained isolates from the N. Atlantic and the other, from the W. Pacific (Japan) and the E.

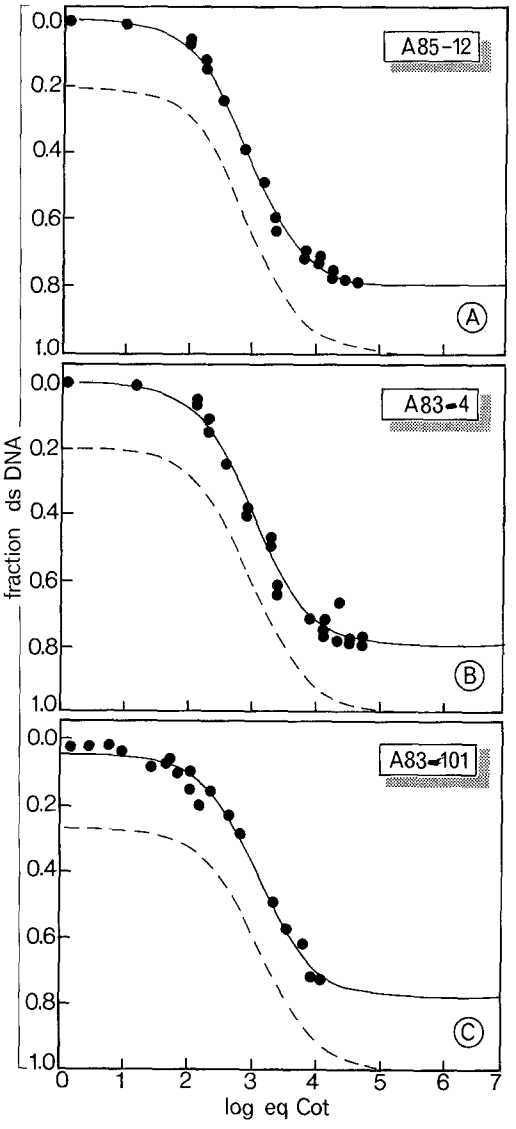

Fig. 2. *Cladophora albida.* A: A85-12; B: A83-4; C: A85-101. Reassociation kinetics of isolated single copy sequences in the presence of total cellular DNA. Continuous line: best non-linear regression analysis of data. Dashed line: fit for one kinetical component. RMS for A85-12, A83-4 and A85-101 were 0.013, 0.018 and 0.021, respectively

Table 3. Genetic relatedness among geographic isolates of *Cladophora albida* based on DNA-DNA hybridization. Tritium labeled tracer DNAs were mixed with a 2 000 fold excess of 450 bp driver DNA, denatured (5 min 100 °C) and allowed to reassociate to Cot 20 000 mol s^{-1}. Incubation temperature was 25° or 35 °C below the T_m of native DNA. Fragment sizes were as follows: driver DNAs 450 bp; tracer DNAs: 260, 250 and 280 bp for A83-4, A85-12 and A85-101, respectively. Homologous hybridization percentages were between 72 and 80%. Hybridization percentages were normalized to 100% hybridization (NPH) after correcting for unreactable and self-reassociating tracer DNA. *N*: number of hybridizations

Isolate hybridization	NPH	ΔT_{me} °C	*N*
A83-4 (single copy DNA) ($T_{me}-25$ °C)			
× A83-4	100	0	23
× A83-6	98.4±2.6	−0.1±0.3	3
× A83-3	101.2±2.1	+0.3±0.2	6
× A83-c	92.4±1.9	−0.8±0.2	14
× A85-12	24.6±3.4	−5.9±0.4	6
× A85-23	26.2±2.8	−5.7±0.5	6
× A85-101	28.2±3.1	−5.8±0.4	6
× A85-102	28.1±2.3	−5.7±0.4	6
× R83-5	9.4±3.3	−10.2±1.1	6
A85-12 (single copy DNA) ($T_{me}-25$ °C)			
× A85-12	100	0	15
× A85-23	80.2±3.2	−2.2±0.4	6
× A85-101	79.1±1.8	−1.8±0.3	6
× A85-102	80.3±2.9	−1.9±0.3	6
× A83-4	27.5±3.4	−5.6±0.5	6
× A83-c	29.1±3.1	−5.3±0.3	6
× R83-5	7.3±2.9	−12.1±0.9	6
A85-101 (single copy DNA) ($T_{me}-25$ °C)			
× A85-101	100	0	6
× A85-102	98.6±4.1	−0.2±0.4	3
× A85-12	85.8±3.1	−1.7±0.3	6
× A85-23	79.0±3.4	−4.6±0.3	3
× R83-5	6.1±1.1	−12.8±2.3	3
A85-12 (single copy DNA) ($T_{me}-35$ °C)			
× A85-12	100		6
× A85-23	96.8±3.8		3
× A85-101	98.4±4.2		3
× A85-102	95.3±2.7		3
× A83-4	28.7±1.9		6
× R83-5	8.9±2.4		3

Indian Ocean (W. Australia). No significant divergence could be observed between the French isolates, but the reduction of both the NPH (92%) and T_{me} (0.8 °C) in hybridizations between isolate A83-4 and A83-c from the W. Atlantic coast, indicated that some differentiation has occurred between the opposite sides of the Atlantic Ocean.

Isolates of the Indo-W Pacific group showed more variation. The NPH of reciprocal hybridizations between Australian and Japanese isolates ranged down to about 80% and the ΔT_{me} varied between 1.7° and 2.2 °C. The Australian isolate A85-23 from an estuary in W. Australia, represented the single exception to the trend for higher divergence with increasing geographical distance. Divergence between A85-23 and A85-12 (from Rottnest, an island in W. Australia), was of the same order of magnitude as between A85-12 and the two Japanese isolates. When A85-23 was hybridized with A85-101 single copy tracer, we observed a NPH of about 80%. However, the significant reduction of the thermostability (ΔT_{me} 4.6 °C) suggests that A85-23 has diverged more from the Japanese isolate than A85-12.

Cladophora albida and *C. rupestris* share a small number of highly diverged sequences which demonstrate that these species are only very distantly related.

In Fig. 3 ΔT_{me} values of the hybridizations were plotted against their corresponding NPH. The overall tendency is that a reduction of the NPH corresponds to increasing mis-

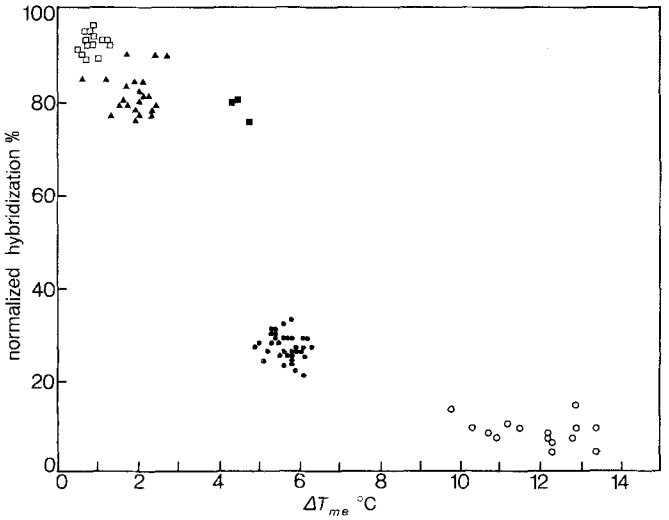

Fig. 3. Extent of NPH versus ΔT_{me} □: A83-c × A83-4; ▲: A85-101 × A85-12; A85-102 × A85-12; A85-23 × A85-12; A85-12 × A85-101; ■: A85-23 × A85-101; ●: A85-12 × A83-4; A85-101 × A83-4; A85-102 × A83-4; A85-23 × A83-4; A83-c × A85-12; A83-4 × A85-12; ○: R83-5 × A83-4, A85-12 and A85-101. For abbreviations of *Cladophora* isolates see Table 1

match in the hybridized sequences. However, the data for isolate A85-23 form an exception to this trend.

The degree of apparent mismatch in hybridized DNA molecules is partly determined by the criteria of stringency under which the DNA is reassociated. Under the standard hybridization condition (T_{me}-25 °C), a base pairing of at least 75 to 80% is required for the formation of stable DNA hybrids. Therefore, DNA fragments comprised of highly diverged sequences will remain single stranded. At a reduced criterion (T_{me}-35 °C) greater mismatch is tolerated. Hybridizations conducted under this more permissive criterion, indicate that the mechanisms of divergent DNA evolution are not uniformly distributed between the genomes of the *Cladophora albida* isolates. The significant increase of the NPH in hybridizations between Indo-Pacific isolates (Table 3) demonstrates that their genomes contain a subset of sequences which have less stability, with respect to nucleotide substitutions than those that account for the observed ΔT_{me}. This indicates that within this group, genomic variation is primarily based on alterations in the primary nucleotide sequence. No such marked increase could be detected in the intergroup hybridizations. This suggests that the conformity between the genomes of the Indo-Pacific and the N. Atlantic *C. albida* isolates is the product of distinct sets of, probably conserved, single copy sequences.

Discussion

An interesting aspect of the DNA comparisons is the incongruity between genetic identity and the current taxonomy of *Cladophora albida*. The NPH reported for hybridizations between N. Atlantic and Indo-W. Pacific isolates is comparable to values observed between orders of birds (Sibley and Ahlquist 1983), sub-families of rodents (Brownell 1983), and genera of angiosperms (Belford and Thompson 1981). Biochemical evidence has shown for quite some time that the rate and amount of morphologic change are not closely related to the rate and amount of genomic change (Wilson et al. 1977, Kimura 1981). However, in the case of *C. albida* the discrepancy is extreme. In the genus *Cladophora* the species are mainly distinguished on the basis of thallus architecture and cell dimensions (van den Hoek 1963, 1982c). The consistency of these morphological traits, despite the high degree of genomic change, suggests that they are highly conservative. The reported NPH pertains to sequences which, over evolutionary time, retained enough similarities to hybridize. This fraction may include genes that code for these conservative traits, but it is far from clear whether this kind of relation can be made. Eukaryotic genomes are complex and the pathways that lead from genes to morphological traits are long and largely unknown. Moreover, we cannot exclude the possibility that the two *C. albida* groups have independently developed similar morphological traits from their once common ancestral genome. The morphological "species" *C. albida* apparently comprises genetically widely divergent populations and it can perhaps be better regarded as a species complex.

The Japanese and W. Australian isolates of *Cladophora albida* exhibit a small but significant genetic divergence. This could possibly be related to this species amphiequatorial distribution in temperate to warm-temperate climatic sectors and its rarity in the tropics (van den Hoek 1982c). Valentine (1984) postulates that this type of distribution was induced by a rise of temperature in the tropics, concomitant with cooling in the middle and high latitudes, from the Miocene onwards. However, this theory is not consistent with the results of culture experiments, which showed that the temperature requirements and tolerances of *C. albida* do not exclude its growth and survival in tropical waters (Cambridge et al. 1984). Moreover, both the DNA hybridizations as well as preliminary results from allozyme electrophoresis, yield no sign of a major genetic break in the tropical sector of the Indo-W. Pacific. The divergence between Australian and Japanese *C. albida* isolates probably reflects the clinal lag in the transfer of genetic change between members of the same population at opposite edges of their distribution area. The apparent absence of *C. albida* in the tropical belt of the Indo-W. Pacific may only indicate lack of collecting.

The magnitude of divergence between the Atlantic and Indo-W. Pacific isolates of *Cladophora albida* supports the idea that the two groups evolved as a consequence of allopatric subdivision of a formerly continuous distribution area. Two important historical connections with the Indo-W. Pacific were interrupted during the geological history of the N. Atlantic Ocean. One was a marine contact with the central Indian Ocean through the Mediterranean, W. Asia and N. India. This seaway was part of the Tethys Ocean, and finally ceased to exist about 12 million years ago (Rögl and Steininger 1984). The other dates from a more recent period and concerns the final closure of the Isthmus of Panama,

about 3.5 million years ago (Kennett 1982). Based on the time lapse since these two separation events, the average rate of base pair change in the sequences shared by both *C. albida* groups, can be estimated. This yields 0.22 to 0.25% and 0.76 to 0.84% per million years for the ancient and the more recent subdivision, respectively. However, some caution is required in interpreting these estimates. First, they do not cover the effects of genetic isolation predating the disruption of the above marine connections. Second, the estimates probably represent the rates of change in conserved sets of single copy sequences. Finally, these estimates are based on the assumption that the melting temperature reduction of hybridized DNA sequences is solely a function of nucleotide substitutions.

There is no information on the rates of divergent DNA evolution in the genus *Cladophora*. However, if it is assumed that the sequences shared by the Atlantic and the Indo-W. Pacific *Cladophora albida* groups diverge at rates comparable to those for animals, tentative estimations can be made about the time of their divergence. In animals, rates of base pair change per million years vary between 0.13% in higher primates and some birds lineages, and 0.66% for rodents, sea urchins and Drosophila (Britten 1986). Only the rates estimated for a subdivision of *C. albida* by the final closure of the Tethys Ocean (0.22 to 0.25%) lie within this range. This is not unreasonable in the light of existing theories about the separation of Indo-W. Pacific and Atlantic Rhodophytan floras (van den Hoek 1984) and coral reef faunas (Rosen 1984). We want to emphasize that the procedure used to transform DNA hybridization data in divergence time is quite provisional, and further investigations are required to test whether the genetic relationships found in this study can be reproduced in seaweeds with comparable distribution patterns. However, apart from the absolute time estimates, the geographical pattern of the major genetic break within *C. albida* remains clear and deserves consideration.

The divergence between *Cladophora albida* isolates from both sides of the Atlantic, may appear to be a case of DNA polymorphism within the same region (discussed below), but could also be the result of interactions between dispersal capacity and barriers to gene flow. In a previous paper (Bot et al. 1989) we reported the lack of differentiation in the DNA of *C. sericea* isolates from both sides of the N. Atlantic. It was hypothesized that the geographical and physical conditions in the northernmost part of this ocean may facilitate transatlantic dispersal. *C. albida* is in contrast to the cold tolerant *C. sericea*, excluded from the far northern waters of the Atlantic Ocean and from Iceland by the cool summer temperatures (Cambridge et al. 1984). This suggests that the divergence reflects blocked, or more likely, attenuated gene flow with the increasing geographical distance which had to be bridged after the increased severity of climatic cooling in the course of the past million years (Shackleton and Opdyke 1977).

Unexpected are the results obtained with the two Australian *Cladophora albida* isolates (A85-12 and A85-23). These isolates were collected ca 50 km apart and, the observed divergence indicates that geographical distance is not a prerequisite for genetic dissimilarity. A85-23 may reflect the fluctuating conditions of its estuarine habitat. This isolate appears to be less sensitive to increased salinity than the other *C. albida* isolates (unpublished results) and so would be better adapted to its estuarine environment (Spencer 1956). It is debatable whether this adjustment reflects the impact of environmental selection pressure, but if so, the biological implication would be that mutation rates are not constant for a species. This raises an important question concerning the utility of DNA hybridizations. When evolutionary distance, defined as the amount of accumulated molecular change, shows no relation to the elapsed evolutionary time, the technique loses its claim as a direct indicator of divergence time.

In conclusion, the genomic relationships indicated by the DNA-DNA hybridizations in this study, exemplify the role that historical biogeographic factors can play in the distribution of a seaweed species. At the same time it is important to recognize that at present, we suffer from an almost complete lack of understanding about the mechanisms that generate genomic changes in a seaweed genus like *Cladophora*. Consequently, assumptions based on the relationship between DNA distance and time of divergence remain conjectural without the support of geological evidence that a present distribution may be the result of a vicariant subdivision.

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