

SEXUAL HYBRIDIZATION EXPERIMENTS AND PHYLOGENETIC RELATIONSHIPS  
AS INFERRED FROM RUBISCO SPACER SEQUENCES IN THE GENUS  
*ALARIA* (PHAEOPHYCEAE)<sup>1</sup>

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*Alaria* (Alariaceae, Phaeophyceae) is a common genus of kelps generally found in the lower intertidal and shallow subtidal regions of rocky shores subject to strong wave exposure. Fourteen species are currently recognized, of which 11 are found in the cold-temperate North Pacific Ocean. *Alaria esculenta* (L.) Greville, the type species described from the North Atlantic, exhibits a range of biogeographically correlated morphotypes indicating the possibility of multiple species, subspecies, and/or hybrids. This has led to an unstable taxonomy. We compared five species from the Atlantic and Pacific, including six North Atlantic isolates of *A. esculenta*. Phylogenetic analyses based on Rubisco spacer sequences resulted in a well-resolved topology of these five species, but did not distinguish between the six biogeographic isolates of *A. esculenta*. Laboratory hybridization experiments among four *A. esculenta* isolates showed partial intrafertility. Among five tested *Alaria* species, interfertility as well as fertility barriers were encountered, inconsistent with reproductive isolation. The data reject both a biological and morphological species concept and support only a phylogenetic species concept for *Alaria*, demonstrating that morphological variation has evolved independently of molecular variation in the genes under investigation in the species of the genus *Alaria*.

**Key index words:** *Alaria esculenta*; brown algae; chromosomes; cross experiments; hybridization; interspecific; intraspecific; phylogeny; Rubisco spacer

**Abbreviations:** bp, base pair; CTAB, hexadecyl trimethyl ammonium bromide; EtBr, ethidium bromide; TAE, Tris-acetic acid–EDTA

Since the generic name *Alaria* was proposed by Greville (1830), more than 108 specific and subspecific names have been ascribed to the genus, which highlights the considerable amount of morphological plasticity inherent in species of this genus (Yendo 1919, Widdowson 1971a). Based on morphological features, Widdowson (1971a) recognized 14 species, of which the North Pacific harbors 11 and only 3 species, *A. esculenta* (L.) Greville, *A. pylaii* (Bory de Saint-Vincent) J. Agardh, and *A. grandifolia* J. Agardh, are

generally considered North Atlantic. Lüning (1990) considered *A. pylaii* and *A. grandifolia* to be “northern forms” of *A. esculenta*. In addition, subspecies, ecotypes, and hybrids have been described for species of the genus *Alaria*, leading to considerable taxonomic confusion (Yendo 1919, Widdowson 1971b). Statistical analyses of morphological variation in the North Atlantic *A. esculenta* revealed three different groups (Widdowson 1971b). In the North Pacific, four morphological groups of *A. marginata* Postels et Ruprecht were recognized (Widdowson 1971b), which suggests the possible existence of multiple species, subspecies, or hybrids, or both. Lüning (1990) categorized *A. esculenta* as an Arctic–cold temperate amphioceanic species occurring in both oceans, although Selivanova and Zhigadlova (1997) after surveying the marine algae of the Komandorskiye Ostrova (Commander Islands) in the Bering Sea concluded that *A. esculenta* is strictly an Atlantic representative.

*Alaria* species richness in the North Pacific Ocean suggests that its radiation center was in this area and that only *A. esculenta* migrated, via the Arctic, into the North Atlantic; this follows a similar pattern proposed for certain *Laminaria* species (Estes and Steinberg 1988, Saunders and Druehl 1992) and other organisms (Lüning 1990). Hybridization experiments in the Laminariaceae (Bolton et al. 1983, tom Dieck 1992) have shown interfertility, inconsistent with normally expected reproductive isolation between different geographically separated species (Manhart and McCourt 1992). Based on these experiments, the biological species concept is not valid in the family Laminariaceae. Only morphological criteria have been applied for species separation in *Laminaria*. Hybridization studies in Pacific and Atlantic *Alaria* species have not been carried out and species-level differences are based on morphological characters only.

The many species described for the genus *Alaria*, the inherent taxonomic confusion, and the importance of *A. esculenta* as a potentially economically important alga (Kain and Dawes 1987, Kain et al. 1990, Guiry and Blunden 1991, Mai et al. 1996) make reassessment of its taxonomy and phylogeny of interest. In the present study, we combined hybridization experiments with Rubisco spacer sequence comparisons among five species of *Alaria*, including six isolates of *A. esculenta* from different biogeographical regions in the North Atlantic Ocean.

The plastid-encoded Rubisco spacer separates the large and the small subunits of the ribulose-1,5-bis-

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phosphate carboxylase/oxygenase genes; it has been used to examine relationships among populations, isolates, and species in red algae (e.g. Destombe and Douglas 1991, Maggs et al. 1992, Goff et al. 1994) and in brown algae (e.g. Stache-Crain et al. 1997, Siemer et al. 1998).

The objectives of the present study were (1) to assess the usefulness of hybridization experiments in species differentiation in *Alaria*; (2) to correlate geographical variation in *A. esculenta* with Rubisco spacer sequence differences; (3) to evaluate phylogenetic relations in 5 species of the genus *Alaria*; and (4) to test the hypothesis that recent divergence in the genus *Alaria* follows a pattern similar to that described in *Laminaria*.

#### MATERIAL AND METHODS

**Hybridization studies.** Male and female gametophyte cultures isolated from zoospores were obtained from the geographical locations listed in Table 1 according to methods described by South (1970) and Nakahara and Nakamura (1973). Cultures were kept in a vegetative state in red fluorescent light, 16:8 h L:D, at 10°C, 20 µmol photons·m<sup>-2</sup>·s<sup>-1</sup> in glass dishes containing sterile enriched seawater as modified by Guiry and Cunningham (1984), which was changed monthly. Red light was used, as it inhibits the formation of eggs and sperm (Bolton et al. 1983).

Hybridization experiments, 50 per cross combination, were carried out according to methods described by tom Dieck (1992) and incubated at 10, 15, 20, and 25°C, 16:8 h L:D, 30 µmol photons·m<sup>-2</sup>·s<sup>-1</sup> of white light to initiate the formation of eggs and sperm (Lüning et al. 1978). The length and width of 25 successful hybrid sporophytes were measured with a dissecting microscope, and blade, haptera, general appearance, and cultivation time were recorded. In addition, controls consisting of 25 self-crosses and 25 isolated male and female gametophytes

were incubated in parallel with each crossing experiment to identify possible parthenogenesis or apogamy (Nakahara and Nakamura 1973).

**DNA extraction and purification.** Plants from the sites listed in Table 1 were transported to the laboratory in a cool-box or were sent by airmail alive or dried in silica gel. Upon arrival, plants were cleaned and immediately processed for DNA extraction or quick-frozen in liquid nitrogen and stored at -70°C. All DNA was extracted from sporophytes. DNA was extracted using the CTAB extraction protocol as described by Doyle and Doyle (1987) and modified by Siemer et al. (1998). RNA was removed by incubation (30 min, 37°C) with 10 µL RNase (Boehringer, Mannheim, Germany). DNA concentrations were measured in a spectrophotometer at 260 nm according to standard methods (Sambrook et al. 1989).

**Double-stranded PCR.** Target sequences were amplified in an Omn-E thermal cycler (Hybaid Ltd., England) with an initial denaturation step of 95°C for 3 min followed by 30 cycles with the following temperature profile: 1 min 95°C, 2 min 55°C, and 2 min 74°C, ending with one extension step of 72°C for 5 min. The reaction volume was 100 µL, comprised of 10–100 ng genomic DNA, 50 µM of each dATP, dNTP, dCTP, and dGTP, 0.5 µM of each primer, 10 µL of 10× reaction buffer, 6 µL 25 mM MgCl<sub>2</sub>, and 2.5 units of Taq (Sigma). Primer sequences and annealing positions are shown in Table 2. Amplifications were checked for correct length, purity, and yield on 1.5% agarose TAE gels stained with EtBr in accordance with the methods of Sambrook et al. (1989).

**Sequencing and analysis.** Double-stranded PCR products were custom-sequenced using the LI-COR 4200 system using a nested amplification technique (MWG Biotech UK Limited, Milton Keynes, UK). Sequences were initially aligned and edited using GENEDOC (Nicholas and Nicholas 1997); final alignments were made by eye. Another representative of the family Alariaceae, *Undaria pinnatifida* (Harvey) Suringar, was chosen as an outgroup. The alignment is available at GenBank.

The maximum parsimony, maximum likelihood, and distance matrix methods were used to construct phylogenetic trees. Maximum parsimony analysis was implemented with the PAUP program (Swofford 1993) using the exhaustive search

TABLE 1. Origin of *Alaria* species and *Undaria pinnatifida* used for DNA extractions and *Alaria* and *U. pinnatifida* gametophyte cultures used in the present study. Voucher specimens are deposited in the Martin Ryan Institute, N.U.I., Galway. Accession numbers are recorded in the GenBank sequence database. M = male, F = female.

Species	Code	Chromosome number ( <i>n</i> ) <sup>a</sup>	Gametophyte culture	Origin	Collector and collection date	GenBank accession no.
<i>Alaria esculenta</i>	IRL	28	M & F <sup>d</sup>	Trácht Each, Inis Oírr, Ireland	S. Kraan 04-1996	AF 109795
<i>Alaria esculenta</i>	SCL	28	DNA only	Hind Rock, St Andrews Bay, Scotland	S. Draisma 02-1997	AF 109796
<i>Alaria esculenta</i>	NOR	28	M & F <sup>c</sup>	Ålesund, Norway	J. Rueness 06-1996	AF 109797
<i>Alaria esculenta</i> <sup>b</sup>	ICL	28	M & F <sup>c</sup>	Tjörnes, Iceland	K. Lüning 01-1996	AF 109798
<i>Alaria esculenta</i>	CAN	28	M & F <sup>c</sup>	Sandy Cove, Halifax, Canada	C. Bird 08-1996	AF 109799
<i>Alaria esculenta</i>	FRC	28	DNA only	Roscoff, France	J. Cabioch 09-1996	AF 109800
<i>Alaria praelonga</i>	AP	22	M & F <sup>c</sup>	Katsurakoi, Kushiro, Hokkaido, Japan	M. Masuda 05-1996	AF 109801
<i>Alaria crassifolia</i> <sup>b</sup>	AC	22-28	M & F <sup>c</sup>	Katsurakoi, Kushiro, Hokkaido, Japan	M. Masuda 05-1996	AF 109802
<i>Alaria tenuifolia</i>	AT	14	F <sup>d</sup>	Bamfield, Vancouver, Canada	L. Druehl 06-1996	
<i>Alaria nana</i>	AN	14	M & F <sup>d</sup>	Bamfield, Vancouver, Canada	L. Druehl 06-1996	AF 109803
<i>Alaria marginata</i>	AM	14	M & F <sup>d</sup>	Bamfield, Vancouver, Canada	L. Druehl 06-1996	AF 109804
<i>Undaria pinnatifida</i>	UND	30	M & F <sup>d</sup>	Munseom Island, Korea	O. Yoon Sik 05-1996	
<i>Undaria pinnatifida</i>	UND	30	DNA only	Oshoro, Japan	H. Kawai 05-1996	AF 109805

<sup>a</sup> Lewis (1996).

<sup>b</sup> DNA extracted from sporophytes grown from gametophyte cultures.

<sup>c</sup> Male and female gametophyte cultures were sent by the collectors.

<sup>d</sup> Male and female gametophyte cultures were reared from zoospores.

TABLE 2. Primer sequences used for polymerase chain reaction amplification and sequencing of the Rubisco spacer region.

Code	Seq. <sup>a</sup>	Sequence	Anneals to
BLSrbcL1124F <sup>b</sup>	F	5'-ATGGATTGGGCAGCTCTTAG-3'	1124–1144 <sup>c</sup>
BLSrbcS153R <sup>b</sup>	R	5'-CATCAAATAAAGGAAGACCCC-3'	132–153 <sup>d</sup>

<sup>a</sup> Sequencing direction: F = forward, R = reverse.

<sup>b</sup> Siemer et al. (1998).

<sup>c</sup> Alignment site in the large subunit of the Rubisco gene of *Pilayella littoralis* (Linnaeus) Kjellman (Assali et al. 1991).

<sup>d</sup> Alignment site in the small subunit of the Rubisco gene of *P. littoralis* (Assali et al. 1991).

options. All sites were weighted equally and alignment gaps were excluded or handled as a single evolutionary event. Bootstrap analysis (1000 replications) was performed on the parsimony analysis. Maximum likelihood analyses were performed with the DNAmI program from the PHYLIP package (Felsenstein 1995).

A distance matrix was generated using the computer program MEGA (Kumar et al. 1993). Pairwise distances were calculated using the Tajima–Nei distance (Tajima and Nei 1984), which gives a better estimate in cases where nucleotide frequencies deviate from 25%. A neighbor-joining distance network was generated from the distance matrix using the program NEIGHBOR (NJ method; Saitou and Nei 1987).

The between-sequences transition/transversion ratios (ts/tv) were calculated with the computer program MEGA (Kumar et al. 1993) to check for saturation effects.

#### RESULTS

*Hybridization experiments.* The results of the hybridization experiments between Atlantic × Atlantic, Atlantic × Pacific and Pacific × Pacific *Alaria* species are shown in Tables 3 and 4. Parental codes are listed in Table 1. Plants crossed successfully only at 10° C. Intergeneric cross combinations of *U. pinnatifida* with all of the *Alaria* species were completely unsuccessful.

All crosses and reciprocal crosses between *A. esculenta* isolates of four Atlantic populations were successful except for the male Canada × female Iceland cross (Table 3). Sporophytes derived parthenogenically in the female parallel cultures were easily identified because of their small, round, clump-like appearance and the lack of a hapteron.

Within the *A. esculenta* × Pacific *Alaria* crosses, the largest and best-developed plants were produced by the *A. esculenta* × *A. tenuifolia* Setchell cross; however, the reciprocal cross could not be tested, as no male *A. tenuifolia* gametophytes were available. A cross between *A. esculenta* and *A. praelonga* Kjellman resulted in healthy and well-developed sporophytes with sporophylls. Crosses between female *A. esculenta* and male *A. crassifolia* Kjellman gave progeny that grew very slowly; the reciprocal cross producing small (~1 cm long) stunted sporophytes that resembled the parthenogenic sporophytes from the control. Crosses and reciprocal crosses of *A. esculenta* with *A. marginata* and *A. nana* Schrader resulted in a few elongated stipes or no sporophyte formation.

TABLE 3. Hybridization matrix among four North Atlantic strains of *Alaria esculenta*. Success of the crossings in percentages is indicated as morphology and maximum length that was achieved during cultivation. Normal = development of the sporophytes as seen in the field. Small/broad = sporophytes grow slowly in length and faster in width producing triangular shaped sporophytes. Long/thin = sporophytes grow fast in length and slow in width producing very thin ribbon like sporophytes. Small = sporophytes of normal shape but grew 2 to 4 times slower than normal sporophytes. N = no progeny, + = more than 3 roots as haptera, +/- = 1–3 roots as haptera, – = no haptera, sp = sporophylls, CNT = crossing not further tested after sporophyte formation, months = cultivation period. For origin codes see Table 1.

Female	Male			
	IRL	ICL	NOR	CAN
IRL	100%, normal sp, + 430 mm, 7 months 70%, small/broad	60%, small/broad – 65 mm, 6 months	100%, normal +/- 460 mm, 6 months 60%, small	100%, normal sp, + 420 mm, 6 months 0%
ICL	+/- 80 mm, 6 months 100%, long/thin	CNT	+/- 30 mm, 16 months	N 12 months 100%, small
NOR	sp, +/- 270 mm, 6 months 100%, normal/missing midrib	sp, + 700 mm, 9 months 90%, small	CNT	+/- 210 mm, 9 months
CAN	+ 130 mm, 6 months	+ 180 mm, 9 months	– 25 mm, 9 months	CNT

TABLE 4. Hybridization matrix of North Atlantic *Alaria esculenta* with Pacific *Alaria* species and within Pacific species. Success of the crossing in percentage is indicated as morphology and maximum length that was achieved during cultivation. Normal = development of the sporophytes as seen in the field. Few stipes = sporophytes produced a midrib only, without blade material. Long/thin = sporophytes grow fast in length and slow in width producing very thin ribbon like sporophytes. Stunted = sporophytes resemble parthenogenetic derived sporophytes in parallel cultures. Clumps = sporophytes <1 cm were round and clump-like without haptera development. Months = time in months of cultivation of the sporophytes. Bleached = half of the surface area of the blade without pigmentation. N = no progeny, + = over 3 roots as haptera, +/- = 1–3 roots as haptera, - = no haptera, sp = sporophylls, CNT = crossing not further tested after sporophyte formation, months = cultivation period. For origin codes see Table 1.

Female	Male				
	IRL	AP	AC	AN	AM
	100%, normal	100%, normal	85%, normal	20%, few stipes	25%, few stipes
IRL	sp, + 430 mm, 7 months	sp, + 210 mm, 6 months	- 60 mm, 7 months	- 80 mm, 6 months	- 80 mm, 7 months
	100%, normal	100%, normal/long thin	Clumps <10 mm	Clumps <10 mm	Clumps <10 mm
AP	sp, + 110 mm, 6 months	+ 130 mm, 8 months	- 9 months	- 12 months	- 9 months
	100%, stunted	100%, normal		70%, stunted	clumps, <10 mm
AC	<10 mm bleached, 7 months	sp + 250 mm, 9 months	CNT	bleached, - 10 mm, 7 months	- 12 months
	35%, few stipes	100%, normal	100%, normal		0%
AN	- 60 mm, 6 months	+/- 50 mm, 6 months	+/- 60 mm, 6 months	CNT	N 20 months
	Clumps, <10 mm	100%, normal	Clumps, <10 mm	0%	85%, long/thin
AM	- 14 months	sp, + 600 mm, 10 months	- 12 months	N 20 months	+ 220 mm, 8 months
	100%, normal	100%, normal	60%, stunted	50%, stunted	100%, normal
AT	sp, + 610 mm, 6 months	+ 125 mm, 9 months	- <10 mm, 6 months	- <10 mm, 6 months	+ 560 mm, 9 months

Within the Pacific crosses, *A. praelonga* male gametophytes hybridized with all other Pacific *Alaria* species, but the reciprocal did not result in any progeny. *Alaria tenuifolia* female gametophytes hybridized with *A. praelonga* and *A. marginata* male gametophytes, and the progeny of *A. tenuifolia* × *A. marginata* produced sporophylls. In terms of ability to cross, the least successful species was *A. nana*, which hybridized only with *A. praelonga* and *A. crassifolia* male gametophytes.

Control cultures with isolated female or male gametophytes sometimes produced parthenogenetically or apogamously derived sporophytes. These sporophytes had a high mortality rate and never exceeded 1 cm in length. Healthy and viable hybrids could thus be easily distinguished from parthenogenic or apogamous sporophytes. In some experiments, the parthenogenic or apogamous sporophytes survived up to a year without much growth.

*Alignment of the Rubisco spacer.* The Rubisco spacer of each species was sequenced entirely on both strands. This resulted in an aligned data set of 816 positions, of which 363 presented the 3' end of the Rubisco large subunit, 283 the spacer, and 170 the Rubisco small subunit. All *A. esculenta* isolates and *A. praelonga* sequence alignments were 815 bp long and shared with all the other *Alaria* species one basepair deletion at position 463 in the spacer region. The remaining Pacific species were 805 bp, due to a 10-bp

deletion gap in the spacer region at position 402–411. The outgroup species, *U. pinnatifida*, had a 13-bp gap at position 475–487 in the spacer and was therefore 802 bp long. The Tajima–Nei distance matrix between species based on this alignment is given in Table 5, where the upper right panel represents the Tajima–Nei distance (proportion of nucleotide differences in percentages) and the lower left panel the absolute distances (nucleotide changes). Of the aligned data set, 51 sites (excluding gaps) are variable (6.4%), of which 14 are phylogenetic informative (1.8%). Within *A. esculenta* isolates, the strains from Iceland and Canada show most sequence divergence (see Table 5). The nucleotide composition within sequences was unbalanced: A = 31.7%, T = 35.5%, C = 13.8%, G = 18.9%. The ts/tv ratios between sequences were in general >1, suggesting an absence of saturation (Bakker 1995). Within *A. esculenta* isolates and between *A. praelonga* and *A. esculenta*, the tv/ts ratios were relatively low (<0.5). These values are probably not very reliable because they are based only on a small number (≤6) of substitutions (data not shown).

*Parsimony analysis.* Parsimony analysis (exhaustive search and all gaps removed) revealed 4 most parsimonious trees (MPTs) out of 67 (data not shown). Parsimony analysis (exhaustive search), where the 10-bp gap in the spacer was treated as a single evolutionary event, revealed 2 MPTs of 62 steps. Both MPTs are

TABLE 5. Pairwise distances in Rubisco spacer sequences between *Alaria esculenta* strains, *Alaria* species and *Undaria*. Upper right panel, Tajima–Nei sequence distances (in percentages); lower left panel, absolute distances (nucleotide changes). For codes see Table 1.

	1	2	3	4	5	6	7	8	9	10	11
1 <i>A. esculenta</i> –IRL	—	0.00	0.13	0.25	0.38	0.13	0.89	1.41	1.02	1.28	4.61
2 <i>A. esculenta</i> –SCL	0	—	0.13	0.25	0.38	0.13	0.89	1.41	1.02	1.28	4.61
3 <i>A. esculenta</i> –NOR	1	1	—	0.38	0.51	0.25	1.02	1.54	1.15	1.41	4.75
4 <i>A. esculenta</i> –ICL	2	2	3	—	0.64	0.38	1.15	1.67	1.28	1.54	4.61
5 <i>A. esculenta</i> –CAN	3	3	4	5	—	0.51	1.28	1.80	1.41	1.67	4.87
6 <i>A. esculenta</i> –FRC	1	1	2	3	4	—	1.02	1.54	1.15	1.41	4.74
7 <i>A. praelonga</i> –AP	8	8	9	10	11	9	—	1.80	1.28	1.15	5.02
8 <i>A. crassifolia</i> –AC	11	11	12	13	14	12	15	—	1.67	1.93	4.33
9 <i>A. nana</i> –AN	8	8	9	10	11	9	11	13	—	0.38	4.61
10 <i>A. marginata</i> –AM	10	10	11	12	13	11	10	15	3	—	4.88
11 <i>U. pinnatifida</i> –UND	35	35	36	35	37	36	38	33	35	37	—

shown in Fig. 1A and 1B and differ in the position of *A. praelonga* only, that is, closer to the Atlantic clade but in a separate clade or in one clade together with *A. nana* and *A. marginata*. A strict consensus tree based on the 2 MPTs is shown in Fig. 1C. Bootstrap analysis on the parsimony analysis (BP, 1000 replicates) revealed that the *A. esculenta* isolates are grouped together 88% of the time. Within the Pacific species, there is good support (BP = 91%) for a separate clade consisting of *A. nana* and *A. marginata* and a clade (BP = 84%) comprising *U. pinnatifida* and *A. crassifolia*. The MPTs are robust, reflected by the low number of MPTs, high bootstrap values, and low levels of homoplasy (HI = 0.065). The tree length distributions were good, showing a left negative skewed g1 value of  $-0.880435$ , which indicates a relatively high phylogenetic signal (Hillis and Huelsenbeck 1992). Phylogenetic trees obtained with the ML method showed identical tree topology (data not shown). A neighbor-joining distance network (data not shown) supported the relatively short distance between *A. praelonga* and the *A. esculenta* isolates and the long distance between *A. crassifolia* and all other *Alaria* species as shown in the MPTs. Within isolates of *A. esculenta*, the distance is largest between the western and eastern Atlantic (Table 5).

#### DISCUSSION

*Intraspecific relationships.* Cross combinations between *A. esculenta* isolates from different geographical locations in the North Atlantic show almost complete interfertility, which provides biological species concept support for current taxonomic opinion based mainly on morphological similarity, but does not explain the sterility barrier in the female Iceland × male Canada cross. Müller (1979) found that in the filamentous brown alga *Ectocarpus siliculosus* (Dillwyn) Lyngbye sterility barriers do exist within isolates of the same species from different geographical locations. Other cross combinations with female Iceland game-

tophytes and the female Canadian with male Norway cross combinations produced small or poor developed sporophytes, indicated by a missing midrib or no hapteron formation. Kamiya et al. (1998) demonstrated in the red alga *Caloglossa leprieurii* (Montagne) J. Agardh that the ability to hybridize correlates with genetic, rather than geographic, distance. They suggested that breeding incompatibility, as demonstrated in the laboratory, functions as a real barrier to mixing between different gene pools in nature. This contrasts with our hybridization results between different geographically distant isolates of *A. esculenta*, where the ability to hybridize is related neither to geographical distance nor to genetic distance. It is possible that the Icelandic population is genetically isolated and in a process of reproductive isolation, or the poor hybridization results from Icelandic plants might be caused by ecotypic variation, such as different temperature tolerance of the gametophyte and/or sporophyte and temperature responses of growth (see Breeman 1988). The summer isotherm at the sample locations is different: 5–10° C for Iceland versus 10–15° C for Ireland, Norway, and Canada (Lüning 1990). Fertilization and zygote and young sporophyte development in all crosses was induced at 10° C, which is the upper summer isotherm boundary for Iceland. This relatively high temperature for crosses with Iceland gametophytes may be inhibitory, resulting in poor hybridization or no progeny in these crosses. Influences of different temperatures on fertility success in *Laminaria* have been shown in a study by tom Dieck and de Oliveira (1993). It is, however, possible that local and genetically fixed temperature ecotypes have evolved in *A. esculenta*, resulting in reproductive isolated populations.

*Alaria esculenta* isolates from Canada and Iceland showed the most sequence divergence, compared with other *A. esculenta* isolates examined (Fig. 1 and Table 5). Bhattacharya et al. (1990) and Coyer et al. (1997) found distinguishable biogeographic popula-

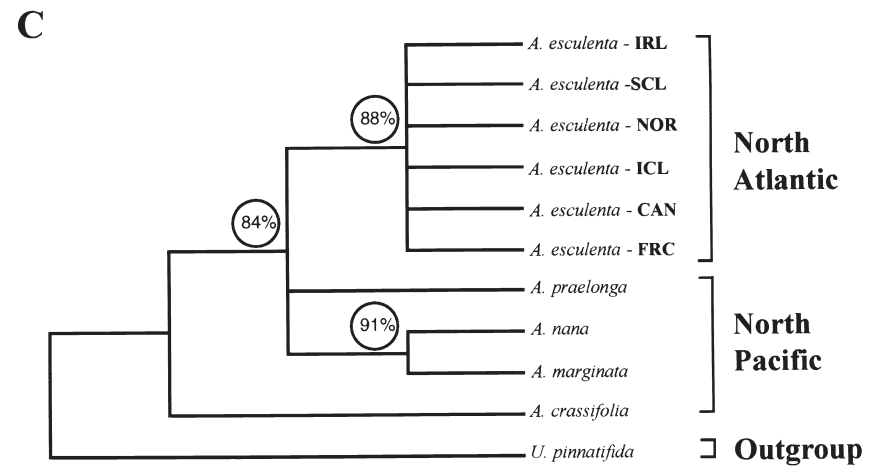
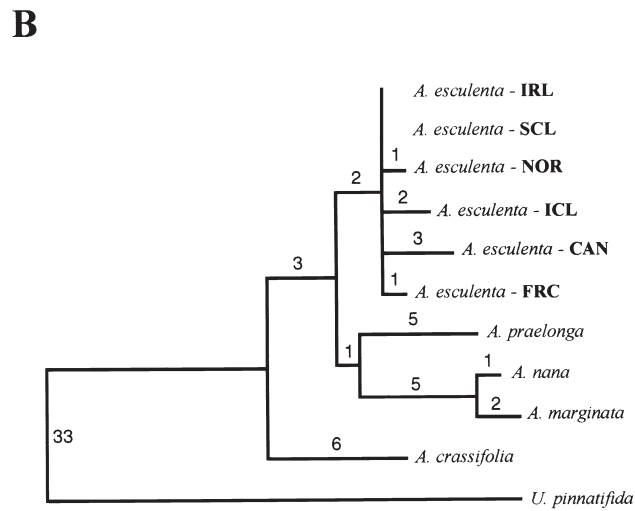
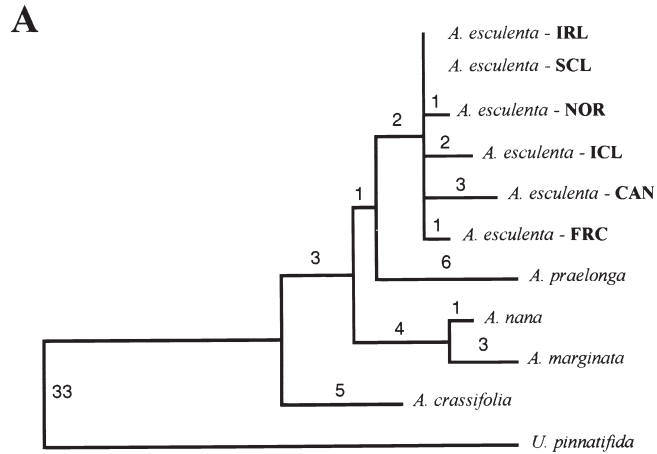


FIG. 1. Unweighted parsimony analysis of Rubisco spacer sequences among *Alaria* species with *Undaria pinnatifida* as outgroup and the 10-bp gap treated as one single evolutionary event. (A, B) Two MPTs obtained by exhaustive search ( $L = 62$  steps,  $CI = 0.935$ ,  $RC = 0.757$ ) in which branch lengths are proportional to the number of changes. Numbers above the branches indicate the number of base changes. (C) Strict consensus tree for all species based on the two MPTs; numbers in circles are bootstrap values (1000 replicates).

tions along the northeast Pacific coast of the kelps *Costaria costata* (C. Agardh) Saunders and *Postelsia palmaeformis* Ruprecht, respectively, and demonstrated decreasing genetic relatedness with increasing distance between populations using RFLPs, M13 fingerprinting, and RAPDs, which is consistent with our results.

*Interspecific relationships.* *Alaria esculenta* from the Atlantic does hybridize with some Pacific *Alaria* species (Table 4), indicating that there is a close relationship between *Alaria* species throughout the Northern Hemisphere, although some fertility barriers are apparent. Similarly close relationships and fertility barriers have been found in other Atlantic and Pacific kelp species (Bolton et al. 1983, Lewis et al. 1986, tom Dieck and de Oliveira 1993, tom Dieck 1993). A biological species concept in kelps is therefore not particularly practical, and may reflect their relatively recent evolution (Druehl and Saunders 1992).

Hybridization among Pacific *Alaria* species showed that they hybridize in many cases. By contrast, *A. nana* × *A. marginata* crosses did not produce progeny, which is contrary to the observations of Widdowson (1971b), who reported plants in the wild that he considered hybrids between *A. nana* and *A. marginata*. Figure 1 and Table 5 show, however, that the *A. nana* and *A. marginata* strains isolated and characterized by us are closely related, supported by a high BP value (91%) and a low amount of sequence divergence (0.38%), which is in discrepancy with our own hybridization results. Druehl (1990) showed that environmental factors caused distinctive species morphotypes in *A. nana*, *A. marginata*, and *A. tenuifolia*. Using restriction enzyme digestion of the small subunit rDNA and intergeneric spacers, he found three restriction map variants indicating separate, genetically isolated populations of the three species, which also contradicts the findings of Widdowson (1971b). Druehl and Saunders (1992) concluded that there is no consistent relationship between morphological and rDNA variation in Pacific species of *Alaria*. After sequencing the entire 18S ribosomal gene for *A. nana* and *A. marginata* they found a nucleotide divergence of only 0.05%, indicating virtually negligible genetic divergence. These observations and our results demonstrate that fertility barriers, which in most cases indicate complete reproductive isolation, may arise without affecting genetic divergence in the particular genes under investigation (Hillis 1987).

Remarkably, the interspecific sequence divergence between *A. nana* and *A. marginata* (0.38%) is smaller than the intraspecific sequence divergence between *A. esculenta* isolates from Iceland and Canada (0.64%). In this case, there is more variation within a species than between two species of the same genus, which casts doubt on the morphological and biological species concepts employed in *Alaria* and the usefulness of hybridization studies in assessing species-level differences (see Harrison 1991, Guiry 1992, Manhart and McCourt 1992, Medlin et al. 1995). Moreover, the shar-

ing of a single common sex pheromone, lamoxirene, in the order Laminariales facilitates interfertility (Müller et al. 1985, Yarish et al. 1990); however, intergeneric cross combinations of *U. pinnatifida* with other *Alaria* species were not successful. Consequently, hybridization studies are able to show species relatedness but they should always be used in combination with other methods to assess species-level differences, particularly in brown algae.

The low level of sequence divergence suggests that *A. nana* and *A. marginata* have diverged relatively recently. Conflict between speciation based on morphological or molecular characteristics result from different rates at which molecular and morphological changes accumulate (Medlin et al. 1995). Consequently, the use of a morphological and biological species concept to separate *Alaria* species is not sufficient and does not fully reflect their phylogenetic relationships.

*Alaria evolution and divergence.* Analyses of the Rubisco spacer sequences suggest that the genus *Alaria* has evolved relatively recently, given the relatively small distances between species, easy alignment, and the absence of saturation. The ability of the Atlantic *A. esculenta* to hybridize with several Pacific *Alaria* species indicates a close relationship between the species in the two oceans and is further support for the original hypothesis of Stam et al. (1988), that species of Laminariales diverged from a common ancestor 15–19 Ma ago, most probably in the north Pacific, and then invaded the North Atlantic after inundation of the Bering Land Bridge ca. 3.5 Ma ago. It is possible that *Alaria* species with  $n = 14$  chromosomes (see Table 1) diverged first from a common ancestor of the genus *Alaria*, representing the oldest assemblage of *Alaria* species during the Miocene. This relative long period of existence of the  $n = 14$  assemblage may have resulted in strict reproductive isolated species, hence the poor crossability results (Table 4). Extensive radiation in North Pacific Laminariales took place, probably during the mid-Miocene steepening of the temperature gradient between high and low latitudes (Stam et al. 1988). This major cooling step in the Tertiary leading to glaciation of the higher latitudes was perhaps the driving speciation force in the Laminariales (Lüning 1990). Coupled with plate tectonics and sea-level fluctuation in the North Pacific, the climatic disturbance could provide the ecological opportunity in which polyploids (amphiploids) might exploit their inherent genetic advantages (Grant 1981). Climatic changes in general bring about changes in the distribution of the species, altering genetic isolation; new contacts are made, natural hybridization occurs, and hybrid polyploids are formed (Grant 1981). Lewis (1996) suggested that the evolution of most taxa of brown algae was accompanied by polyploidy. It is therefore possible that  $n = 22$ – $28$  *Alaria* species are polyploid entities and diverged from hybrids of  $n = 14$  species and resulted in several new species during the mid-Miocene cooling period. Possible donors for

the polyploid hybrids might be *A. tenuifolia* and *A. marginata*, because of the ability of these donors to hybridize with the postulated polyploid *Alaria* species and between themselves (Table 4). Not until the opening of the Bering Land Bridge were *Alaria* species and the Laminariales in general able to invade the Atlantic Ocean. Because polyploids often exhibit superior vigor, homeostatic buffering, and environmental adaptability compared with their diploid partners, they are likely to be the most suitable candidates for an invasion of the Atlantic and were well able to adapt to their new environment (Grant 1981). Most likely candidates to invade the Atlantic were *A. praelonga*, *A. crassifolia*, and *A. esculenta*, of which only *A. esculenta* has apparently done so. The average sequence divergence between *A. praelonga* and the other *A. esculenta* strains is 1.04%. Setting a molecular clock can provide an insight in *Alaria* divergence. If we apply a chloroplast-DNA divergence rate of 0.3% per 1 Ma (Saunders and Druehl 1992), *A. praelonga* and *A. esculenta* have diverged 3.47 Ma ago, which coincides almost exactly with the opening of the Bering Land Bridge 3.5 Ma ago. The maximum sequence divergence in the *Alaria* species used in this study is 1.93%, which roughly equals 6 Ma. Such data are speculative; however, they do provide an insight into the aspects of *Alaria* evolution. In conjunction with the phylogenetic analyses, the hybridization experiments, and chromosome numbers, our findings lead us to postulate that radiation and hybridization led to a new polyploid species assemblage in the mid-Miocene from which *A. esculenta* successfully invaded the Atlantic Ocean after inundation of the Bering Land Bridge. It is possible that other *Alaria* species may have invaded the Atlantic but did not survive the severe Pleistocene glacial conditions.

Maximum sequence divergence between *A. esculenta* from the western and eastern Atlantic is 0.64% (ca. 2.0 Ma). Although speculative, it suggests that *A. esculenta* must have survived the severe climatic changes during the Pleistocene glacial–interglacial cycles (Lüning 1990). During the last Ice Age 18,000 years ago, the ice sheet reached as far as Massachusetts in the western Atlantic and to southwestern Britain and northern France, which coincides with the southern lethal limit for this alga today (Munda and Lüning 1977, CLIMAP 1998), although temperatures during the last glaciation were 10° C lower than today (Lüning 1990). We hypothesize that *A. esculenta* most probably survived at the western Atlantic as well as at the eastern Atlantic coast and could recolonize the coasts as soon as the ice retreated, as indicated by the low level (<0.51%) of sequence divergence between the west-coast and east-coast *A. esculenta* isolates (Table 5).

In conclusion, analyses of the Rubisco spacer sequences are useful for species separation in *Alaria*, but not at the population level. This is in agreement with other brown algae studies (Stache-Crain et al. 1997, Siemer et al. 1998). In tandem with hybridiza-

tion experiments, it provides us with a powerful tool for phylogenetic studies, although more rigorous sampling, particularly in the Pacific, is necessary to provide a more complete assessment of speciation in the genus *Alaria*. Rubisco spacer sequences of and hybridization experiments with the putative *A. esculenta* from the Pacific could help to resolve some important question about radiation and invasion of the Atlantic Ocean in the geological past. A recommended combination with sequences of the intertranscribed spacers of the ribosomal DNA (Siemer et al. 1998) might be preferred.

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