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Phase II: Strain hybridisation field experiments and genetic fingerprinting of the edible brown seaweed *Alaria esculenta*.

By

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ABSTRACT

Under phase II of the Marine Research Measure several field trials were performed with five biogeographical dispersed North Atlantic strains and their hybrids of the edible brown alga *Alaria esculenta* at Ard Bay, Carna, Co. Galway. The weight, length, width, biomass per meter rope, growth rate and protein level were measured.

The fastest-growing crosses were produced with female Norway gametophytes together with male gametophytes of other strains. The Canadian selfcross produced most biomass of over 45 kg per meter rope. The female Iceland x male Ireland crossing produced the second highest biomass figure of 13.75 kg wet weight per meter rope, while the Irish self cross using a strain from the Aran Islands produced 7.4 kg wet weight per meter.

The Canadian selfcross expressed the highest protein level followed by the female Newfoundland x male Norway hybrid. These strains and hybrids are well suited to be included in a protein rich macro-herbivore diet. The Irish native strain showed a lower protein level of 8% of the wet weight.

Genetic fingerprinting using RFLPs did not show any genetic differences amongst the strains in respect of the DNA examined. A detailed sequencing study on the Rubisco spacer region showed a negligible 3 bp difference between the Irish and Canadian strains

In conclusion, the Canadian strain or hybrids derived from female Canada gametophytes or female Iceland gametophytes produce more biomass per meter rope and grow larger and wider in size compared to the native Irish strain. They Canadian strain also showed the highest protein values in the field trials and hence are most suited to be applied in Irish aquaculture of the brown seaweed *Alaria esculenta*.

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1. INTRODUCTION

The brown alga *Alaria esculenta* (Linneaus) Greville, (which literally means 'edible wings'), is a large brown seaweed belonging to the family *Alariaceae* and the order Laminariales (Kelp). It is perennial seaweed that grows in exposed areas near or below the low-water mark. Growth is restricted to regions bounded by the 16º C summer isotherm and the species may thus be found all around the Irish coast. It is rich in sugars, iodine and vitamins and can be used for a variety of purposes from human consumption to fodder and cosmetic products. It also has potential as a foodstuff in aquaculture for herbivorous molluscs such as abalone (Mai *et al.*, 1996). Besides *A. esculenta* there are said to be 13 other *Alaria* species in the Atlantic and Pacific Oceans (Madlener, 1977; Widdowson, 1970; Munda & Lüning, 1977; Kain & Dawes, 1987).

Young *A. esculenta* (the only native species in Ireland) can be used as a substitute of *Undaria pinnatifida* (Harvey) Suringar (Wakame) a very popular seaweed in Asian countries with numerous applications and a yield over 500,000 t fresh weight per annum (Nisizawa *et al* 1987; Indergaard & Minsaas, 1991; Druehl, 1988; Yamanaka & Akiyama, 1993). *Alaria esculenta* was used in the past in both Scotland and Ireland for human consumption and fodder. It was also gathered and spread on infertile land and used as fertiliser (Newton, 1931; Guiry and Hession, 1996). Experiments have shown that within 3 months a harvest of 9 t per hectare is possible. *Alaria esculenta* is a fast-growing seaweed and can grow at up to 10 cm a day. The commercial potential is thus considerable. Finding a suitable strain of *A. esculenta* will provide a huge potential for cultivation of a new aquaculture species, with the potential for job creation from the use of native natural resources in farming and aquaculture (Kain & Dawes, 1987; Kain, Holt & Dawes, 1990).

From the scientific literature it is known that members of the *Laminariaceae* are able to cross inter- and intraspecifically (tom Dieck, 1992; Egan *et al.*, 1990). Bolton *et al.* (1983) produced hybrids between Atlantic and Pacific *Laminaria* species with different growth rates and other morphological characteristics. They concluded that the ability to produce these characters appeared to be genetically determined. Lüning *et al.*(1978) also produced crosses between *Laminaria* species from both sides of the Atlantic. These later authors suggested that blade width in hybrid offspring could be sex linked to male parents. The same observation was made by Egan *et al.* (1990).

Under phase I of the Marine Research Measure (IR.95.MR.011), strains of *A. esculenta* were collected from a number of sites in the North Atlantic and established in culture at N.U.I., Galway. Laboratory hybridisation experiments at the Martin Ryan Institute have shown statistical differences in growth rate performances between Irish, Norwegian, Canadian and Icelandic hybrid strains of North Atlantic *A. esculenta* species. Ireland x Iceland crosses showed the slowest growth rates and least biomass, whereas Ireland x Norway crosses produced the fastest growth rates. In addition, and very significantly, all crosses with Icelandic

A. esculenta strains produced poorly developed haptera which, of course, make them less suitable for rope cultivation.

Differences could be a result of ecotype variation or genetic differences in the *A. esculenta* strains. However, these results are from laboratory experiments performed under controlled conditions and do not reflect field conditions. It is therefore necessary to test the different hybrid strains in the field in order to find the most suitable hybrid for cultivation in Ireland.

Genetic fingerprinting by means of Restriction Fragment Length Polymorphisms (RFLPs) and sequencing will be carried out to detect genetic differences among the different strains. RFLPs are a commonly-used molecular tool to distinguish morphologically similar populations (Hillis *et al*., 1996). They have also been used in numerous algal studies (e.g., Bhattacharya and Druehl, 1989; Rice and Bird, 1990; Bhattacharya *et al*., 1991; Lehman and Manhart, 1997).
The nuclear ribosomal DNA internal transcribed spacer regions (ITS1 and ITS2) have 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 plastid encoded Rubisco spacer separates the large and the small subunits of the ribulose-1,5-bisphosphate 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).

Recently, *A. esculenta* has become of interest as a foodstuff in aquaculture for herbivorous molluscs, urchins shrimp and fish (Yone *et al*., 1986; Moss, 1994; Stuart & Brown, 1994; Mai *et al.*, 1996; Nakagawa *et al*., 1997). Biochemical constituents, growth rates and biomass will therefore be very important features of the different strains and their hybrids.

For example, in China, extensive studies on hybridization and inbreeding has led to stable and improved strains of *Laminaria japonica* Areschoug. Large-scale production experiments showed that the new varieties yield up to 40% more biomass and an iodine content 20-58% higher than the control plants. These new varieties are introduced to *Laminaria* farms and grown in large quantities in North China (Wu, 1998).

One of the applications for *A. esculenta* is the inclusion in macroherbivore or fish feed. Intensive production of abalone, urchins or fish relies upon many factors, one of which is a nutritionally complete, cost-effective, environmentally friendly, formulated feed (Hardy, 1994). These diets must be relatively high in protein (40-50%), which is one of the most expensive components (Haard, 1994; Elangovan and Shim, 1997). Other essential main components in feed are polyunsaturated fatty acids of the n-3 and n-6 series, minerals and vitamins, which are relatively expensive (Ikenoue & Kafuku, 1992; Jobling, 1995). The biochemical composition of marine macroalgae has received relatively little attention (Wahbeh, 1997). In general, the protein proportion in algae accounts for as much as 25% of the dry weight (Madlener, 1977) but may be as little as 2-3%. *Alaria*

possesses excellent binding proportions and could replace conventional binding agents like starch and cellulose. Inclusion of *Alaria* in traditional diets to partially replace fish meal, fish oils, vitamins and trace minerals could lower the overall costs considerably, and create a more organic produced end-product. To find the most suitable strain to be used in aquaculture diets protein levels in the different strains and their hybrids need to be measured systematically.

2. OBJECTIVES

The overall objective of the project is to find the most suitable strain of the edible brown seaweed *Alaria esculenta* for field cultivation by selection and hybridisation and to produce a scientific method for strain selection and hybridisation in kelps.
There were three main objectives for this study:

(1) Establish growth performances of all hybrid strains and identify the most suitable hybrid for cultivation purposes. Develop a scientific method for strain selection and hybridisation in kelps.

(2) Detection of genetic differences between strains with RFLPs and sequencing.

(3) Measure protein levels in the different hybrid strains in order to screen for the most suitable strain to be used for aquaculture diets.

3. MATERIALS AND METHODS

3.1 Hybridization techniques and rope seeding

Hybrid crosses were made using individual male and female gametophyte seed stocks (Fig. 1) isolated from spores released by parent sporophytes previously described in final report IR.95.MR.011 Phase I Strain selection in the edible brown seaweed *Alaria esculenta:* Genetic fingerprinting and hybridization studies under laboratory conditions.

A total of 5 Atlantic strains listed in Table 1 were used for field trials resulting in 25 hybrid cross combinations. Hybrid cross combinations were made by thoroughly mixing 10 ml of a male gametophyte seed stock with 10 ml of a female gametophyte seed stock using a house hold blender. This mix of male and female gametophyte fragments of different parental plants was then seeded onto fine twine or string. Seeding consisted of smearing or spraying the mixture over the ropes that were tightly wrapped around a small plastic seed collector coil (10 cm length and 4 cm diameter). The coil with seed string was incubated and aerated in small containers at 10° C, 16:8 h L:D (light: dark), 30 µmol Photons$\cdot m^{-2} \cdot s^{-1}$ of white light to initiate the formation of eggs and sperm (Figs. 2 and 3). A total of 25 seed string collectors were made for all possible strain combinations (Table 2).

Table 1. Origin of *Alaria esculenta* species used for DNA extractions, gametophyte cultures and hybridization field experiments 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	Gametophyte culture	Origin	GenBank accession no
Alaria esculenta	IRL[a, b, c]	M & F	Trácht Each, Inis Oírr, Ireland	AF 109795
Alaria esculenta	SLH[b]	M & F	Slea Head, Co. Kerry, Ireland	
Alaria esculenta	CBH[b]	M & F	Corbet Head, Co. Down, Northern Ireland	
Alaria esculenta	BFB[b]	M & F	Belfast Bay, Co. Down, Northern Ireland	
Alaria esculenta	ACH[b]	M & F	Keem Beach, Achill Island, Co. Mayo, Ireland	
Alaria esculenta	MRB[b]	M & F	Mulroy Bay, Co. Donegal, Ireland	
Alaria esculenta	SCL[c]	DNA only	Hind Rock, St Andrews Bay, Scotland	AF 109796
Alaria esculenta	NOR[a, c]	M & F	Ålesund, Norway	AF 109797
Alaria esculenta	ICL[a, c]	M & F	Tjörnes, Iceland	AF 109798
Alaria esculenta	CAN[a, c]	M & F	Halifax, Canada	AF 109799
Alaria esculenta	CAN[a]	M & F	Newfoundland, Canada	
Alaria esculenta	FRC[c]	DNA only	Roscoff, France	AF 109800

[a] Plants used for hybridization field experiments, [b] Plants used for RFLPs,[c] Plants used for sequencing

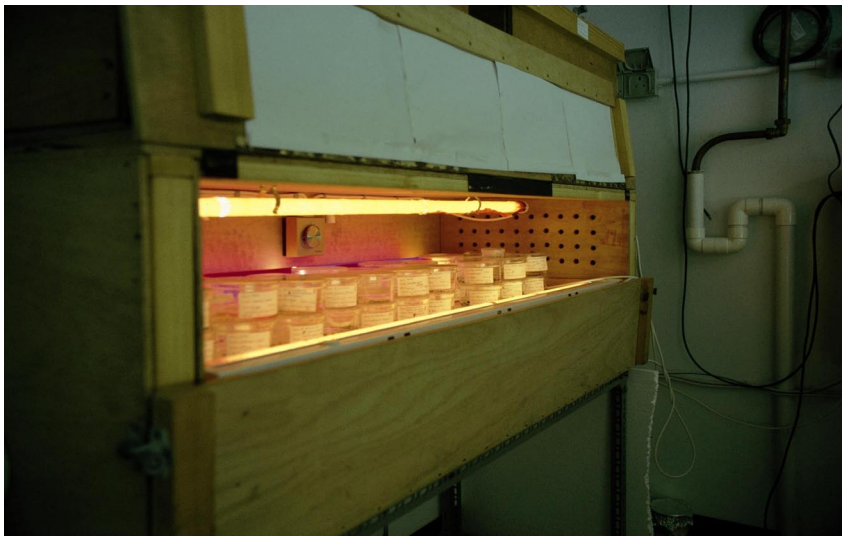


Fig. 1. Red light cabinet with seed stocks of female and male gametophytes of different *A. esculenta* strains and other *Alaria* species.

Fig. 2. Seed string with gametophtes of different strains, incubated and aerated in small containers at 10° C, 16:8 h L:D, 30 µmol Photons·m^{-2}·s^{-1}

Table 2. Different hybrid cross combinations of 5 Atlantic strains of *A. esculenta* listed in Table 1.

Strain number	Hybrid combination
1	Selfcross Newfoundland
2	Selfcross Norway
3	Female Newfoundland X Male Ireland
4	Female Newfoundland X Male Canada
5	Selfcross Ireland
6	Female Norway X Male Newfoundland
7	Selfcross Iceland
8	Female Norway X Male Iceland
9	Female Norway X Male Canada
10	Female Newfoundland X Male Iceland
11	Selfcross Canada
12	Female Norway X Male Ireland
13	Female Ireland X Male Iceland
14	Female Canada X Male Newfoundland
15	Female Canada X Male Ireland
16	Female Canada X Male Iceland
17	Female Canada X Male Norway
18	Female Ireland X Male Canada
19	Female Ireland X Male Newfoundland
20	Female Ireland X Male Norway
21	Female Newfoundland X Male Norway
22	Female Iceland X Male Ireland
23	Female Iceland X Male Newfoundland
24	Female Ireland X Male Norway
25	Female Iceland X Male Canada

Fig. 3. Seed string removed from coil. Gametophytes as brown tufts are clearly visible.

Seed strings were ready to use after 2 weeks when gametophytes had attached properly to the string and started to form small plantlets (Fig. 4). Seed strings were kept at 10 ° C, 16:8 h L:D, 30 µmol Photons·m^{-2}·s^{-1} for 3 months before they were out-planted in autumn. Different types of seed string have been used of which polyamide string, and soft fibre breaded nylon twine (2-8mm) were best for gametophyte attachment.

Fig. 4. Detail of nylon breaded twine with gametophytes of which some showed small 1-2 mm plantlets

Von Stosch's medium (quarter strength and modified as given in Guiry and Cunningham, 1984) was used as the culture medium and contained equal amount of seven nutrients (Table 3). The culture medium was changed monthly.

Table 3. Ingredients of Von Stosch's medium (Guiry & Cunningham, 1984).

NUTRIENT	CONCENTRATION
$FeSO_4.7H_2O$	0.278 g/L
Na_2 β-Glycerophosphate	5.36 g/L
$NaNO_3$	42.52 g/L
Na_2-EDTA.$2H_2O$	3.72 g/L
Vitamin B12	0.4 g/L
Thiamine (Aneurine-HCL)	0.2 g/L
$MnCL_2.4H_2O$	1.96 g/L
Biotin	0.1 g/L

3.2 Site location and out-planting

Two sites were selected for out-planting of seed strings and for out-growing the *A. esculenta* hybrids and self crosses.

Site 1 was at an existing commercial seaweed farm of the company Sliogeisc Mhic Dara in Ard Bay, Carna, Co. Galway. The farm is located just opposite Mweenish Island. The site was relatively sheltered at a depth between 5 and 10 m and benefited of a good water exchange. Three 25 m polypropylene long-lines were incorporated into the existing seaweed farm structure (Fig. 5). A length of 4 m seeded string tied into four 1-m strings

Fig. 5. Seaweed farm, Sliogeisc Mhic Dara at Ard Bay

of every cross combination (Table 2) were wrapped around the polypropylene long-lines (Fig. 6) and fastened with cable ties and labeled with plant labeling tags (Fig. 8). In this way each strain took up about 1 m of long-line. Seed strings of the 25 hybrid cross combinations were outplanted in November 1998, May 1999 and November 1999. The long lines were adjusted to 1-1.5 m under the surface.

Fig. 6. Wrapping the seed string around the poly propylene long line

Site 2 was at New Quay, Co Clare in cooperation with the company Catron Point/Red Bank Shellfish. This site was at 6-8 m depth with strong tidal currents and excellent water exchange. At this site only the native *A. esculenta* strain was used as a control for the Ard Bay site. For the purpose of cultivation a simple long-line structure was used (Fig 7). To seed the 25-m polypropylene long-line a 60-cm seed coil (diameter 10 cm) with 25-m string was seeded in the laboratory. The seeded string was out-planted and wrapped around the long-line in November 1999. Due to strong currents the long-line was placed 0.5 to 1m below the surface. During regular checks it appeared that plants closer to the mouth of the tidal inlet grew larger and wider. For that reason the long-line was sampled in 3 parts, i.e., the seaward, middle and inner site.

3.3 Harvest and length and weight measurements

Ropes were regularly checked using a small boat or kayak. Ropes were harvested in April 1999 for the rope seeded in November 1998 and in May 2000 for the rope seeded in November 1999. The rope seeded in May 1999 did not yield any progeny. Ropes were harvested by pulling off plants or cutting off plants with a sharp knife. Wet weight in kilograms per meter rope was determined of the different strains in the Laboratory using scales and the length and width of the plants were measured using a stainless steel ruler.

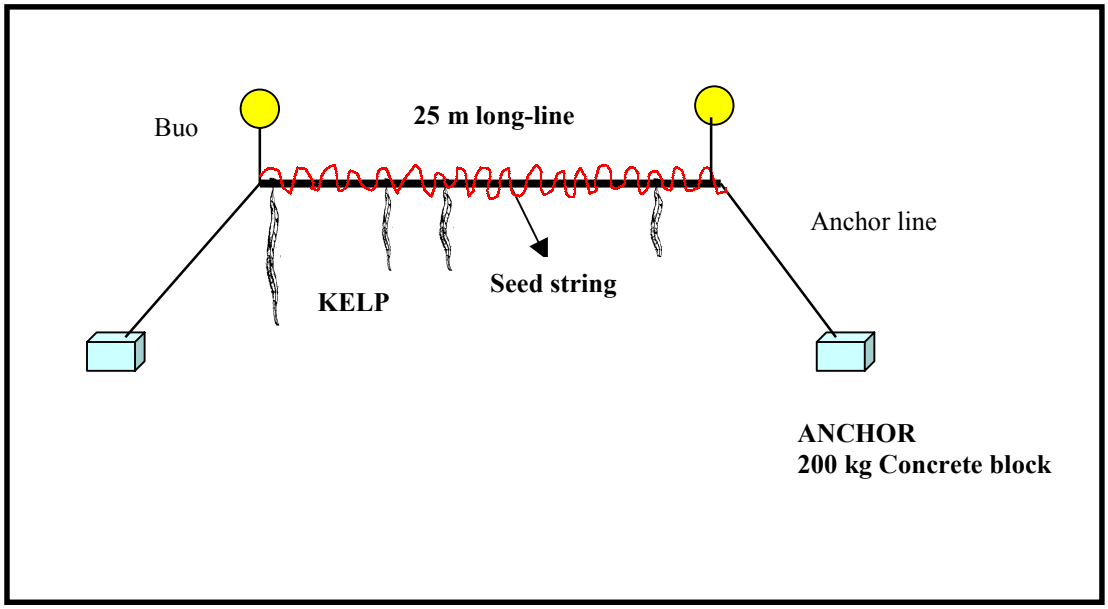


Fig. 7. A simple long-line system used for native *Alaria esculenta* cultivation at New Quay, Co. Clare.

Fig. 8. Different cross combinations per meter long line labelled with plant labels and fastened with tie cables.

Fig. 9. Small plants are measured and marked for subsequent sampling.

3.4 Growth rates

The growth rates of the strains were measured using the punch-hole method (Parke, 1948). A small hole is punched at the base of the plant with a cork borer (Fig. 10). As new tissue is formed at the base of the stipe the hole will travel upwards. By measuring the distance the hole has travelled upwards devided by the days, a growth rate per day can be calculated. For the plants at New Quey growth was measured from mid-April to mid-May. For Ard Bay growth rates of strains and hybrids were measured from early May to early June. An average of 5 plants per strain were marked with large white cable ties for growth rate measurement; however, it was very difficulty to trace back plants, as plants were lost or simply disappeared.

3.5 Protein measurements

Proteins were measured on fresh or frozen algae of all strains and hybrid crosses according to the method given by Lowry (1957). Measurements were done in duplicate. Results are given in percentages of fresh weight. Proteins were measured in April 2000 and again in May 2000 for the plants from the site in New Quey. For Ard Bay proteins were measured in May 2000 and again in June 2000.
Proteins were extracted as follows : An aliquot of known fresh weight of tissue (2 g blotted dry tissue) and 1 g of fine sand was placed in a mortar. Five mL of a 0.3 M phosphate buffer (pH 6.8-7.0) was added and grind for 10 min. Twenty mL of distilled water was added and ground again briefly. The tissue extract was pored into two 15 ml centrifuge tubes and centrifuged at high speed (9000 rpm) for 20 min. The supernatant was decanted and diluted with the same volume of distilled water (diluted 1:1). Absorbance was measured against distilled water with a spectroFig.meter at 750 nm. Two standard curves were used for high (> 0.5 A) and low (<0.5 A) values. Proteins were measured twice for each sample.

Fig. 10. The punch-hole technique. A small hole is punched with a cork borer.

3.6 DNA extraction, purification and double stranded PCR

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, 37º C) with 10 µL RNase (Boehringer Mannheim, Germany). DNA concentrations were measured in a spectroFig.meter at 260 nm according to standard methods (Sambrook *et al*. 1989).

The intertranscribed spacers ITS1 and ITS2 of the ribosomal DNA were PCR amplified in an Omn-E thermal cycler (Hybaid Ltd., U. K.) for the purpose of genetic fingerprinting with the RFLP method to compare Irish strains listed in Table 1 for genetic differences. Primer pair P1 and G4 were used to amplify the region in the ribosomal cistron from position 1542 in the 18S across the ITS1, the 5.8S, and ITS2 to position 42 in the 26S (Saunders and Druehl, 1993), using the following temperature profile: a denaturation step of 95 °C for 3 min followed by 30 cycles of 1 min 95 °C, 2 min 50 °C, and 2 min 72 °C, and ending with one extension step of 72 °C for 5 min.

The RFLP method did not detect genetic differences amongst Irish *A. esculenta* strains in respect of the DNA examined. Therefore the more powerful sequencing

method was applied on another part of the DNA of several *A. esculenta* strains of the North Atlantic listed in Table 1. For this purpose the Rubisco spacer region of the chloroplast DNA was PCR amplified using primer pair BLSrbcL1124F and BLSrbcS153R (Siemer *et al*., 1998), using the following temperature profile: 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 and ended with one extension step of 72° C for 5 min.

The reaction volume for ITS1and 2 and the Rubisco spacer was 100 µL and 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 10x reaction buffer, 6 µL 25 mM $MgCL_2$ and 2.5 units of Taq (Sigma). 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).

Isolation of PCR products from amplification reactions was carried out using the High Pure PCR Product Purification Kit of Boehringer Mannheim according to the manufacturer's protocol.

3.7 Genetic fingerprinting (RFLP method)

Genetic fingerprinting of the *A. esculenta* isolates was performed with the RFLP technique using restriction endonucleases according to the manufacturer's protocol (Boeringer Mannheim). The following restriction endonucleases were applied: PST I, RSA I, MSP I, HAE III, CFO I, CLA I, SCA I, BAM HI, ECO RV, DRA I, TAQ I, XBA I, ALU I, ECO RI, and BCL I.

Restriction fragments were run on 3% TAE agarose gels at 20 V for 20 h and stained afterwards with EtBr. The stained restriction fragments were visualised by UV-fluorescence, captured with a video camera and printed with a thermal printer. Fragment sizes were determined according to standard methods (Hillis *et al*., 1996). All digestions were performed twice to assure accurate calculation of size of the fragments. Presence and absence of restriction fragments were scored manually from the thermal prints and a presence/absence matrix was constructed for all individuals.

3.8 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) and final alignments were made by eye. All sequences were submitted to GenBank. Table 1 shows the GenBank accession numbers. A distance matrix was generated using the computer program MEGA (Kumar *et al*. 1993). Pair-wise distances were calculated using the Tajima-Nei distance (Tajima & Nei 1984), which gives a better estimate in cases where nucleotide frequencies deviate from 25%.

4. RESULTS

4.1 Weight, length and width measurements

4.1.1 Ard Bay Site

First seeded long-line of November 1998

The first seed strings were seeded in the laboratory during May 1998 and out-planted and wrapped around the long-line in November of the same year (Figs. 6 and 8). An early examination of the long-line in January revealed no growth of *A. esculenta.* The long-line appeared to be covered with a thin layer of brownish deposit. A follow up check at the end of March showed spectaculair growth (Figs. 12 and 13) of almost all strains with plants reaching 2.5 m length and 35 cm width. Unfortunatelly due too strong gales (Force 11) in the months November and December, many of the labels (Fig. 8) had broken off and numbering had dissappeared. Only the labels of 5 strains had survived and could be recognized as such (Fig. 11).

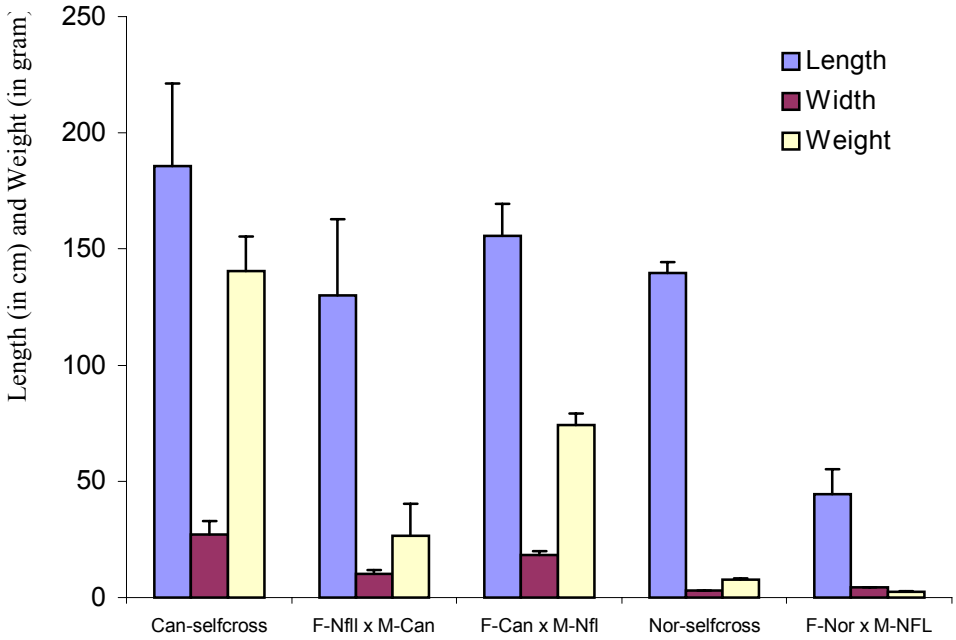


Fig. 11. Length, width and weight measurements of 10 individual plants of 5 strains of the seeded long-line of November 1998.

The measurements show that the strain from Canada (Halifax) consisted of the largest and heaviest plants. Hybrids with the female Newfoundland gametophytes were slighly smaller and lighter. The Norwegian strain showed very long and narrow blades. The female Norway x male Newfoundland cross was very poor in weight and length for all measurements. The total wet weight per meter rope was

determined for the 5 crosses (Table 4). The Canadian selfcross produced most biomass; the female Norway x male Newfoundland cross produced the lowest weight.

Second seeded long-line of May 1999 at the Ard Bay site

A second long-line was seeded in May 1999 to produce a crop in winter 1999 and a second crop in spring 2000. However, the seeded long-line did not produce any viable progeny during autumn and winter 1999 or spring 2000.

Fig. 12 (left) and **Fig.13** (right). Checking long-lines at Ard Bay in March 1999. The long-lines are densly covered with *A. esculenta* strains and hybrids.

Third seeded long-line of November 1999 at the Ard Bay site

A third long-line was seeded in Novemeber 1999, at a time period similar to the first trial. For this field trial no plant labels were used but a simple numbering system from left to right for the place on the long-line. In this way crossings could always be identified. No growth was detected on the long-line in January and February 2000. A check in March however revealed substantial growth of most crosses.

Table 4. Five identifiable crosses from the first out-planted seed strings of November 1998 with their biomass in kg wet weight per meter rope.

Crosses	Wet weight ($kg.m^{-1}$ rope)
Canada selfcross	15.16
Female Newfoundland x male Canada	6.8
Female Canada x male Newfoundland	9.33
Norway selfcross	1.3
Female Norway x male Newfoundland	0.8

The different strains and their hybrids were partially harvested in May. Weight, length and width and wetweight per meter rope were determined and protein levels measured (Figs. 16, 17 and 25). Of every viable strain plants were marked and growth rates were determined over the course of one month (Fig. 22).

All crosses of the first seeded long-line of November 1998 produced off spring again in March 2000. Of these crosses the largest was measured for length, width and weight, protein levels, wet weight per meter rope and growth rate of strain (Figs. 16, 17, 19 and 20). This cross was likely to be the Canadian selfcross due to its position on the long-line, however, fertilization with other strains might have taken place and therefore we cannot state with certainty that the cross measured in the second year was indeed this cross.

Figs. 16 and 17 show that crossings no's 1, 8, 15, 16, 18, 23, 24 and 25 did not produce any viable progeny. The most productive strain appears to be the supposedly selfcross from Canada (Halifax) growing for the second year with 45.70 kg wet weight per metre of rope and plants with lengths of 2.5 m and 20 cm width average. Other high productive strains in terms of biomass are crosses 12, 13 and 22 with 5.23, 6.65 and 13.75 kg wet weight per meter rope respectively.

Fig. 14 (left). Norwegian selfcrosses. Plants are long and thin. Ruler = 45 cm
Fig. 15 (right). Canadian selfcross (left) compared to Irish selfcross (right). Ruler = 45 cm.

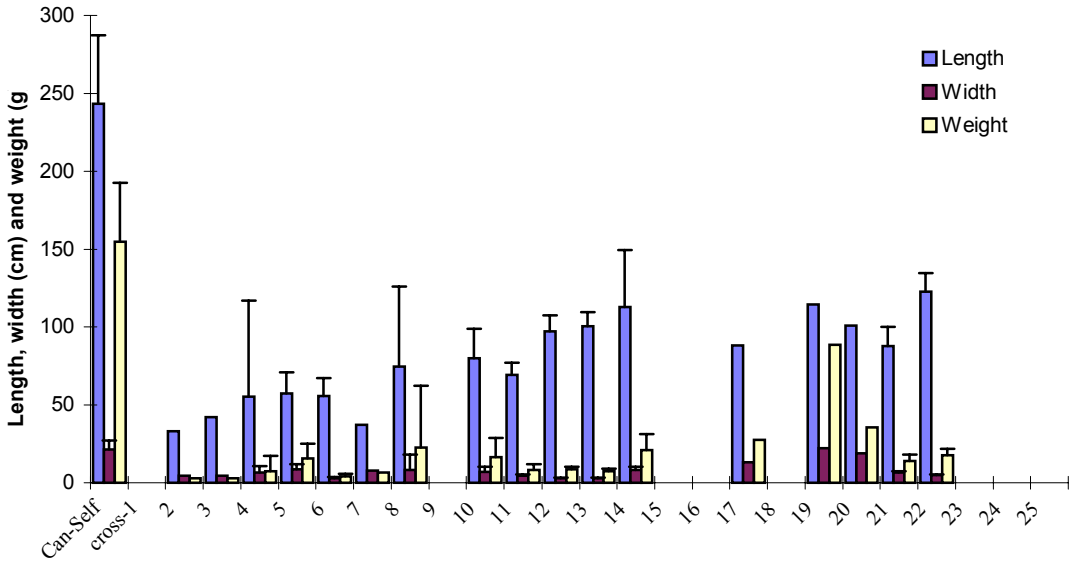


Fig. 16. Length, width and weight (with 95% confidence intervals) of all viable *A. esculenta* strains and their hybrids of the third seeded long-line in November 1999, Ard Bay. Can-Self = Canadian selfcross from 1998 seeded rope harvested in May 2000. Cross 1-25 are strains from the November 1999 seeded long-line (for numbers see table 2).

The poorest strains are the 2nd, 3rd, 7th, 17th and 20th in which the 2nd, 3rd and 7th have the smallest lengths, widths and weights. These crossings produced only a few plants in total.

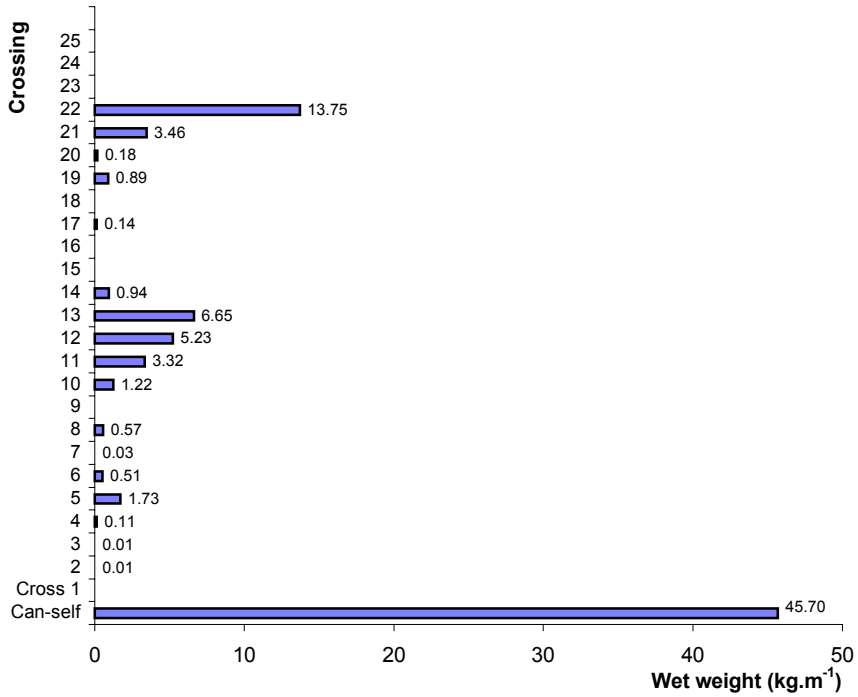


Fig. 17. Total wet weight of *A. esculenta* strains and their hybrids per one meter rope of the third seeded long-line in November 1999, Ard Bay. Can-Self = Canadian selfcross from 1998 seeded rope harvested in May 2000. Cross 1-25 are strains from the November 1999 seeded long-line (for numbers see Table 2).

4.1.2 New Quay site

Seeded long-line of November 1999

At New Quay a 25 m long-line was seeded with seed string prepared from gametophyte seed stock from Inis Oírr, Aran Islands in May 1999 consisting of a native Irish strain only. The seed string was outplanted in November 1999 and checked in January for growth. First growth was detected during a second visit at 22th of March. The long-line was sampled on the 17th of April and 18th of May (Fig. 18).

Weight, length and width and wetweight per meter rope were determined and protein levels measured (Figs. 3, 4 and 8). Plants of the seaward, middle and inner position were marked and growth rates were determined over the course of one month (Fig. 7).

Fig. 18. *Alaria esculenta* growing at the middle site on the long-line at New quey, April 2000

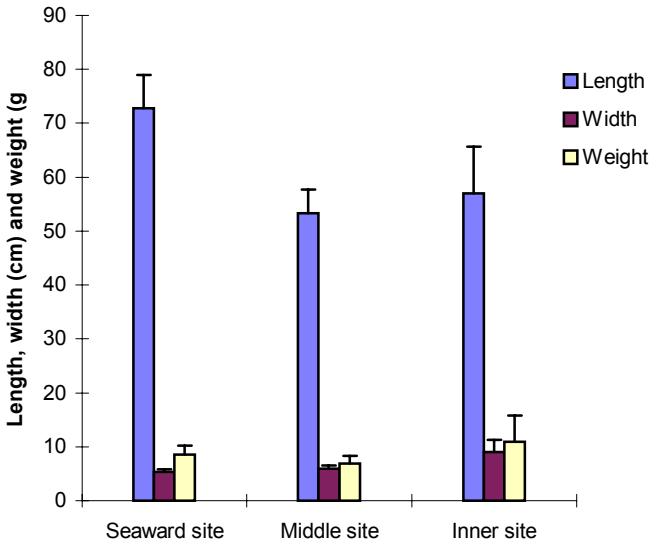


Fig. 19. Length, width and weight (with 95% confidence intervals) of *A. esculenta* plants seeded on a long-line in November 1999at New Quay, Co. Clare and measured on the 18th of April 2000.

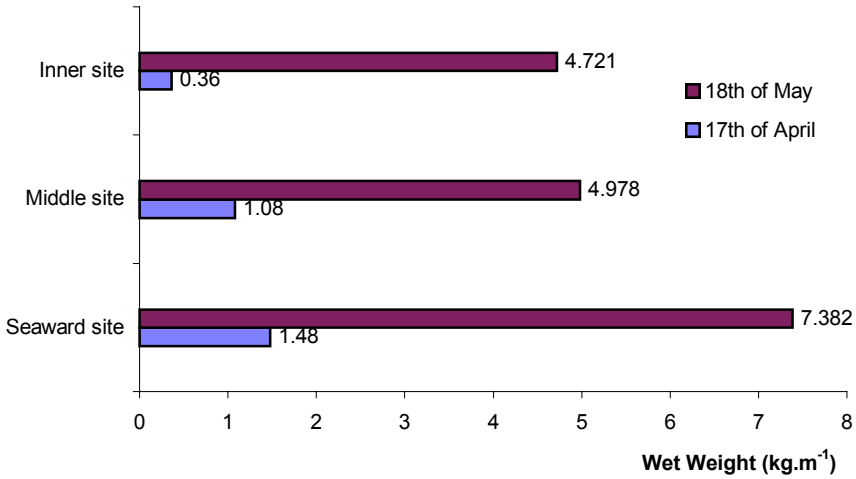


Fig. 20. Total wet weight of *A. esculenta* plants per one meter rope of the seeded long-line in November 1999 at New Quay, Co. Clare sampled in the month April and May 2000.

Figs. 19 and 20 appareantly show that the long-line produces more wet weight of *A. esculenta* per meter rope at the seaward site compared to the middle or the inner site. This trend was observed for April and May although there was a seven time increase in wet weight over the course of one month for all the three sample positions on the long-line. The number of plants growing per meter in April were 135 for the seaward site, 128 for the middle site and 25 for the inner site. In May plants had almost double their size up to 1.5 m (Fig. 14).

4.2 Growth rates

Growth rates were measured over the course of 1 month (Fig. 10). Growth rates of the New Quey and Ard Bay sites are plotted together and are shown in Figure 22.

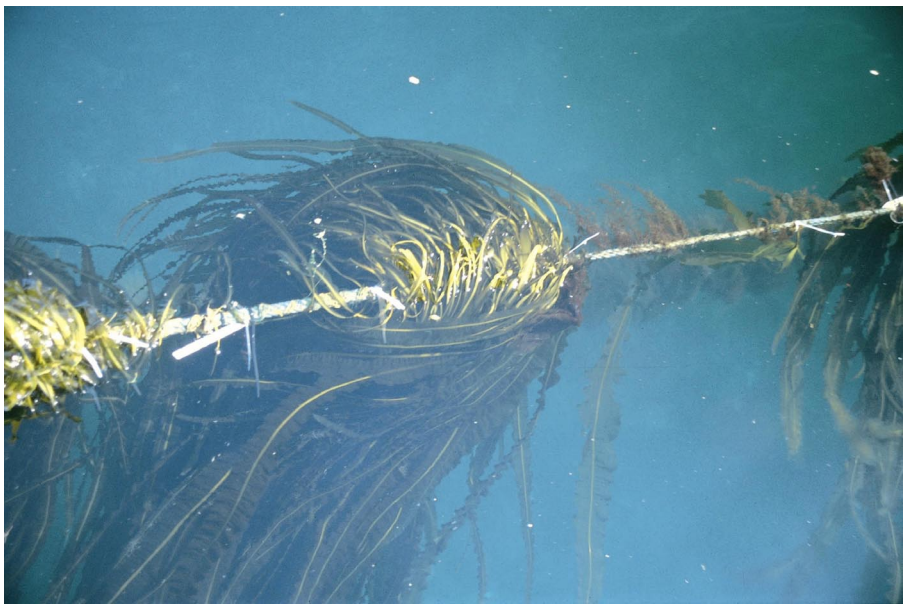


Fig. 21. The third seeded long-line at Ard Bay showing abundant growth and one gap of a Newfound land crossing with no progeny.

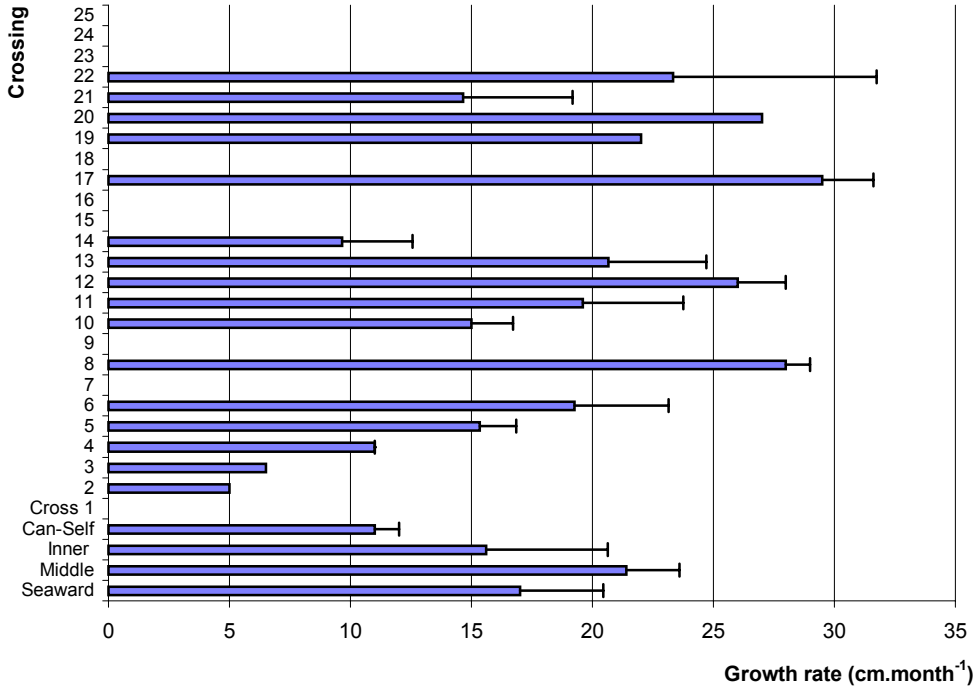


Fig. 22. Mean growth rates with standard deviations of *A. esculenta* strains and hybrids measured over one month at the sites New Quay and Ard Bay.

The growth rates of the native Irish strain at New Quey are similar to the growth rate of cross 5 that is the same Irish strain only grown in Ard Bay (see Fig. 7). It appears that the Middle sample site grows faster than the other sample positions; however, considering the standard deviations, they are all approximately similar.
The growth rates for the for the strains and their hybrids at Ard Bay can be separated in four categories:

- Growth rate > 25 cm.month^{-1}: crossings 8, 12, 17, 20 and 22
- Growth around 20 cm.month^{-1}: crossings 6, 11, 13 and 19
- Growth around 15 cm. month^{-1}: crossings 5, 10 and 21
- Low growth < 10 cm.month^{-1}: crossing 2 and Canadian selfcross

The results show that the fastest growers are crosses with female Norway gametophytes together with male gametophytes from Iceland, Newfoundland and Ireland, the female Canada and male Norway cross and the Female Iceland with male Ireland crosses. Crosses not working or performing poorly are crosses with female Newfoundland gametophytes (see Fig. 15). A comparison of the most remarkable growth forms of four different crossings is shown in Fig. 16. The Canadian x Irish hybrid was 11 times wider than the Norwegian selfcross

Fig. 23. A comparison of growth forms of four different crossings. Outer left plant represents a Norwegian selfcross, second from left represents a female Canadian x male Irish cross, third form left represents an Irish selfcross and outer right represents a Canadian selfcross. Ruler = 45 cm.

4.3 Protein levels

Protein levels are given in percentage wet weight. Levels were measured in duplicate in April and May for the plants at New quey, and in May and June for plants at the Ard Bay site.

4.3.1 New Quay

Protein levels in April are lower compared to the May values. The highest value is measured in samples from the Inner sample location. However, over the course of a month a sevenfold increase was noticed in the seaward sample and a three- to fourfold increase in the other samples (Fig. 24).

Protein levels are lowest in May in all crossings except for crossing 14 and 19, which showed higher levels in May compared to June. The highest levels were measured in crossing 11 and 22, the Canadian selfcross and the female Iceland x male Ireland cross. The protein levels may be separated in three categories (see Fig. 9).

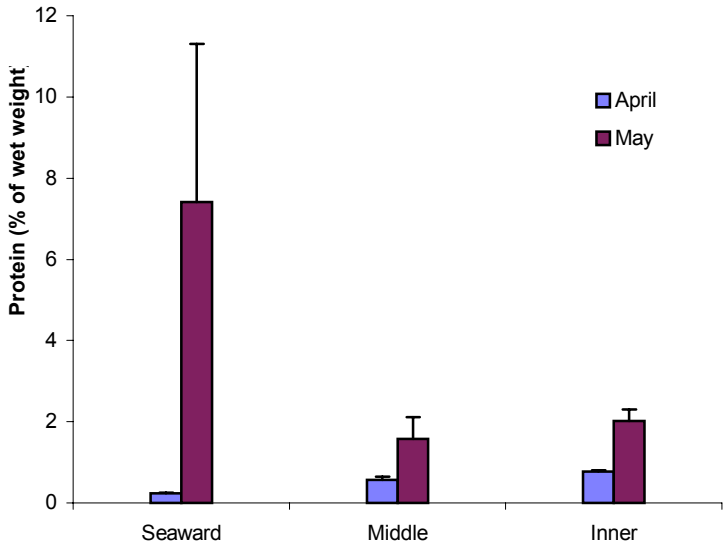


Fig. 24. Protein levels with standard deviations in *A. esculenta* from New Quay taken from three sample locations on the long-line in April and May 2000.

4.3.2 Ard Bay

- High (>15 % protein): crossing 11 and 21
- Medium (~ 10 % protein): crossing 2, 8, 10, 12, 14, 19 and 20
- Low (~ 5%, <5% protein): Canadian selfcross, 4, 5, 13, 17, and 22.

The protein content of strains 3 and 7 were not measured in June due to a lack of sample tissue.

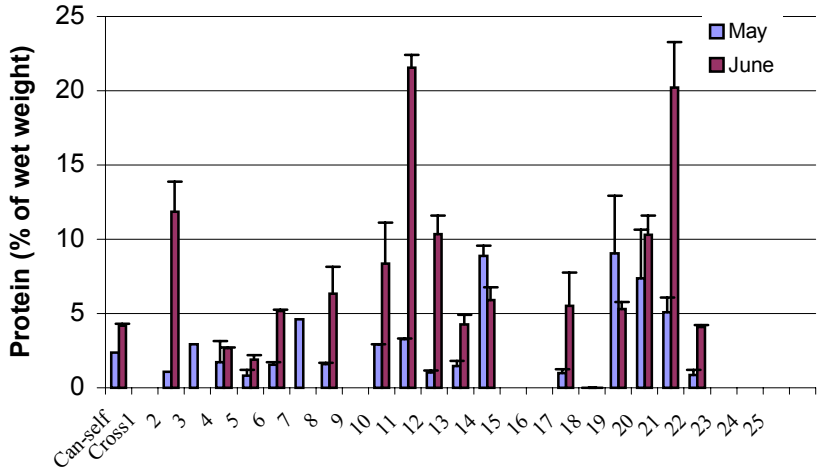


Fig. 25. Protein content with standard deviations of *A. esculenta* in Ard Bay measured in May and June.

A final check of the long-lines in Ard Bay in mid-July showed that most plants were weatered away with sporphylls and part of the midrib remaining. All fertile tissue of the sporophylls had sporulated as noticed by their light brownish appearance, assuring a new crop of *Alaria* in winter 2001; however, it is not known which strain or crossings these will be as gametophytes of all different tested strains can mix freely. The plants checked in July were covered with hydroids and bryozoans and are not useful for any application, see Fig. 26.

Fig. 26. *Alaria esculenta* fronds from early June at the third long-line at Ard Bay showing tattered tips with hydroids and bryozoan growth on the fronds.

4.4 Genetic fingerprinting

PCR amplification of the ITS 1 and ITS 2 region resulted in a single product of 1004 bp. Of the 15 restriction enzymes used, only Rsa I (4 fragments), Msp I (7 fragments), Cfo I (7 fragments), Taq I (4 fragments), Alu I (4 fragments) and Eco RI (2 fragments) proved informative and produced 28 scorable bands. The total number of base pairs recognised by the six informative REs was 116 bp, which is 11.6 % of the amplified spacer region. Comparison of the restriction enzyme digestion patterns of the five tested isolates of *A. esculenta* (Table 1) showed no polymorphisms, resulting in similar patterns (see Fig. 27).

4.5 Sequencing

The Rubisco spacer of each strain was sequenced entirely on both strands (Fig. 28). 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* strain alignments were 815 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 7 sites are variable (0.86 %) of which none are phylogenetically informative. 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). Amongst strains of *A. esculenta* the distance is largest between the western and eastern Atlantic (Table 5).

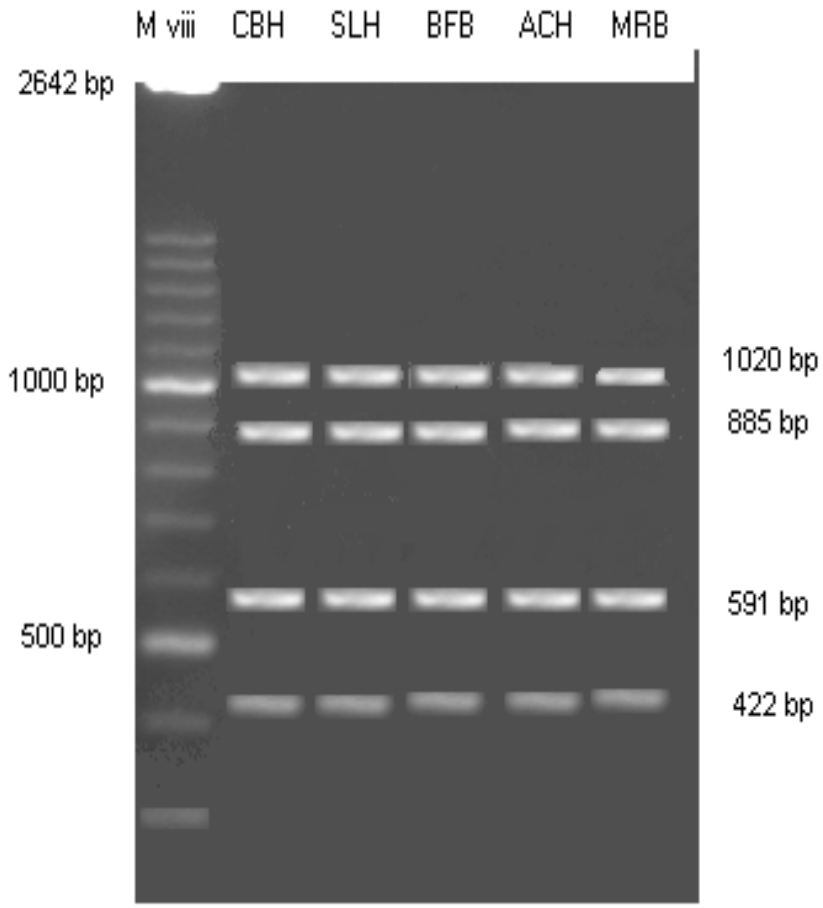


Fig. 27. Example of restriction fragment pattern on agarose gel resulting from digestion of the ITSI and ITSII PCR-product with restriction enzym Taq I. (bp = base pair, M VIII = 100 bp ladder, CBH = Corbet Head, SLH = Slea Head, MRB = Mulroy Bay, ACH = Achill island, BFB = Belfast Bay, see Table 1).

Table 5. Pairwise distances in Rubisco spacer sequences between *Alaria esculenta* strains. 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
1 *A. esculenta*-IRL	-	0.00	0.13	0.25	0.38	0.13
2 *A. esculenta*-SCL	0	-	0.13	0.25	0.38	0.13
3 *A. esculenta*-NOR	1	1	-	0.38	0.51	0.25
4 *A. esculenta*-ICL	2	2	3	-	0.64	0.38
5 *A. esculenta*-CAN	3	3	4	5	-	0.51
6 *A. esculenta*-FRC	1	1	2	3	4	-

5. DISCUSSION AND CONCLUSIONS

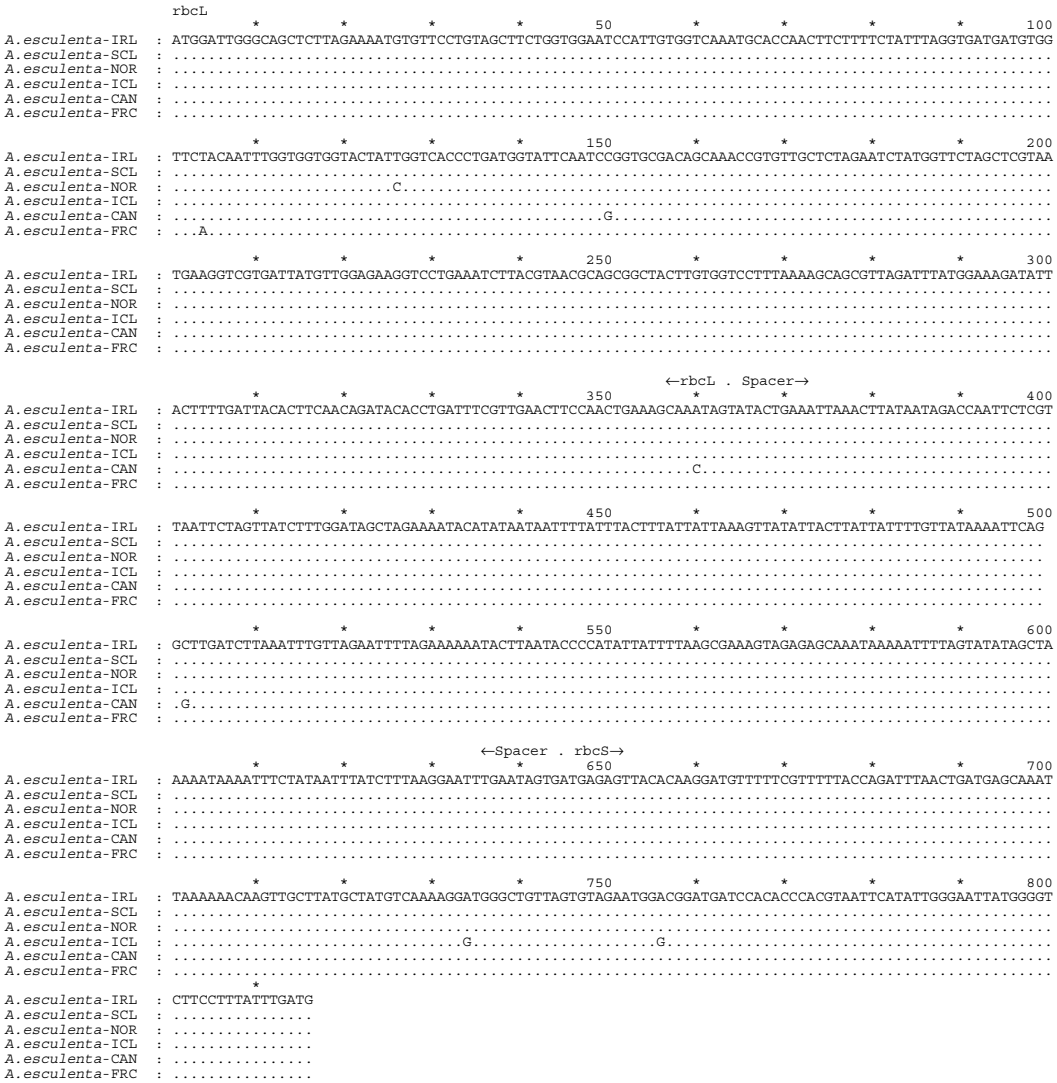
Strain selection through hybridization or simply selecting the most suitable varieties has been practised in a number of macroalgal genera, including *Porphyra*, *Chondrus* and *Gracilaria* (Patwary and van der Meer, 1992), *Laminaria* (Wu, 1998) and *Undaria* (Pang *et al*., 1997). So far, several cultivation trials with *A. esculenta* have been carried out with success, although no attention has been paid to strain selection (Kain and Dawes, 1987). Due to the recent possible applications of *A. esculenta*, the use of *A. esculenta* in aquaculture has become of economic interest (Yone *et al*., 1986; Mai *et al.,* 1996; Moss, 1994; Stuart and Brown, 1994; Nakagawa *et al*., 1997), and strain selection will undoubtedly become important.
The results presented in this field study for crosses between *A. esculenta* strains from different geographical locations show that the five strains are interfertile, which is in agreement with Kraan and Guiry (2000) who showed that *A. esculenta* populations throughout the North Atlantic Ocean were interfertile in the laboratory.

5.1 Growth rate, weight, length and width measurements

Substantial differences were encountered amongst crossings at the Ard Bay site (Fig. 16 and 17). The fastest-growing crosses were produced with female Norway gametophytes together with male gametophytes of other strains. However, these crossings grew rapidly in length but not in width, resulting in ribbon-like plants with a low biomass (Fig. 14). The supposedly Canadian (Halifax) self cross produced most biomass of over 45 kilogram per meter rope. However, this strain was harvested from the first rope for the second year and therefore we cannot state with certainty that this cross consisted of only male and female Canada (Halifax)

gametophytes. Nevertheless, high biomass results were reported for the Canadian self crosses from the first seeded rope and the Canadian self crosses from the third rope (crossing 11).

Fig. 28. Sequences of the Rubisco spacer and flanking regions rbcL and rbcS of 6 geographically dispersed strains of A. esculenta. FRC = France, SCL = Scotland, for other codes see Table 1.



Remarkably, the crossings with female Iceland or female Canada gametophytes either produced the largest plants with most biomass or did not produce any progeny (crossings 15, 16, 23 and 25). Plants from Iceland or Canada are most probably adapted to lower winter and summer temperatures compared to the Norwegian and Irish strains used in this field trial (U.S. Navy, 1981). Seeded under warmer and more favourable conditions, like the site in Ard Bay, these plants might grow faster and therefore larger and bigger. On the other hand, the higher temperatures might perhaps also inhibit fertilization in certain crossings with the Canadian and Icelandic female gametophytes resulting in no progeny as seen in crossings 15, 16, 23 and 25.

The female Iceland x male Ireland crossing produced the second highest biomass figure of 13.75 kg wet weight per meter rope. The Irish self cross using a strain from the Aran Islands produced 1.73 kg wet weight per meter. The same strain used at the site in New Quay produced a higher biomass of up to 7.4 kg.

Differences between the two sites might be caused due to a high water exchange and strong current regime at New Quay, hence providing a higher nutrient input and therefore a better growth.
Hybridization and strain selection with *Undaria pinnatifida* gametophytes from different locations in Japan and China produced F1 sporophytes with substantial size and weight variation (Pang *et al*., 1997). These authors found that some selfcrosses resulted in plants with the largest weight and longest size as well as plants with the lowest weight and smallest size, similarly to what was observed in the *A. esculenta* selfcrosses during the present study at Ard Bay and New Quay.
Substantial variation was also encountered at one long line within the same native Irish strain at New Quay. The long line situated in a tidal channel showed over a length of 25 m larger and heavier plants at the seaward side compared to the inner site. It is not clear what causes these differences.

No progeny was found on the May 1999 seeded long line. A possible explanation for inhibition of fertilization or a 100% mortality of young sporophytes perhaps might be exposure to UV radiation. Han and Kain (1993) showed a 100% mortality-rate of young, 24-day-old, *A. esculenta* sporophytes after 180 s of UV exposure in the laboratory. These exposure times can easily be reached in the field during wrapping of seed strings around the long line. The long line with seed strings was out planted in May 1999 on a very sunny day (Fig. 6). In the vessel the long line with seed strings ware exposed to air and UV for about 20 min before sufficient area of the long line was covered with seed string, after it was released and submerged again (Figs. 6 and 8). This problem can be avoided by seeding the long line indoors, transporting the long line in a small tank, submerging the long line directly and then fastening it between the seaweed farm structures.
A second explanation for the absence of progeny on the May seeded long line could be a temperature effect. The upper temperature tolerance of *A. esculenta* gametophytes is 20° C (Tom Dieck, 1993). The warm air temperature or water surface temperature in May might have been to high causing cell damage and hence inhibiting proper fertilization or growth of the young sporophytes. It is therefore recommended that long-lines be seeded from October to February.

In conclusion, the results clearly show that the Canadian strain or hybrids derived from female Canada gametophytes or female Iceland gametophytes produce more biomass per meter rope and grow larger and wider in size compared to the native Irish strain.

5.2 Protein levels

The Canadian selfcross expressed the highest protein level followed by the female Newfoundland x male Norway hybrid. These strains and hybrids are well suited to be included in a protein rich macro-herbivore diet. The Irish native strain at Ard

Bay showed a low protein level of less than 5% of the wet weight. This value is comparable to the New Quay site were values were measured between 2.5-8 %.
Over one month a strong increase in the protein level was recorded at both trial sites. The increase is probably a seasonal effect with higher protein levels towards the end of the growing season at the end of June. Therefore, if these strains or hybrids are to be harvested for their protein content it is best to wait until the end of the growing season just before plants start to deteriorate and are overgrown by epiphytes.

5.3 Genetic fingerprinting and sequencing

The restriction fragment analysis of *A. esculenta* isolates from around the Irish coast, showed that they were genetically identical in respect of the DNA region examined. Sequencing of the Rubisco spacer sequences of *A. esculenta* isolates from elsewhere in the North Atlantic (Table 1) confirms the results of the restriction fragment analysis. The sequencing results showed a maximum of three-base-pair difference amongst *A. esculenta* isolates from Halifax, Canada and Inis Oírr, Ireland, showing negligible genetic variation. By contrast, Coyer *et al*. (1997) showed distinguishable biogeographic populations along the north-east Pacific coast for the kelp *Postelsia palmaeformis* Ruprecht; however, for ecological reasons, gene flow may be more restricted in this species. These authors showed decreasing genetic relatedness with increasing distance of 25 m or more between populations assessed with M13 Fingerprinting and 16 to 250 km with RAPDs. Bhattacharya *et al*. (1990) showed with RFLPs 8 distinct populations of *Costaria costata* (C. Agardh) Saunders over a range of 400 km. They showed that these populations appeared to be discreet breeding groups.

Despite relative isolation of individual *A. esculenta* populations at headlands or islands around the Irish coast and even throughout the North Atlantic, differences observed in the hybridization field trials are not likely to be caused due to genetic variation between the populations considered. The different protein levels, growth rates and biomass in kg wet weight per meter rope encountered in the crosses are most probably caused by ecotypic variation such as different temperature tolerance of the gametophyte and/or sporophyte and temperature responses of growth (see Breeman, 1988).

In conclusion, the most suitable strain for use in Irish aquaculture of the edible brown alga *Alaria esculenta* is the Canadian (Halifax) strain or hybrids derived from female Canadian gametophytes. They showed the highest protein and biomass values in the field trials and hence are most suited to be used in or for other applications.

Acknowledgements

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