PRIMARY RESEARCH PAPER

Phylogeography of the Northern Atlantic species *Chondrus crispus* (Gigartinales, Rhodophyta) inferred from nuclear rDNA internal transcribed spacer sequences

Zimin Hu · Xiaoqi Zeng · Alan T. Critchley · Steve L. Morrell · Delin Duan

Abstract Eighteen isolates of the red algae *Chondrus crispus* were collected from Northern Atlantic sites, together with *C. ocellatus*, *C. yendoi* and *C. pinnulatus* from the North Pacific. The nuclear rDNA internal transcribed spacer (ITS) was sequenced and compared, spanning both the ITS regions and the 5.8S rRNA gene. Percentage of nucleotide variation for *C. crispus* ranged from 0.3% to 4.0%. Phylogenetic analyses were performed using maximum parsimony (MP), neighbor-joining (NJ) and minimum evolution methods. They showed that two main clades existed within the *C. crispus* samples examined and that suggested *C. crispus* had a single Atlantic origin. The clustering however did not follow the geographic origin. We hypothesized that the current distribution of *C. crispus* populations might be a result of three main factors: temperature boundaries, paleoclimate and paleoceanography. ITS data exhibited abundant molecular information not only for phylogeographical investigation but also for systematics studies.

Keywords *Chondrus crispus* · Internal transcribed spacer · Phylogeography · Red algae

Handling editor: C. Sturmbauer

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Introduction

Chondrus crispus Stackhouse belongs to the division Rhodophyta, class Florideophyceae, order Gigartinales and family Gigartinaceae (Dixon & Irvine, 1977), and was first separated from the wide-embracing genus *Fucus* by Stackhouse (Stackhouse, 1797), and it was the basis of a multi-million dollar phycocolloid industry (Levring et al., 1969; Michanek, 1983). Usually, *C. crispus* flourishes in the lower shore and is distributed mainly on Atlantic coasts of Europe, such as Germany (Franklin et al., 2001), France (Gall et al., 1990; Boyen et al., 1994; Donaldson et al., 2000), Spain (Gutierrez & Fernández, 1992), the Netherlands (Groen et al., 1997), and Northern America like Canada (Chopin et al., 1996, 1997; Donaldson et al., 1998) and USA

(Carrington et al., 2001; Zertuche-González et al., 2001), as well as in the Northern Russia, Western Baltic and Iceland (Dixon & Irvine, 1977). These surveys indicate that *C. crispus* is a cold temperate species restricted to the Northern Atlantic, and its distribution may be correlated with seasonal global isotherms (van den Hoek, 1984). This paper attempts to explain the contemporary distribution patterns of *C. crispus* based on other factors, such as climatological changes and vicariant events (Rögl & Steininger, 1984), and paleobiogeography (Bakker et al., 1995b), which greatly avail the recognition of global biogeographic disjunctions in marine organisms.

Nuclear ribosomal DNA (rDNA) sequences have been extensively used over the past decades to investigate phylogeographic questions among taxa at different hierarchical ranks in algae. At or below the species level, however, rDNA coding regions reach their limit or resolution (Hillis & Moritz, 1990; Bakker et al., 1992). For this reason, the greater sequence divergences of the internal transcribed spacer (ITS) regions of the rDNA are of interest (Bakker et al., 1992). Located between the three regions within the nuclear rDNA cistron (5′-18S-ITS1-5.8S-ITS2-28S-3′), the ITS regions are part of the initial transcript but spliced out later during maturation of pre-RNA (Gerbi, 1995; Mattai et al., 1993; van Nues et al., 1994). These regions are virtually free to evolve at a faster rate, as reflected in the number of substitutions and well-documented length variation (Yokota et al., 1989; Gardes et al., 1991). These characteristics allow to the usage of ITS for phylogenetic and biogeographic studies (Bakker et al., 1992, 1995a). In this study, we present a comparison of ITS of *C. crispus* sampled from eighteen sites along the North Atlantic Ocean, and three out-group isolates of *C. ocellatus*, *C. yendoi* and *C. pinnulatus* from the Northern Pacific Ocean. Our aims were to describe phylogenetic relationships among distant isolates of *C. crispus*, to compare the ITS sequence divergence of morphologically different isolates from various sites, and to analyze the dispersion mechanisms of *C. crispus* populations in the view of Tethyan events.

Materials and methods

Sample collection

Eighteen samples of *C. crispus* Stackhouse were collected from seventeen sites in the Atlantic Ocean, ranging from Lepreau, Canada, situated at 45° N, 66° W, to Helgoland, situated at 54° N, 8° E in Germany (Fig. 1). Three Pacific species (*C. ocellatus* Holmes, *C. yendoi* Yamada & Mikami and *C. pinnulatus* (Harvey) Okamura) were also included. Voucher specimens were prepared and lodged in KEMB (Key Laboratory of Experimental Marine Biology, Institute of Oceanology, Chinese Academy of Sciences). A mid portion of each specimen was selected for DNA extraction and further analysis. Where isolates collected from the same locality exhibited similar morphology, only one individual was used for sequencing. The two morphological variants of *C. crispus* (marked with #1 and #2) from Roscoff, France were analyzed separately. All steps including DNA extraction, PCR and sequencing were repeated three times. Voucher images of representative isolates of each entity are presented in Figs. 2–14.

DNA extraction

DNA was isolated according to the procedure described by Hu et al. (2004). In this procedure, about 0.12 g fresh tissue was frozen in liquid nitrogen, ground into fine powder and quickly transferred to a 1.5 ml tube containing 0.7 ml extraction buffer (0.1 M Tris–HCl, pH 8.0, 0.05 M ethylene diamine tetracetic acid (EDTA), 0.5 M NaCl, 1.6% sodium dodecyl sulphate [SDS], 0.2% polyvinyl-polypyroolidone [PVPP] and 2% β-mercaptoethanol) and incubated at 37°C for 1 h. An equal volume of ice-cold potassium acetate (5.0 M, pH 7.5) was added and mixed. The content were kept on ice for 20 min and was centrifuged at $10,600 \times g$ for 15 min. The aqueous phase was collected and extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and chloroform:isoamyl alcohol (24:1) for two times. The supernatant was collected and RNAse was added to a final concentration of 0.1 mg ml^{-1}. The upper aqueous was extracted

Fig. 1 A map indicates
the collection sites of
Chondrus crispus in this
study

with chloroform:isoamyl alcohol (24:1) and 2/3
volume of ice-cold isopropanol was added to
precipitate DNA. Finally, the DNA was washed
with 70% EtOH and stored at –20°C.

ITS region amplification and sequencing

The nuclear ITS rDNA was amplified using the
synthetic primers TW18: 5′GGGATCCGTTTC
CGTAGGTGAACCTGC3′ (Annealing to 3′ end
of 18S rDNA) and AB28: 5′GGGATCCA-
TATGCTTAAGTTCAGCGGGT3′ (Annealing
to the 5′ end of 28S rDNA) according to Goff &
Moon (1993). Amplification was performed on an
Eppendorf Masterthermocycler Personal (Ger-
many). The reaction mixture (20 μl) contained
10 mM Tris · HCl (pH 8.3), 50 mM KCl, 1.5 mM
$MgCl_2$, 2.0 mM dATP, dCTP, dGTP, dTTP
respectively, 50 ng nuclear DNA, 1.0 mM of each
primer and 1.0 U *Taq* DNA polymerase (Takara
Biotechnology Co., Ltd. Japan). The PCR

parameters were 5 min at 95°C; 1 min at 90°C
and 2 min at 50°C for 5 cycles; 72°C for 1 min;
followed by 30 cycles of 1 min at 90°C, 1 min at
60°C, 1 min at 72°C; finally left at 72°C for
10 min. The size, purity and yields of PCR prod-
ucts were assessed by electrophoresis through a
1.5% agarose gel for 1 h at 80 V and stained with
ethidium bromide. Reaction products were puri-
fied with QIAquick PCR purification kit (QIA-
GEN, Germany) and sequenced on an ABI Prism
377 automatic sequencer (Perkin ElmerApplied
Biosystems, China) utilizing standard methods.
Sequencing primers were the amplification primer
AB28 and revised TW18-1: 5′CGTTTCCGT
AGGTGAACC3′.

Sequence identification and alignment

The boundaries of ITS regions and the 18S, 5.8S,
and 28S rDNA were determined by comparing
aligned sequences of the ITS spacer region of
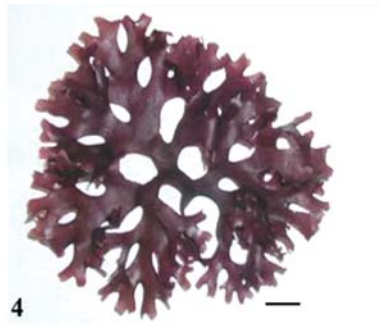
◀ **Figs. 2–14** (2) Prince Edward Island, Canada; (3) Helgoland North sea, Germany Eastney, Portsmouth, UK; (4) Eastney, Portsmouth, UK; (5) Hayling Island, UK; (6) New Brunswick; (7) Portaferry, Northern Ireland; (8) Portsall, France; (9) Roscoff, France (isolate #1); (10) Roscoff, France (isolate #2); (11) St. Vaast La Hougue, France; (12) Sune, Porto, Portugal; (13) Wembury beach, Devon, England; (14) Buarocos bay, Portugal. The scale bar shows 1 cm

Gracilariopsis and *Gracilaria* with GenBank accession no. U21339-U21345 (Goff et al., 1994), *Mazzaella* species with AY225230-AY225290 (Ross et al., 2003) and some other Gigartinales species with AF400051-AF400062, and AF398529-AF398547 (Hughey et al., 2001). Sequences were compared using the GCG software package (Genetics Computer Group, 1994) and aligned with Clustal X (Thompson et al., 1997). The final adjustments were conducted manually. In order to assess nucleotide substitution patterns, substitutions were counted in pair-wise comparison among all sequences using MEGA 3.0 (Kumar et al., 2004) regarding the gaps as missing data. Three other *Chondrus* species *C. ocellatus*, *C. yendoi*, *C. pinnulatus* from the Pacific Ocean, and *M. splendens* (Setchell *et* Gardner) Fredericq (Ross et al., 2003) with accession no. AY225290 were used as out-groups.

Phylogenetic analysis

For minimizing information loss, gaps and missing data were deleted only on a pair-wise basis and not down an entire column. Tamura–Nei model was used to estimate the genetic divergence with software MEGA 3.0. Pair-wise distance, Tajima's Neutrality Test (Tajima, 1989) and Interior branch test using minimum evolution (ME) (Rzhetsky & Nei, 1993) and neighbor-joining (NJ) methods were calculated with 1,000 replications, respectively. Phylogenies were inferred with maximum parsimony (MP) and NJ (Kimura-2 model) methods, gaps/missing data were treated as complete deletions. For maximum likelihood (ML) analysis, 1,000 replicate data sets generated by SEQBOOT from PHYLIP 3.57c (Felsenstein, 1995) were subject to distance analyses, the DNAPARS and DNAML program was used to analyze the 1,000 replicate data sets by parsimony

(jumble = 5). Bootstrapping was also performed with 1,000 re-samplings for MP analyses in PHYLIP. The three phylogenetic trees were drawn by Tree View program. FOLD programs from the UWGCG package and visual inspection were used to investigate the presence of secondary structures in the ITS sequences (Bakker et al., 1992).

Results

Sequence data

GenBank accession numbers for the ITS sequences of each isolate identified in the study, along with the collection information are listed in Table 1. Alignment of complete variable nucleotides of ITS1 and ITS2 including the 5.8S rRNA gene were obtained for all isolates (Fig. 15). ITS length ranged between 148 and 151 base pairs for ITS1, and between 399 and 404 base pairs for ITS2. Average nucleotide composition of all isolates was approximately 34.3% and 44.5% GC in ITS1 and ITS2. The total ITS length was about 710 base pairs. The final in-group alignment including *C. ocellatus*, *C. yendoi* and *C. nipponicus* contained 745 positions, of which 101 are variable, among them 39 are phylogenetically informative, and the maximum sequence divergence is 13.56% and 5.24%, respectively.

Transitions were about equal to transversions, and the percentage of nucleotide variation for *C. crispus* ranged from 0.3% to 4.0%. Table 2 shown that pair-wise distances varied from 0.0% to 3.6%, the minimum distance was among the four isolates from France (PS, DE, RS #1 and #2), and the maximum value was among the two isolates from Canada (NB) and France (SVLH), respectively.

Phylogenetic analysis

The three phylogenetic reconstruction methods (NJ, MP and ML) produced similar tree topologies. Maximum parsimony produced 82 equally most parsimonious trees of 517 steps. For ML analysis, the empirical base frequencies were

Table 1 Species, authorities, collection information, and GenBank accession numbers for isolates analysed in this study

Species and authorities	Collection information	Specimen voucher	Accession No.	Sample size
Chondrus crispus Stackhouse	Sune, Porto (SP), Portugal, June 2003, coll. S. Costa	EMBLC H307010	AY699059	2
Chondrus crispus Stackhouse	Buarcos Bay (BB), Portugal, 5th June 2004, coll. L. Pereira	EMBLC H307017	AY830846	2
Chondrus crispus Stackhouse	St. Vaast La Hougue (SVLH), France, 24th Nov. 2003, coll. A. T. Critchley	EMBLC H307015	AY699064	3
Chondrus crispus Stackhouse	Portaferry (PF), Northern Ireland, 20rd June 2004, coll. Matt Dring	EMBLC H307024	AY830845	2
Chondrus crispus Stackhouse	Helgoland North Sea (HNS), Germany, 8th July 2003, coll. L. A. Franklin	EMBLC H307008	AY699057	2
Chondrus crispus Stackhouse #1	Roscoff (RS), France, June 2004, coll. F. Weinberger	EMBLC H307018	AY830842	2
Chondrus crispus Stackhouse	Portsall (PS), France, 4th June 2004, coll. Solene Conan	EMBLC H307020	AY830844	3
Chondrus crispus Stackhouse	Dellee (DE), France, 5th June 2004, coll. Solene Conan	EMBLC H307021	AY830848	2
Chondrus crispus Stackhouse	Institute of Marine Science, Eastney, Portsmouth (EP), UK, 9rd Mar. 2004, coll. W. F. Farnham	EMBLC H307026	AY830849	2
Chondrus crispus Stackhouse	Green Point, Lepreau, New Brunswick (NB), Canada, 19th Nov. 2003, coll. G. W. Saunders	EMBLC H307011	AY699060	3
Chondrus crispus Stackhouse	Black Rock (BR), UK, 20th Jan. 2004, coll. A. T. Critchley	EMBLC H307016	AY699065	2
Chondrus crispus Stackhouse	Fort Bloque (FB), France, 3rd June 2004, coll. Solene Conan	EMBLC H307022	AY830847	2
Chondrus crispus Stackhouse	Prince Edward Island (PEI), Canada, Sep. 2003, commercial sample, coll. A. T. Critchley	EMBLC H307014	AY699063	3
Chondrus crispus Stackhouse	Ireland (I), 9th Feb. 2004, coll. A. T. Critchley	EMBLC H307009	AY699058	2
Chondrus crispus Stackhouse	Wembury Beach, Devon (WBD), England, 5th Dec. 2003, coll. M. Brown	EMBLC H307012	AY699061	3
Chondrus crispus Stackhouse	Langstone Bridge, Hayling Island (HI), UK, 22rd Apr. 2004, coll. W. F. Farnham	EMBLC H307023	AY830850	3
Chondrus crispus Stackhouse	Black Rock, Dale Pembrokeshire (DP), Wales, United Kingdom, 5th Dec. 2003, coll. S. L. Morrell	EMBLC H307013	AY699062	2
Chondrus crispus Stackhouse #2	Roscoff (RS), France, June 2004, coll. F. Weinberger	EMBLC H307019	AY830841	2
Chondrus pinnulatus (Harvey) Okamura	Muroran, Charatsunai, Japan, 22th May 2003, coll. T. Motomura	EMBLC H307004	AY699053	3
Chondrus yendoi Yamada & Mikami	Muroran, Charatsunai, Japan, 22th May 2003, coll. T. Motomura	EMBLC H307003	AY699052	2
Chondrus ocellatus Holmes	Ba Da Guan, Qingdao, China, 26th Mar. 2002, coll. Z. M. Hu	EMBLC H307002	AY699051	2
Mazzaella splendens (Setchell et Gardner) Fredericq	Cape Beale, Bamfield, BC, Canada, coll. A. T. Critchley		AY225290	

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                           [                   11111111111111111111222222233333334444444444555555555555555555556666666666666666666677777777777777777777777777]
                           [344455567777788899999900011233344455677990445780356690112355689000111112333344689900112234445566667789900111222222222333333445556]
                           [245601672456725801234904513446834534123386298155092582560618560015034595178237904056903712328034937167093890134567890123458956 75]
SP, Portugal      TC-CGTTCA-AACTCA---CTTTA-A-TAC-AC-AA-GA-AGGTG-CTGTAGAAGATGAGGTCT-T-T--G-CTTGGGC-A-GTC-AACCTA-AGA-AC--T--G--GTGAAAGGAAAAGACT-AAA-
BB, Portugal      .A-G..C..-.....---.....-.-...C.-..-.-.-.....-.C...G...........G.-.--.-...-.-.....-.-.....--.--...............-..-.-
SVLH, France      .A-G..C..-.....CTTT...-.-..-.-..-.-. AAGA-G.A.....A....AA...-C-.--.-.....A.-.-AC.-G-.-..-.A.-.-.--.-.--...............-..-.-
PF, Northern Ireland  .A-G..C..-.....-TTT...-.-..-.-..-.C.-...-.-.....-.-.....-.-.....-.-.....--C--.--...............-..-.-
HNS, Germany      .A-G..C..-.....-CTT...-.-..-. ACA-...-..-.-.....-.-. -TG..-.-.....T-.....-.-.....--.--...............-..-.-
RS, France(#1)    .A-G..C..-.....-CTT...-.-..-. G.ACA-..-C.CG....G...........-.-.-TG.....T-.....-.-.....--.--...............-..-.-
PS, France        .A-G..C..-.....-CTT...-.-..-. ACA-..-...-.-.....-.-. -TG.....T-.....-.-.....--.--...............-..-.-
DE, France        .A-G..C..-.....-CTT...-.-..-. CC.A.CT.-.-.....-.-. -TG.....T-.....-.-.....--.--...............-..-.-
EP, UK            .A-G..C..-.....-CTT...-.-..-. AC--.C-..C.....-.....A...A..-.-.-.T....T-.....-.-.....--.--...............-..-.-
NB, Canada        .A-G..CGCC..TAAT-CTT.N-.-.-. AC.-.-..C-.....-.-. -TG.....T-.....-.-.....--.--...............-..-.-
BR, UK            .A-GTGCTC-.....-CTT.A-.-..-. C..-..-.-.....-.-. -TG.....T-.....-.-.....--.--...............-..-.-
FB, France        .A-G..C..-.....-CTT...-.-..-.-.....-.-. -TG.....T-.....-.-.....--.--...............-..-.-
PEI, Canada       ..-G..C..-.....-CTT..-.-G-...AC--.-..-.-.....-.-. -TG.....T-.....-.-.....--.--...............-..-.-
I, Ireland        .A-G..C..-.....-CTT...-.-...AC--.-..-.-.....-.-. -TG..A.-.-..CC..T-.G....-G.-..-.--.--.G...............-..-.-
WBD, England      .A-G..C..-.....-CTT...-.-...AC--.-..-.-.....-.-. -TG..A.-......-.-..T--.....-.-.....--.--...............-GGG-
HI, UK            .A-G..C..-.....-CTT...-.-...AC--.-..-.-.....-.-.A.-.G.....C.C.-..C...T.-...G..G-.-.--.--...............-GGG-
DP, Wales         .A-G..C..-.....-CTT.--.-..-...AC--.-..-.-.....-.-.-.G...T-.-.....T-.....-.-.....--.--...............-..-.-
RS, France(#2)    .A-G..C..-.....-CTT..-.-..-...AC--.-..-.-.....-.-.-.G...-.-.....T-.....-.-.....--A..............-..-.-
C. pinnulatus     CAAG.CC.-.....----C.AT-.-...AC--.-..-.-.........A..........-.-.--A-T-.....-.C.-..T-G-...-.--..--T---....G....G.-....-.-
C. yendoi         .A-G..C.-.....----C...-.-...ACC.-.-..-.-.....A.A..G.A....TC-.-.-.-T.A..T-G-.C.-..-T-GCG..T.-.G.TT.T-....G....G..ACG.T...T
C. ocellatus      .G-G..C.-.--AC..---.....-.A-.ACC.-.-..-.-.....-...AGACC...AG.....-C-CGA.-T..A...-G-.C.-..T-G-..G-.G--.-.TT.CTG.GAAG.G....A-...-
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Fig. 15 Alignment of ITS region of seventeen *C. crispus* isolates and three outgroups, numbers indicated variant nucleotides in different sites and the identical nucleotides were not shown

unequal (*A* = 0.27621, *C* = 0.18420, *G* = 0.25328, *T* = 0.28361), –Ln Likelihood = 2956.7969. In three phylogenetic dendrograms, there were two major clades within the *C. crispus* group (Figs. 16, 17). The first clade contained two isolates (SP, BB) from Portugal, PF from Northern Ireland, DP from Wales, NB from Canada, three isolates (SVLH, RS #2 and FB) from France and three

Table 2 Pair-wise distance comparison of ITS sequence variation in *Chondrus crispus* and outgroups (Kimura 2-parameter has been used)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1 *C. crispus* (SP, Portugal)																					
2 *C. crispus* (BB, Portugal)	.006																				
3 *C. crispus* (SVLH, France)	.026	.026																			
4 *C. crispus* (PF, Nor-Ireland)	.006	.006	.026																		
5 *C. crispus* (HNS, Germany)	.005	.005	.024	.005																	
6 *C. crispus* (RS, France) #1	.010	.010	.029	.010	.005																
7 *C. crispus* (PS, France)	.005	.005	.024	.005	.000	.005															
8 *C. crispus* (DE, France)	.005	.005	.024	.005	.000	.005	.000														
9 *C. crispus* (EP, UK)	.006	.006	.026	.006	.002	.006	.002	.002													
10 *C. crispus* (NB, Canada)	.016	.016	.036	.016	.011	.016	.011	.011	.013												
11 *C. crispus* (BR, UK)	.010	.010	.029	.010	.008	.013	.008	.008	.010	.014											
12 *C. crispus* (FB, France)	.003	.003	.023	.003	.002	.006	.002	.002	.003	.013	.006										
13 *C. crispus* (PEI, Canada)	.005	.008	.028	.008	.003	.008	.003	.003	.005	.014	.011	.005									
14 *C. crispus* (I, Ireland)	.008	.008	.024	.008	.003	.008	.003	.003	.005	.014	.011	.005	.006								
15 *C. crispus* (WBD, England)	.010	.010	.029	.010	.005	.010	.005	.005	.006	.016	.013	.006	.008	.008							
16 *C. crispus* (HI, UK)	.010	.010	.028	.010	.005	.010	.005	.005	.006	.016	.013	.006	.008	.006	.003						
17 *C. crispus* (DP, Wales, UK)	.006	.006	.026	.006	.002	.006	.002	.002	.003	.013	.010	.003	.005	.005	.006	.006					
18 *C. crispus* (RS, France) #2	.005	.005	.024	.005	.000	.005	.000	.000	.002	.011	.008	.002	.003	.003	.005	.005	.002				
19 *C. pinnulatus*	.021	.021	.038	.021	.016	.021	.016	.016	.018	.028	.023	.018	.019	.019	.021	.021	.018	.016			
20 *C. yendoi*	.034	.034	.051	.034	.029	.034	.029	.029	.031	.041	.038	.031	.033	.033	.034	.034	.028	.029	.029		
21 *C. ocellatus*	.046	.046	.062	.048	.043	.048	.043	.043	.044	.053	.051	.044	.044	.046	.048	.048	.044	.043	.053	.053	
22 *M. splendens*	.121	.121	.140	.121	.115	.121	.115	.115	.117	.128	.124	.117	.119	.119	.121	.121	.117	.115	.123	.129	.126

Fig. 16 The NJ tree generated from ITS sequences of *C. crispus* using interior branch test. Numbers above the branches are bootstrap values of 1,000 replicates, *C. pinnulatus*, *C. yendoi*, *C. ocellatus* and *M. splendens* were used as outgroups

isolates (WBD, BR and HI) from England, respectively with moderate bootstrap support (55%); while the three isolates (RS #1, DE and PS) from France, PEI from Canada, EP from UK, I from Ireland and HNS from Germany formed the second clade with moderate support (58%). In clade one, WBD and HI had closest relationships (89%), and in group two, three isolates (EP, HNS and RS #1) had sister relationships with high bootstrap support (96%). Outgroups,

Fig. 17 The consensus tree inferred from interior branch test with ME method (1,000 replications)

C. ocellatus, *C. pinnulatus* and *C. yendoi* from North Pacific Ocean were clearly distinguished from *C. crispus*.

Discussion

As shown in Figs. 2–14, it was interesting that *C. crispus* exhibited conspicuous morphological variability which was not reflected by the ITS sequence divergence or pair-wise distance (Table 2), although base mutation including transition/transversion and insertion/deletion might be responsible for the variability. More likely is that the diversity of morphology of *C. crispus* was due to external environmental parameters like tidal variation, exposure to water current etc. (Gutierrez & Fernandez, 1992). For an instance, at low intertidal and exposed sites, *C. crispus* is characterized by fewer dichotomies and exhibits filiform morphology, while at high intertidal sites in sheltered areas, *C. crispus* is characterized by more dichotomies, smaller sizes and narrow fronds (Gutierrez & Fernandez, 1992). It seems likely that the morphological variation seen in *C. crispus* mainly from external environmental factors.

ITS data and phylogeny

The ITS regions were chosen for analysis because they provided a measure of DNA variation that was appropriate for species or subspecies levels of comparisons (Gardes et al., 1991; Lee & Taylor, 1992). In plant genomes, nuclear ribosomal genes are organized in clusters of tandemly repeated units, and thousands of ITS copies are present and located in one or several chromosomes. Concerted evolution of intra- and interchromosomal loci, a process that takes 1–2 million years, is believed to result in homogenization of individual repeats (Won & Renner, 2005). So, it plays an important role in phylogenetic applications (Zimmer et al., 1989; Hillis & Moritz, 1990; Li & Graur, 1991).

Chopin et al. (1996) examined seven different isolates of *C. crispus* from different locations across both the Maritime Province, Canada and Europe by sequencing the ITS region. They found 2.18% sequence divergence for the two isolates from Nova Scotia and PEI, which exceeded by far the average intraspecific sequence divergence (1.3%) of ITS1 region for five various red algal genera (Goff et al., 1994, 1996). They suggested that the genetic diversity of *C. crispus* neither correlated with the phenotype for the plants investigated nor the geographic origin. However, in this research we found that the intraspecific sequence in *C. crispus* varied from 0.3% to 4.0%, this is adequate for the study of phylogeography. It was interesting that there were relatively low pair-wise distances in *C. crispus* species (0–0.16%) except the isolate (SVLH) from France (Table 2), which showed higher distances (0.23–0.36%) to all other isolates. The ITS sequence may not be correlated with the geographic distance of the isolates, and further studies are needed to explain the divergence.

It is interesting that, in some ITS regions of *C. crispus*, short repetitive motifs were found. The presence of these motifs is most likely due to the process of slip-strand mispairing events, causing the addition or deletion of repeat units during replication (Levinson & Gutman, 1987), i.e. an intrahelical event rather than an interhelical event and displacement of the strands of a DNA duplex followed by mispairing at the site of an existing short tandem repeat. Over time, this leads to insertions or deletions of one or several short repeats that can form distinctive nested patterns. The process can be self-accelerating and maintained for long periods in specific populations. It has also been hypothesized that this might provide the raw material for the evolution of regulatory elements, especially when located near genes (Levinson & Gutman, 1987; Bakker et al., 1992).

One factor that may affect ITS evolution is secondary structure. Bakker et al. (1995b) studied the *Cladophora albida*/*sericea* complex and found the conservative regions within both ITS1 and ITS2 correlated with double-stranded regions in the ITS secondary structure (within the pre-rRNA molecule). Van Nues et al. (1994) demonstrated that distinct conserved structures existed within ITS1 directly processing of pre-rRNA (precursor RNA). No such clear secondary structural elements, however, could be found in the *C. crispus* ITS sequences. Another factor is the variability of the ITS sequence, which has been proved to strongly correlate with sexual isolation in the Volvocacean algae *Pandorinum morum* Bory and *Gonium pectorale* Mueller (Coleman et al., 1994). Geographically disparate isolates of *G. pectorale* can interbreed and this species has an ITS region that is remarkably uniform with a world-wide distribution. Isolates of *P. morum* behave quite differently, and syngens (breeding group) of worldwide distribution or those restricted to one small geographical area show great similarity for ITS sequences. Since little is known about reproductive patterns or sexual isolation in *C. crispus*, we cannot decide whether a similar explanation applies to the ITS evolution in this species.

Biogeography

The topology and level of divergence expressed in the trees (Figs. 16, 17) is consistent that of a relative old species in which *C. crispus* from Northern Atlantic are of more recent origin. The most general biogeographic conclusion that can be drawn from the consistently more basal position of three *Chondrus* speices from the Pacific, is of a Pacific origin. However, while a general

theory explaining the pattern of biogeographic distribution for algae along the Northern Atlantic coasts is presently lacking, we can implicate with confidence the roles of paleoclimate and paleoceanography as prime factors influencing the biogeographic distribution of *C. crispus*. Neumayr (1885) hypothesized a primordial ''Central Mediterranean'', which arose ca. 160 million years ago and extended from Mexico to the Himalayas. Suess (1893) renamed this great equatorial ocean the Tethys Sea, which provided an open east–west connection between the present-day Atlantic and Pacific Oceans via the ''Mediterranean'' until roughly 12 Ma ago (Tarling, 1980; Rögl & Steininger, 1984; Por, 1989). Also, biogeographic disjunctions observed today may be explained by Tethyan closure events (Silva, 1962; Rögl & Steininger, 1984). So, the biogeographical consequences of plate movements, interactions, the rearrangement of continental landmasses and island areas, the opening and closing of sea and ocean basins initiated by these movements and interactions have profoundly affected the distribution of organisms on land and in the ocean. In this study, we assumed that both in clade one and two, the isolates from Canada grouped with the isolates from France, and United Kingdom. This affinity showed that the isolates from Canada, located in the western Atlantic were closely related to the isolates from France, England, Portugal and Germany, located in eastern Atlantic. This is congruent with the Tethyan hypothesis that an early equatorial cosmopolitan marine flora gradually became dispersed.

From this point onward in the discussion, we can therefore assume that an important paleoceanographic event was the closure of the warm Tethys Sea at around 12 Ma ago, thus ''pinching off'' the major Pacific and Atlantic domains, and the subsequent further division of the eastern and western Atlantic marine floras (van den Hoek, 1984; Joosten & van den Hoek, 1986). Tethyan divergence has been suggested for many green algae in which large genetic distances have been found, such as *Dictyosphaeria cavernosa* Boergesen (Olsen et al., 1987), *Cladophora* and related species (Kooistra et al., 1992; Bakker et al., 1995a, b). Given the large uncertainties about the divergence times, the estimates are useful as a qualitative pre- or post-Tethys closure divergence estimate at most. At the same time, we can understand the distribution of the algae and the formation of the algal regions and provinces from a historical point of view (van den Hoek, 1975). The Atlantic Ocean opened up in the Cretaceous (135–65 Ma ago), so that a distinct Caribbean province could originate in the tropical Tethyan Realm. To the south and to the north of this tropical Tethyan Realm, (a belt between the 50° S to 50° N), conditions were warm and temperate. During the Cretaceous polar temperatures fluctuated between 10°C and 16°C. By the early tertiary polar temperatures may have dropped to the point where the development of cold temperature flora could have begun (with winter surface temperature at Spitsbergen of 5–8°C) (van den Hoek, 1975). From this period onwards, there were a number of climatic temperature fluctuations. A gradual cooling culminated in the Pleistocene ice ages (2–3 Ma ago), this affected the distribution of marine floras.

Tropical taxa were largely unaffected, while temperate floras were forced south or became extinct depending on their eurythermic abilities. Retreat of the ice sheets meant a mass recolonization of the middle and North Atlantic (and Mediterranean) of taxa whose distribution patterns follow isotherms rather than latitude (MacIntyre, 1981). This may then explain the present complex dispersion of *C. crispus* in Atlantic Ocean. About 135–65 Ma ago, North America and Europe were moving apart slowly. After that, the warm Tethys Sea was closed at around 12 Ma ago, thus ''pinching off'' the major Pacific and Atlantic domains, and the subsequent further division of the eastern and western Atlantic marine floras. The British Isles was also separated from European continent, then *C. crispus* formerly distributed in Northern Pangaea dispersed to both east and west of the Atlantic Ocean.

Further speculations might include the effects of vicariance, temperature boundaries and dispersal. The correlation between thermal barriers and vicariance events seen indicate that the former also has play a role because each would have predicted a correlation between biogeographic distribution and tree topology (Bakker et al.,

1995a). Joosten & van den Hoek (1986) divided the distribution of 613 red algal genera into 22 climatically defined coastal regions two of which were the cold temperate Northern (CN3 and CN4) and the warm temperate Western (WN3 and WN4) Atlantic region (70–30° N). *C. crispus* has a range similar to this (northern Russia (65° N) to southern Spain (38° N)) (Dixon & Irvine, 1977). We think the most environmental factor responsible for the distribution over these areas is temperature. In New Hampshire, USA, *C. crispus* grows abundantly in waters with an annual variation in surface temperature from –1°C to 19°C (Mathieson & Burns, 1975), and its optimum temperature for growth has been reported as 10–15°C (Fortes & Luning, 1980), 15–17°C (Tasende & Fraga, 1999) and 20°C (Simpson & Shacklock, 1979). Prince & Kingsbury (1973) reported cessation of growth in *C. crispus* culture at 26°C, first mortality of spores at 21.1°C and total mortality of spores at 35°C, even if exposed for just 1 min. Maximum sea surface temperature in the Northern Atlantic rarely exceeds 20°C (Joosten & van den Hoek, 1986). It is unlikely that *C. crispus* will colonize the middle tropical East and West Atlantic regions, where the summer temperature is 25°C. *C. crispus* is not capable of completing all the stages of its life cycle at these temperatures.

In conclusion, ITS sequence data suggests a single phylogenetic Atlantic origin for *C. cripus* (Atlantic origin). The present distribution is attributable to temperature barriers, vicariance, paleoceanography and paleoclimate. The results indicate that *C. crispus* distributed from cold to warm-temperate areas, might be useful in understanding historical vicariance in marine algae, and it may provide further illumination on continent drift.

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