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Nucleotide diversity within and between four species of *Laminaria* (Phaeophyceae) analysed using partial LSU and ITS rDNA sequences and AFLP

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In order to estimate the nucleotide diversity of the four kelp species *Laminaria digitata*, *L. hyperborea*, *L. saccharina* and *L. faeroensis*, three approaches were used. First we sequenced 1320 base pairs (bp) of nuclear-encoded large subunit (LSU) rDNA, which included some of the most variable domains. These sequences discriminated between *L. digitata*, *L. hyperborea* and *L. saccharina*, but *L. faeroensis* was identical to *L. saccharina*. A phylogeny inferred based on 672 bp of the nuclear-encoded non-coding internal transcribed spacer regions (ITS1 and ITS2) rDNA, which are more variable than LSU rDNA, likewise failed to resolve a monophyletic *L. saccharina* with respect to *L. faeroensis*. Thirdly, we tested the usefulness of the amplified fragment length polymorphism (AFLP) technique to provide a resolved phylogeny for 43 *Laminaria* specimens. *Laminaria digitata* and *L. hyperborea* were resolved as strongly supported monophyletic groups. A third robust clade consisted of *L. saccharina* plus *L. faeroensis*. Within this clade, *L. faeroensis* samples were grouped with bootstrap support of 80%, but the *L. saccharina* clade was less well supported. The AFLP data in combination with rDNA sequences indicated subspecies status for *L. faeroensis*. AFLP analyses of different morphotypes of *L. digitata* and *L. saccharina* from Danish coastal waters did not reveal any differences. However, *L. digitata* from the Faroe Islands was genetically distinct from other samples.

Key words: AFLP, kelps, *Laminaria digitata*, *Laminaria faeroensis*, *Laminaria hyperborea*, *Laminaria saccharina*, LSU rDNA, morphotypes, nucleotide diversity, rDNA ITS

Introduction

Lamouroux (1813) erected *Laminaria* (Laminariales, Phaeophyceae) and the genus was later lectotypified with *L. digitata* (L.) Lamouroux (Farr *et al.*, 1979). The genus is one of the most important marine benthic brown algal genera in terms of ecology and economy (Kain, 1979; Dring, 1982; Yarish *et al.*, 1990; Sjøtun, 1995). *Laminaria* is mainly distributed in temperate to polar regions in the Northern Hemisphere (Kain, 1979). Members of Laminariales have a strongly heteromorphic, diplohaplontic life cycle, with an alternation of a highly differentiated diploid sporophyte and a microscopic haploid gametophyte (Sauvageau, 1896). The sporophyte can be several metres long and is differentiated into three parts: the holdfast, simple stipe and lamina, which also constitute the generally accepted diagnostic characters of *Laminaria*. The genus is divided into two sections, Simplices with an undivided lamina, and Digitatae with the lamina split into fingers (Kain, 1979).

In *Laminaria*, species delineation has always been difficult because only a few consistent morphological characters are available (see Kain, 1979 for a comprehensive review). Furthermore, studies have shown plasticity in the morphological characters previously used for species delineation, such as shape of lamina, stipe length, hollowness of stipe, and mucilage ducts in stipe and/or blade. Burrows (1964) showed that the extent and development of mucilage ducts in *L. saccharina* (L.) Lamouroux and *L. agardhii* Kjellman were temperature dependent. Chapman (1973) found that stipe length and hollowness decrease with increasing exposure to wave action, indicating that environmental factors can influence morphological features. Hence, independence from morphological characters should be sought in delineating species of *Laminaria*.

To elucidate interfertility between species, many hybridization experiments have been conducted but with ambiguous results. Cosson & Olivari (1982)

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reported success in producing hybrids of *L. digitata* × *L. saccharina* (including reciprocal crosses). Schreiber (1930) reported parthenogenetic sporophytes of *L. saccharina, L. digitata* and *L. hyperborea* (Gunnerus) Foslie, but found the three species to be non-interfertile.

Molecular studies have shown that neither the genus *Laminaria* nor the order Laminariales form monophyletic groups (Draisma *et al*., 2001; Rousseau *et al*., 2001; Yoon *et al*., 2001). Furthermore, Yoon *et al*. (2001) found that *L. digitata* and *L. hyperborea* have identical sequences in the plastid-encoded RuBisCo spacer regions separating the genes coding for the large and small subunits of the ribulose-1,5-bisphosphate carboxylase-oxygenase enzyme and of the nuclear-encoded ribosomal ITS regions.

In this study we concentrated on delineating the *Laminaria* species and morphotypes occurring in Danish waters and the Faroe Islands (Fig. 1), viz. *L. digitata, L. saccharina, L. hyperborea* and *L. faeroensis* (Rosenvinge & Lund, 1947). The water system comprising Kattegat, Øresund and the Baltic Sea (Fig. 1) has a decreasing salinity gradient southward, due to the net discharge of surface water with low salinity from the Baltic Sea and entry of water with high salinity from the North Sea. The salinity ranges from c. 30 ppt. in the northern part of Kattegat to 5 ppt. at Bornholm in the Baltic Sea. Because the density of water increases with increasing salinity, this exchange of water also causes stratification with low salinity surface water and high salinity at the bottom. These conditions have consequences for the distribution of the laminarians in this area.

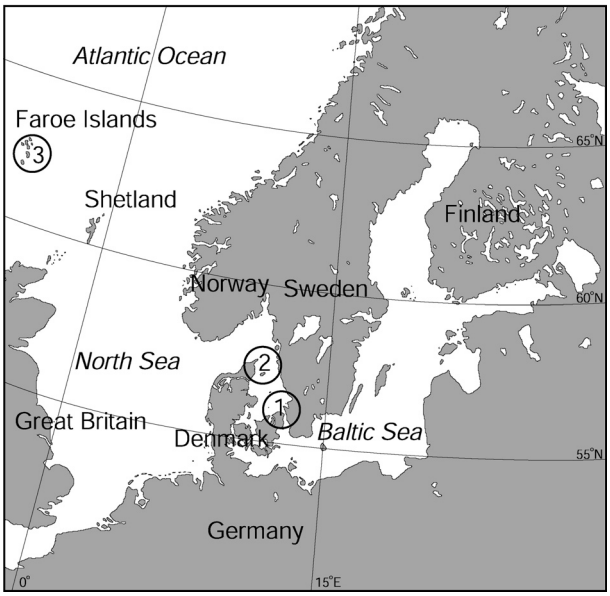

Fig. 1. Map showing the three main locations where the *Laminaria* species were collected (see also Table 1). Area 1 in the southern part of Kattegat comprised Ellekilde Hage, Helsinborg, Hornbæk and Ålsgårde, which are encompassed in a radius of 4 nautical miles. Location 2 in the northern part of Kattegat comprised Deget, Frederikshavn, Sandholm, Hirsholm and Tønneberg Banke, encompassed in a radius of 11 nautical miles. Sites at location 3 at the Faroe Islands were Drátturin, Drelnes and Trongisvágsfjørður, all within 1 nautical mile.

Laminaria hyperborea is confined to the North-east Atlantic Ocean from Portugal to the northern part of Norway (Kain, 1979). In Danish coastal waters, its southern limit is at the middle of Kattegat due to its requirement for high salinity, and it occurs in depths from about 3–30 m (Rosenvinge & Lund, 1947). *Laminaria digitata* and *L. saccharina* are distributed on both sides of the North Atlantic Ocean (Kain, 1979). In Danish waters they are both distributed into the Baltic Sea at Bornholm (Rosenvinge & Lund, 1947). In the northern part of Kattegat both species are distributed from 1–30 m, but in Øresund the plants are submerged into deeper water than normal due to the salinity. In depths of 2.5–3 m only *L. digitata* occurs, whereas both species are found in depths of 14 m (LE, pers. obs.). Both species may show a high degree of phenotypic variability, which earlier led to subdivision of the two *Laminaria* species into different forms or morphotypes based on shape of the lamina. Rosenvinge & Lund (1947) grouped the forms of *L. digitata* into two principal types, the *genuina*-type, that has a deeply divided lamina with many sword-shaped segments and the *intermedia*-type with a slightly thinner and often bullate lamina divided into only a few segments or not divided at all. Between these two principal types of *L. digitata*, many transitional forms were described as forms or varieties of *L. digitata*. They also divided the Danish members of *L. saccharina* into two principal types: the *bullata*-type, which has a thick, coriaceous, bullate, and ribbed lamina, and the *membranacea*-type whose lamina is thin and membranous. As for *L. digitata*, many transition forms between these two types are described as forms or varieties. *Laminaria faeroensis* is only known from the Faroe Islands, where it is only found in sheltered places. One century ago Børgesen (1902) erected *Laminaria faeroensis* as a distinct species based on morphological characters. He had previously described *L. faeroensis* as a variety of *L. longicruris* with which it shared a hollow stipe but it differed from *L. longicruris* by having no mucilage ducts. Today we know that the mucilage ducts and hollow stipe are plastic characters (Burrows, 1964; Chapman, 1974) and crossing experiments and molecular work (Lüning *et al*., 1978; Bhattacharya *et al*., 1991; Cho *et al*., 2000) confirm that *L. saccharina* and *L. longicruris* are conspecific.

Since DNA sequencing has failed to distinguish between some *Laminaria* species and in light of the

ambiguous findings from crossing experiments, the aim of this study was to examine the usefulness of the AFLP technique (Vos *et al*., 1995) in combination with rDNA sequences to delineate the *Laminaria* species in question. AFLP has already been employed for marine and freshwater macroalgae (e.g. Donaldson *et al*., 1998, 2000; Kusumo & Druehl, 2000; Iitsuka *et al*., 2002; Mannschreck *et al*., 2002; Schaeffer *et al*., 2002; Murphy & Schaffelke, 2003). Finally we used AFLP data to estimate nucleotide diversity (π) within and between species using two recently developed methods (Innan *et al*., 1999; Mougel *et al*., 2002).

Materials and methods

Collection and identification of plants

All plants used in this study (Table 1) were diploid sporophytes collected in the field in Denmark and the Faroe Islands (Fig. 1). Plants were collected as drift, by diving or from a boat with a triangular dredge. Unambiguous specimens of each species were identified according to the diagnostic morphological characters given in Børgesen (1902), Rosenvinge & Lund (1947) and Kain (1979). AFLP fingerprints and rDNA sequences of the unambiguous specimens were then used subsequently as references to identify ambiguous specimens.

Laminaria hyperborea (Figs 2, 5) is characterized by having a stiff, rugose, slightly conical stipe, and a divided lamina (Kain, 1979). It is distinguished from *L. digitata* (Figs 3–5, 9, 10–11) by having mucilage ducts in both stipe and lamina; in *L. digitata* and *L. saccharina* these only occur in the lamina. The stipe in *L. digitata* is generally more flexible, cylindrical and compressed distally. Furthermore, in *L. hyperborea* the change of lamina takes place later than the two other species (Rosenvinge & Lund, 1947). *Laminaria saccharina* (Figs 7–8) has a solid stipe and a simple undivided lamina. Generally the lamina has a clear distinction between interior and marginal parts. Some specimens of *L. digitata* have an undivided lamina (Figs 4 and 10) but with no such distinction. *Laminaria faeroensis* (Fig. 6) is characterized by having a hollow stipe but no mucilage ducts (Børgesen, 1902).

Preservation of algae for DNA extraction

Samples were taken from the new blade just above the stipe, and to reduce contamination, they were thoroughly washed in distilled water, removing surface mucus, blotted with paper towels, and preserved in silica gel according to Chase & Hills (1991).

DNA extraction and purification

DNA extraction and purification was performed using the CTAB method (Doyle & Doyle, 1987) and ultracentrifugation through a caesium chloride (CsCl) density gradient as described by Phillips *et al*. (2001) but with a slight modification of the extraction buffer. The extraction buffer consisted of 2% (w/v) CTAB (hexadecyltrimethylammonium bromide), 0.1 M Tris-HCl (pH 8.0), 20 mM EDTA (disodium ethylenediamine tetraacetic acid), 1.4 M NaCl, 0.1% (w/v) SDS (sodium dodecyl sulphate), 3.5 mM DIECA (diethyldithiocarbamic acid), 0.2% (v/v) β-ME (β-mercaptoethanol), 1% (w/v) PVPP (polyvinylpolypyrrolidone). All buffers were made from stock solutions as described in Sambrook *et al*. (1989).

DNA amplification and sequencing

Five to 10 ng of genomic DNA was amplified in a 50 μl reaction containing 5 μl 10 × *Taq* buffer (0.67 M Tris-HCl pH 8.5, 20 mM $MgCl_2$, 166 mM $(NH_4)_2SO_4$, 0.1 M 2-mercaptoethanol), 20 μl 0.5 M dNTP mix, 5 μl 10 μM of each primer, 14 μl sterile H_2O, 1 U *Taq* polymerase. Amplifications were carried out in a PTC-100 thermocycler (MJ Research Inc.). The following profile was used: one initial denaturing step for 2 min at 94°C, followed by 34 cycles of 1 min at 94°C, 1 min at 52°C, 2 min at 72°C, and finally 6 min at 72°C.

After amplification the PCR products were checked on 2% agarose gels stained with ethidium bromide. Before cycle sequencing the PCR products were purified with the QIAquick™ PCR purification Kit 250 (Qiagen, Germany) as described by the manufacturer. Quantities of 5–10 ng of PCR products for ITS sequences and 20–40 ng for LSU sequences were used as template DNA in 20 μl reactions. Primers for PCR and cycle sequencing of partial LSU and ITS rDNA were: D1R-F (5′-ACC CGC TGA ATT TAA GCA TA-3′; Scholin *et al*., 1994), D2C-R (5′-CCT TGG TCC GTG TTT CAA GA-3′; Scholin *et al*., 1994); D3A-F (5′-GAC CCG TCT TGA AAC ACG GA-3′; Nunn *et al*., 1996), D3B-R (5′-TCG GAG GGA ACC AGC TAC TA-3′; Nunn *et al*., 1996); 1483R (5′-GCT ACT ACC ACC AAG ATC TGC-3′; Daugbjerg *et al*., 2000); ITS-1 (5′-TCC GTA GGT GAA CCT GCG G-3′; Mankin *et al*., 1986); and ITS-4 (5′-TCC TCC GCT TAT TGA TAT GC-3′; Lapeyre *et al*., 1993). The cycle sequencing reactions were run on an ABI Prism 377 DNA sequencer, using the Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer).

Alignment and phylogenetic analyses of sequence data

We compiled an alignment of partial LSU sequences (1320 base pairs including gaps) from four species of *Laminaria* (5 specimens). The nucleotide sequences correspond to positions 16–1335 in *L. digitata* (AF331153). Sequences from taxa available in GenBank (Table 2) were included in another alignment, which comprised 13 ITS sequences (672 base pairs including gaps), and corresponds to positions 173–844 in *L. digitata* (AF319014). Partial LSU rDNA and ITS sequences were aligned manually using BioEdit ver. 5.08 (Hall, 1999). The LSU rDNA and ITS data matrices were analysed using PAUP* ver. 4.0b10 (Swofford, 2003). In parsimony analysis of LSU rDNA, we used the exhaustive search option whereas for analysis of the ITS data matrix, we used the heuristic search option with branch swapping (tree bisection reconnection) and 1000 random additions of sequences. For maximum like-

Table 1. Species used in this study (region is keyed to the map in Fig. 1)

Species	ID	Location	Region	Position	Depth	Date	Collector	Accession ITS rDNA	Accession LSU rDNA
Laminaria digitata	05	Hirsholm	2	57° 29,23' N 10° 37,65' E	4.0 m	02-02-01	L. Erting		
Laminaria digitata	06	Hirsholm	2	57° 29,23' N 10° 37,65' E	4.0 m	02-02-01	L. Erting		
Laminaria digitata	08	Hirsholm	2	57° 29,23' N 10° 37,65' E	4.0 m	02-02-01	L. Erting	AY441769	
Laminaria digitata	10	Hirsholm	2	57° 29,23' N 10° 37,65' E	4.0 m	02-02-01	L. Erting	AY441770	
Laminaria digitata	11	Hornbæk	1	56° 05,65' N 12° 28,10' E	Washed ashore	02-01-30	L. Erting		
Laminaria digitata	12	Hornbæk	1	56° 05,65' N 12° 28,10' E	Washed ashore	02-01-30	L. Erting		AY441778
Laminaria digitata	14	Hornbæk	1	56° 05,65' N 12° 28,10' E	Washed ashore	02-01-30	L. Erting		
Laminaria digitata	15	Hornbæk	1	56° 05,65' N 12° 28,10' E	Washed ashore	02-01-30	L. Erting		
Laminaria digitata	16	Hornbæk	1	56° 05,65' N 12° 28,10' E	Washed ashore	02-01-30	L. Erting		
Laminaria digitata	19	Tønneberg Banke	2	57° 28,70' N 11° 16,90' E	15.0 m	02-02-05	J. Damgaard		
Laminaria digitata	34	Ålsgårde	1	56° 05,30' N 12° 30,75' E	Washed ashore	02-04-28	L. Erting		
Laminaria digitata	37	Hornbæk	1	56° 05,65' N 12° 28,10' E	Washed ashore	02-05-07	L. Erting		
Laminaria digitata	48	Frederikshavn Sandholm	2	57° 27,00' N 10° 33,20' E	Washed ashore	02-05-16	L. Erting		
Laminaria digitata	51	Hirsholm	2	57° 29,20' N 10° 37,60' E	1.0–2.5 m	02-05-17	L. Erting		
Laminaria digitata	52	Hirsholm	2	57° 29,20' N 10° 37,60' E	1.0–2.5 m	02-05-17	L. Erting		
Laminaria digitata	53	Hirsholm	2	57° 29,20' N 10° 37,60' E	1.0–2.5 m	02-05-17	L. Erting		
Laminaria digitata	55	Hirsholm	2	57° 29,20' N 10° 37,60' E	1.0–2.5 m	02-05-17	L. Erting		
Laminaria digitata	56	Hirsholm	2	57° 29,20' N 10° 37,60' E	1.0–2.5 m	02-05-17	L. Erting		
Laminaria digitata	57	Hirsholm	2	57° 29,20' N 10° 37,60' E	1.0–2.5 m	02-05-17	L. Erting		
Laminaria digitata	76	Drelnes	3	61° 33,00' N 6° 50,00' W	1.0 m	02-09-21	A.M. Mortensen		
Laminaria digitata	77	Drelnes	3	61° 33,00' N 6° 50,00' W	1.0 m	02-09-21	A.M. Mortensen		
Laminaria faeroensis	71	Trongisvágsfjørður	3	61° 33,00' N 6° 50,00' W	2.0 m	02-06-12	A.M. Mortensen	AY441774	AY441782
Laminaria faeroensis	72	Drelnes	3	61° 33,00' N 6° 50,00' W	5.0 m	02-09-21	A.M. Mortensen		
Laminaria faeroensis	73	Drelnes	3	61° 33,00' N 6° 50,00' W	5.0 m	02-09-21	A.M. Mortensen	AY441775	
Laminaria hyperborea	01	Deget	2	57° 27,20' N 10° 35,05' E	4.5 m	02-02-01	L. Erting	AY441771	AY441779
Laminaria hyperborea	03	Deget	2	57° 27,20' N 10° 35,05' E	4.5 m	02-02-01	L. Erting		
Laminaria hyperborea	04	Hirsholm	2	57° 29,23' N 10° 37,65' E	4.0 m	02-02-01	L. Erting	AY441772	
Laminaria hyperborea	09	Hirsholm	2	57° 29,23' N 10° 37,65' E	4.0 m	02-02-01	L. Erting	AY441773	
Laminaria hyperborea	20	Tønneberg Banke	2	57° 28,70' N 11° 16,90' E	15.0 m	02-02-05	J. Damgaard		
Laminaria hyperborea	21	Tønneberg Banke	2	57° 28,70' N 11° 16,90' E	15.0 m	02-02-05	J. Damgaard		
Laminaria hyperborea	22	Tønneberg Banke	2	57° 28,70' N 11° 16,90' E	15.0 m	02-02-05	J. Damgaard		
Laminaria hyperborea	23	Tønneberg Banke	2	57° 28,70' N 11° 16,90' E	15.0 m	02-02-05	J. Damgaard		

(*continued*)

Table 1. (*continued*)

Species	ID	Location	Region	Position	Depth	Date	Collector	Accession ITS rDNA	Accession LSU rDNA
Laminaria hyperborea	24	Tønneberg Banke	2	57° 28,70' N 11° 16,90' E	15.0 m	02-02-05	J. Damgaard		
Laminaria hyperborea	25	Tønneberg Banke	2	57° 28,70' N 11° 16,90' E	15.0 m	02-02-05	J. Damgaard		
Laminaria hyperborea	26	Tønneberg Banke	2	57° 28,70' N 11° 16,90' E	15.0 m	02-02-05	J. Damgaard		
Laminaria hyperborea	27	Tønneberg Banke	2	57° 28,70' N 11° 16,90' E	15.0 m	02-02-05	J. Damgaard		
Laminaria hyperborea	29	Tønneberg Banke	2	57° 28,70' N 11° 16,90' E	15.0 m	02-02-05	J. Damgaard		
Laminaria hyperborea	30	Tønneberg Banke	2	57° 28,70' N 11° 16,90' E	15.0 m	02-02-05	J. Damgaard		
Laminaria hyperborea	75	Drelnes	3	61° 33,00' N 6° 50,00' W	5.0 m	02-09-21	A.M. Mortensen		
Laminaria saccharina	28	Tønneberg Banke	2	57° 28,70' N 11° 16,90' E	15.0 m	02-02-05	J. Damgaard		
Laminaria saccharina	31	Tønneberg Banke	2	57° 28,70' N 11° 16,90' E	15.0 m	02-02-05	J. Damgaard		
Laminaria saccharina	40	Helsinborg	1	56° 03,31' N 12° 40,46' E	14.0 m	02-05-15	L. Erting	AY441776	AY441780
Laminaria saccharina	54	Hirsholm	2	57° 29,20' N 10° 37,60' E	1.0–2.5 m	02-05-17	L. Erting	AY441777	AY441781
Laminaria saccharina	78	Drätturin	3	61° 32,00' N 6° 40,00' W	2.0 m	02-09-21	A.M. Mortensen		

ID: individual number.

lihood analysis we applied the F84 model available in PAUP* with the option empirical nucleotide frequencies. PAUP* was also used to compute dissimilarity values that were converted to evolutionary distances by correction for multiple substitutions according to the Kimura two-parameter model. The computed distance matrix was used as input to build a tree with the neighbour-joining method. Bootstrap analyses were conducted with 1000 replicates in maximum likelihood, parsimony and neighbour-joining analyses (Felsenstein, 1985).

AFLP analysis

Primers with three selective nucleotide (*Eco*RI-ACT/*Mse*I-CAG), restriction enzymes, adapter oligonucleotides, dNTPs, ligase and *Taq* polymerase for the restriction digestion, ligation and PCR amplification of the AFLP fragments were supplied in the AFLP™ Plant Mapping Kit from PE Applied Biosystems (Perkin Elmer). This protocol mainly follows the original AFLP technique (Vos *et al.*, 1995). The manufacturer's instructions were followed, with a few exceptions. A smaller amount of genomic DNA was used in the digesting ligation step, and slight modifications of the PCR cycles were made. The concentration of the extracted genomic DNA was measured using an Eppendorf BioPhotometer 6131. The protocol in the AFLP™ Plant Mapping Kit suggests using 0.5 μg genomic DNA, but we obtained good results with lesser amounts (5–210 ng) of genomic DNA. Afterwards, the reaction mixtures were diluted with $1 \times$ TE buffer (10 mM Tris-HCl, 0.1 mM EDTA pH 8.0) to a final concentration of 2.5 ng DNA μl^{-1} as described in the standard protocol. Those samples in which the DNA concentration was too low were not diluted, but used directly as template DNA in the preselective PCR amplification. The reactions for the preselective and selective PCR amplifications were carried out in a PTC-100 thermocycler (MJ Research Inc.) with the following PCR cycle profiles. Preselective amplifications were performed with 4 initial cycles of a 2 min extension step at 72°C, a 1 s denaturing step at 94°C, a 30 s annealing step at 56°C, then followed by 20 cycles of 30 s at 94°C, 1 min at 56°C, 2 min at 72°C. To verify the success of the preselective amplification, 5 μl of the PCR product was loaded on a 2% agarose gel stained with ethidium bromide and checked under UV light. Selective amplifications were performed with one initial cycle of 2 min at 94°C, 30 s at 65°C, 2 min at 72°C, followed by 8 cycles consisting of 30 s at 94°C, 30 s at 64°C decreasing 1°C per cycle, 2 min at 72°C, and finally 23 cycles consisting of 30 s at 94°C, 30 s at 56°C, 2 min at 72°C.

The PCR products from the selective amplification were visualized using an ABI Prism 377 DNA Sequencer (Perkin Elmer). Fragments up to and over 500 bp in size were sized with GeneScan-500 and GeneScan-1000 ROX Size Standards, respectively (both from Applied Biosystems).

AFLP data matrix and nucleotide diversity

Sizing and quantification of AFLP fragments from gels were performed with GeneScan® 3.1.2 (Applied Biosys-

Figs 2 – 11. Some of the *Laminaria* plants used in this study all from Denmark unless otherwise indicated. Fig. 2. *L. hyperborea* ID 01 showing a stiff, rugose, slightly conical stipe, and a divided lamina typical for this species. Fig. 3. *L. digitata* ID 52 showing a more flexible, cylindrical and distally compressed stipe typical for this species. Fig. 4. *L. digitata* ID 48 (the larger of the two shown) representing a form of *L. digitata* with undivided lamina. Fig. 5. From left to right *L. hyperborea* ID 04, *L. digitata* ID 05 and *L. digitata* ID 06 illustrating the difficulty in distinguishing between *L. hyperborea* and *L. digitata*. It can be seen that *L. hyperborea* has a more divided lamina than the two *L. digitata* plants and a new lamina has just started to develop, while the two *L. digitata* plants have a much more extended development of the new laminae. Fig. 6. *L. faeroensis* ID 71 from Faroe Islands; the arrow shows the hollow stipe typical for this plant. Fig. 7. *L. saccharina* ID 40 showing the *membranacea*-type. Fig. 8. *L. saccharina* ID 54 showing the *bullata*-type. Fig. 9. *L. digitata* ID 12 developing a new lamina, which probably later will split into a few fingers. Fig. 10. *L. digitata* ID 56 representing a form of *L. digitata* with undivided lamina. Fig. 11. *L. digitata* ID 55 showing an intermediate form of *L. digitata* with a lamina splitting into a few fingers. Scale bars = 10 cm.

Table 2. *Laminaria* sequences from GenBank included in this study

Taxon	Collection site	Locus	Accession	Reference
L. digitata	Dover, Great Britain, Jan. 98	ITS	AF319014	Yoon *et al*., 2001
L. hyperborea	Roscoff, France, Apr. 00	ITS	AF319015	Yoon *et al*., 2001
L. saccharina	Oregon, USA, July 98	ITS	AF319019	Yoon *et al*., 2001
L. saccharina	Canada	ITS	AF362996	Peters, 1998

tems) and the electropherograms were imported into Genotyper® 2.1 (Applied Biosystems). Assignment of AFLP fragments to size categories was performed by manual evaluation of all electropherograms using Genotyper 2.1. The AFLP fragments were aligned after size categories in a data matrix and assigned either value 0 or 1 (0 = fragment absent; 1 = fragment present).

In order to calculate the nucleotide diversity (π), two computer programs were programmed in Turbo Pascal by LE (the compiled programs and the source code are available on request). The programs read the present-absent AFLP matrix and calculated nucleotide diversity based on mathematical models provided by Innan *et al*. (1999) and Mougel *et al*. (2002).

AFLP are dominant markers, so in a diploid organism the fragment may be scored as present in one of its two haplotypes, and absent for the other haplotype. For genomes that have diverged substantially this seems not to be a problem, since fragments that differ between species may tend to have their presence or absence fixed within each species (Felsenstein, 2003). Within species these fragments will not necessarily be fixed between populations, but the problem can be solved as suggested by Innan *et al*. (1999).

This model has different approaches to handling a diploid organism depending on whether it is a selfing species (fixed homozygotes) or an out-crossing species in Hardy-Weinberg equilibrium. Billot *et al*. (1999) found that self-fertilization is possible in *L. digitata*, but it seems that out-crossing prevailed over self-fertilization. However, other studies have indicated a slight heterozygote deficiency in populations of *Laminaria* (Billot *et al*., 2003). Because these conditions are not fully explained in populations of *Laminaria*, we estimated the nucleotide diversity for both conditions, which then gave a range of minimum and maximum nucleotide diversity within the species.

For a diploid selfing species the proportion of shared AFLP fragments (F) between two individuals is given by $F = 2n_{xy}/(n_x + n_y)$ (Nei & Li, 1979). In this equation, which is equal to eq. 20 in Innan *et al*. (1999), n_{xy} is the number of AFLP fragments shared by both individual X and Y and n_x and n_y are the number of fragments in individuals X and Y, respectively. The nucleotide diversity (π) was then obtained from eq. 19 (Innan *et al*., 1999), in which the average of F, obtained from eq. 21a (Innan *et al*., 1999), was used. The model of Mougel *et al*. (2002) is analogous to this approach.

In the case of a crossing species in Hardy-Weinberg equilibrium, the nucleotide diversity was also estimated from eq. 19, but the average of shared AFLP fragments F was obtained from eq. 27 (Innan *et al*., 1999).

Phylogeny based on AFLP fragments

Phylogenetic analyses were performed using MEGA version 2.1 (Kumar *et al*., 2001), PHYLIP (Phylogeny Inference Package) version 3.5c (Felsenstein, 1993) and PAUP* ver. 4.0b10 (Swofford, 2003). An unrooted tree based on nucleotide diversity as a measure of evolutionary distance, from 197 different AFLP fragment size categories, was constructed using neighbour-joining (Saitou & Nei, 1987). Bootstrapped data sets from the original AFLP matrix were generated (1000 replications) based on the method by Mougel *et al*. (2002). These were exported to NEIGHBOR from the PHYLIP package. The tree output file was read into PAUP* ver. 4.0b10, where trees were midpoint rooted. A strict consensus tree with bootstrap value according to the 50% majority rule was constructed.

AFLP fragments can mainly be considered as characters independently drawn at random, a necessary condition for the bootstrap method. However, it is possible that an AFLP fragment might change by point mutations into two detectable AFLP fragments on the gel, which therefore cannot be considered as independent. To compensate for fragment independence as described by Felsenstein (1985) and Mougel *et al*. (2002), reduced bootstraps were also performed by sampling 85% of the AFLP fragments at random with replacement among the original 197 different AFLP fragments. In this way each of the resulting data sets were based on a reduced resampling of the original absent – present AFLP matrix and handled as described above.

Results

Partial LSU rDNA sequence data

In the alignment comprising five sequences, only 18 sites varied among the 1320 bp, of which 15 were parsimony informative. The largest evolutionary distance was between *L. digitata* and *L. saccharina*, a divergence of 1.4% (Table 3). There were no differences between the sequences of *L. saccharina* and *L. faeroensis*, and only 3 bp (0.2%) between *L. digitata* and *L. hyperborea* (Table 3).

ITS rDNA sequence data

The phylogeny of the 13 sequences shown in Fig. 12, including four sequences from GenBank, is based on 672 bp from the ITS rDNA region. The

Table 3. Average pairwise nucleotide divergence (substitutions per site) between partial LSU rDNA sequences of one individual of *Laminaria digitata*, *L. hyperborea*, *L. faeroensis* and two of *L. saccharina* (which were identical)

Species	1	2	3	4
1 *L. digitata*	–			
2 *L. hyperborea*	0.002	–		
3 *L. saccharina*	0.012	0.014	–	
4 *L. faeroensis*	0.012	0.014	0.000	–

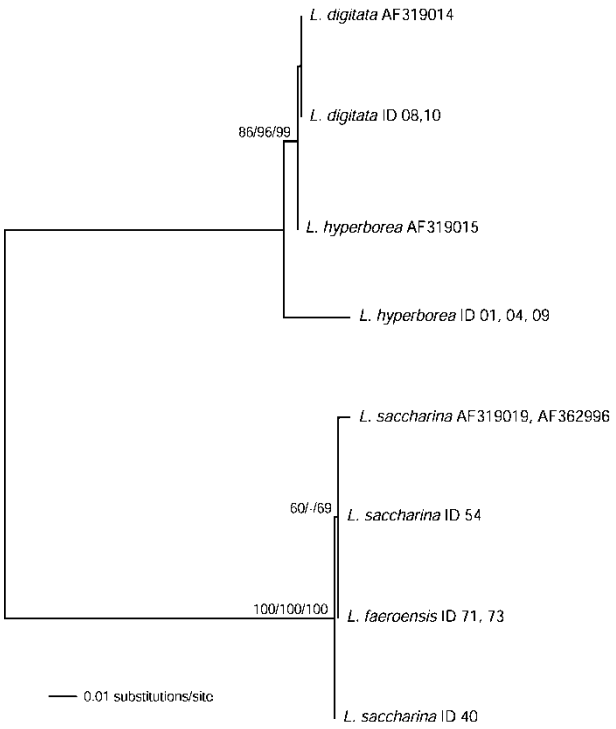

Fig. 12. Unrooted phylogeny based on maximum likelihood analysis of ITS sequences from four species of *Laminaria*. With the F84 model the best log likelihood score was − 1557.39. Parsimony analysis returned 8 equally parsimonious trees, each 153 steps long (CI = 0.993 and RI = 0.997). Bootstrap values based on 1000 replicates are from maximum likelihood, parsimony and neighbour-joining analyses, respectively. Only values above 50% are shown to the left of nodes. Identification numbers (see Table 1) or GenBank accession numbers are given in parentheses. *Laminaria hyperborea* AF319015 seems to be a misidentified *L. digitata* and its identity should be re-examined.

nucleotide composition had an average C and G content of 31.2% and 25.8%, respectively. The ratio of transitions to transversions was 0.5. Across the entire alignment 140 sites were variable, of which 130 were parsimony informative. There were two strongly supported 100% clades representing (1) *L. digitata* + *L. hyperborea* and (2) *L. saccharina* including *L. faeroensis*. The three *L. hyperborea* sequences (ID 01, ID 04 and ID 09) were identical, as were the two *L. digitata* sequences (ID 08 and ID 10). However, the sequence of *L. hyperborea* (AF319015) from Roscoff differed from *L. digitata* ID 08 and ID 10 only by a single nucleotide at position 120. It seems therefore to be a misidentified *L. digitata* and was excluded from further analyses. *Laminaria digitata* (AF319014) from Dover was also identical to *L. digitata* ID 08 and ID 10, except for ambiguities at positions 120, 123 and 579. Sequences of *L. digitata* and *L. hyperborea* differed by 3.1% (21 bp) (Table 4).

In the *L. saccharina* clade all sequences were very closely related. The two *L. faeroensis* and *L. saccharina* (ID 54) were identical, *L. saccharina* ID 40 differed by one base and the two *L. saccharina* sequences available from GenBank (AF362996 from Canada and AF319019 from Oregon) by 3 bp. Divergences in the *L. saccharina* group were up to 0.3% (Table 4). In comparison a nucleotide divergence of 3.1% (Table 4) was observed between *L. digitata* and *L. hyperborea*. *Laminaria faeroensis* and *L. saccharina* showed a divergence of c. 22% from *L. digitata* and *L. hyperborea*.

AFLP data

A total of 197 different AFLP fragment size categories were observed with fragment lengths ranging from 38 bp to 630 bp inclusive of adapter-ligated ends. Only half the number of AFLP fragments was scored in the group comprising *L. faeroensis* and *L. saccharina* compared with *L. digitata* and *L. hyperborea* (Table 5). In the *L. faeroensis* group it was possible to score only 26 unambiguous AFLP fragments for *L. faeroensis* ID 72 due to weak PCR amplification compared to 40 and 41 for the two other specimens in the *L. faeroensis* group. In specimens of *L. digitata* two AFLP fragments 110 bp and 210 bp long were amplified very strongly and are unique for the species. A 273 bp AFLP fragment amplified very strongly in both *L. digitata* and *L. hyperborea* (Fig. 13), whereas a 214 bp fragment was present only in *L. faeroensis* and in *L. saccharina* ID 78 from the Faroe Islands. Even when PCR amplification was weak, e.g. *L. digitata* ID 48 shown in Fig. 13, AFLP fragments specific for *L. digitata* were scored for this individual, e.g. fragments 110 bp and 210 bp long, but the specimen was excluded from further analyses.

In order to test the reproducibility of the AFLP fingerprints, two samples of template DNA extracted from *L. digitata* ID 55 and *L. faeroensis* ID 71 were processed independently through the AFLP technique. The electropherograms of *L. digitata* ID 55 were identical but the electropherograms of *L. faeroensis* ID 71 had a difference of two AFLP fragments. This corresponds to a

Table 4. Average pairwise nucleotide divergence (substitutions per site) of ITS rDNA sequences between *Laminaria* species, based on 2–4 sequences for each species

Species	Samples	1	2	3	4
1 *L. digitata*	3	–			
2 *L. hyperborea*	3	0.031	–		
3 *L. saccharina*	4	0.219	0.227	–	
4 *L. faeroensis*	2	0.221	0.229	0.003	–

Table 5. Average number (m) of AFLP fragments scored per individual

Species	Samples	m	sd	m_{min}	m_{max}
L. digitata	20	63.4	4.35	59	70
L. hyperborea	15	63.7	12.05	42	87
L. saccharina	5	26.4	5.27	21	35
L. faeroensis	3	35.7	8.39	26	41

m = average number of AFLP fragments scored per individual.
sd = standard deviation of m
m_{min} = smallest number of AFLP fragments scored for a particular individual.
m_{max} = largest number of AFLP fragments scored for a particular individual.

proportion of shared fragments (similarity) of F = 0.95 to F = 1.00. Similar results F = 0.95 with an observed nucleotide divergence of 0.3% were observed for *L. digitata* ID 05 and ID 06 (Fig. 5), which probably are clonal individuals.

The methods of Mougel *et al.* (2002) and Innan *et al.* (1999) for estimating nucleotide diversity from AFLP data gave nearly equal values of nucleotide diversity within groups of species (Table 7). The neighbour-joining (NJ) tree (Fig. 14) and bootstrap values constructed based on each model gave the same topology. After resampling 85% of the AFLP fragments randomly chosen among the 197 AFLP fragment size categories with replacement, all clades supported by bootstrap values in Fig. 14 were maintained in the NJ trees (not shown).

Laminaria digitata, L. hyperborea and *L. saccharina* plus *L. faeroensis* were resolved as clades with high bootstrap values ($\geqslant$ 99%). Within the *L. saccharina* clade, the three specimens of *L. faeroensis* formed a monophyletic group with a bootstrap value of 73%, sister to a *L. saccharina* group supported by a relatively high bootstrap value of 93%.

In the *L. hyperborea* and *L. digitata* clades only a few sub-groups were well supported, but the two specimens in the *L. digitata* group from the Faroe Islands were closely related, as were some other specimens collected together (e.g. ID 11 and ID 12). In the *L. hyperborea* clade only one group consisting of two specimens from Tønneberg Banke (ID 27 and ID 29, respectively) received high bootstrap support (97%).

Average nucleotide divergence within samples of *L. digitata* and *L. hyperborea* ranged from 1.4–2.5% and in samples of *L. faeroensis* and *L. saccharina* from 1.5–4.7% (Table 6). Between putative species the smallest evolutionary distance 3.8% was between *L. faeroensis* and *L. saccharina* compared to 6.8% between *L. digitata* and *L. hyperborea* (Table 7). The evolutionary distances between other pairwise combinations were from 10–11% (Table 7).

Our molecular data did not reveal genetic differences despite a large phenotypic variability. *Laminaria digitata* ID 52 in Fig. 3 represents the *genuina*-type, and *L. digitata* ID 48, ID 12, ID 56 and ID 55 (Figs 4, 9, 10, 11) represent the *intermedia*-type (Rosenvinge & Lund, 1947). Extracts from the electropherograms of these specimens are shown in Fig. 13. The NJ tree in Fig. 14 shows that these specimens did not cluster so as to indicate the presence of the *genuina*- or *intermedia*-types.

Rosenvinge & Lund (1947) reported that *L. hyperborea* plants sometimes have laminae divided into only a few segments and, therefore, show resemblance to a form of *L. digitata* described as *L. digitata* f. *cucullata* (Fig. 9). The difficulty in identification is illustrated by the specimens shown in Fig. 5, which have long, relatively stiff stipes, typical for specimens of *L. hyperborea*. The two plants to the right in Fig. 5, which are *L. digitata* (ID 05 and ID 06), resemble the left plant *L. hyperborea* ID 04 which they were attached with, but they can clearly be distinguished by AFLP fingerprints (Fig. 13). This illustrates how difficult it can be to distinguish the two species if taken from the overlapping zone in the sublittoral where these three specimens were found and collected in the beginning of February. It can be seen that *L. hyperborea* has a more divided lamina than the two *L. digitata* plants and a new lamina has just started to develop, while the two *L. digitata* plants have a much more extended development of the new laminae.

Our results show that partial LSU and ITS rDNA sequences were identical for *L. faeroensis* ID 71 (Fig. 6) and *L. saccharina* ID 54 (Fig. 8). However, the AFLP technique distinguished them from each other (Fig. 13). The two principal types of *L. saccharina*, the *membranacea*-type and the *bullata*-type (Rosenvinge & Lund, 1947), are represented by *L. saccharina* ID 40 (Fig. 7) and *L. saccharina* ID 54 (Fig. 8), respectively. In Fig. 13 it can be seen that these two morphotypes have a very similar AFLP fingerprint and clustered together in the NJ tree (Fig. 14).

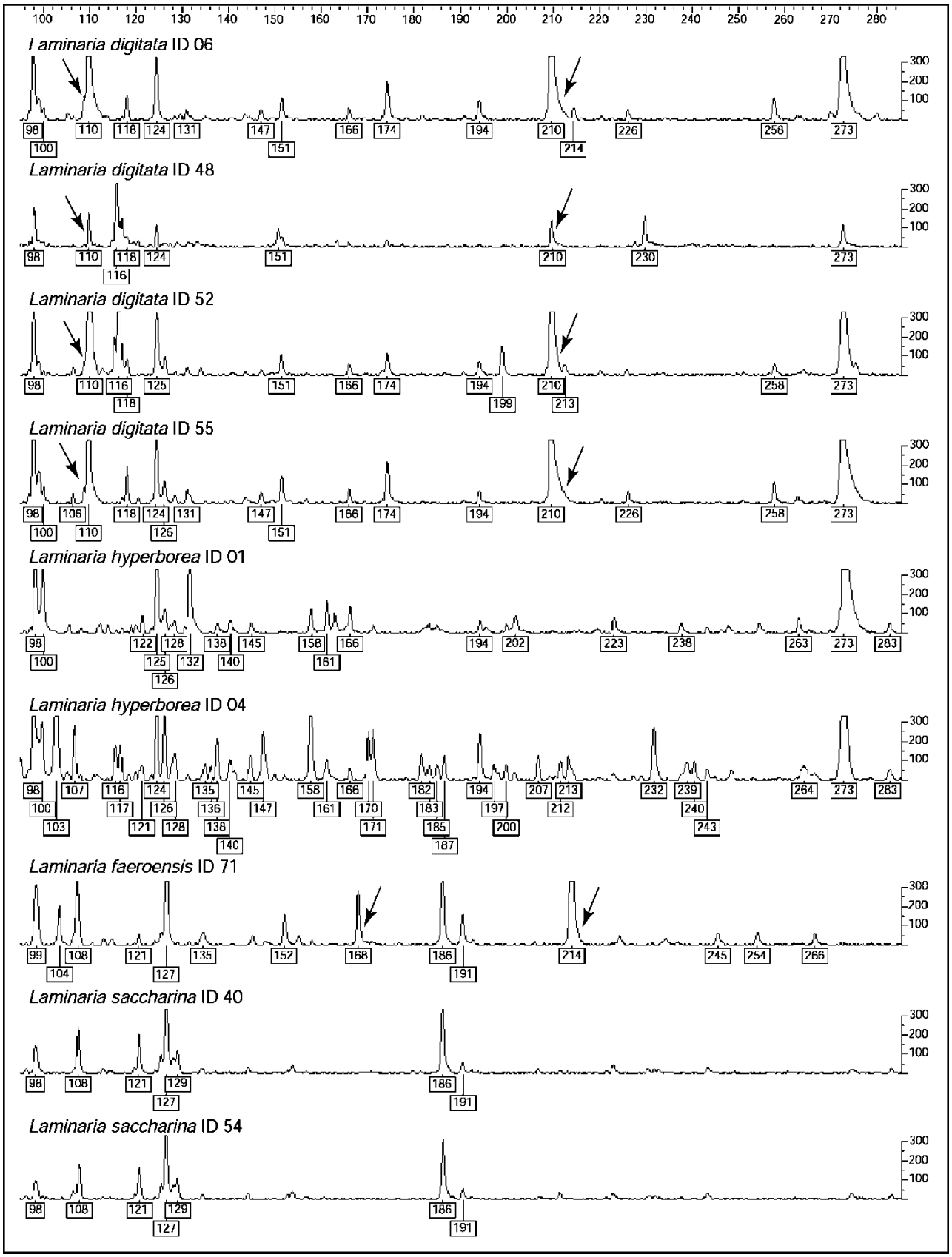

Fig. 13. Extracts from electropherograms from the ABI Prism 377 DNA sequencer for nine selected specimens. X-axis: fragment size in bp. Y-axis: intensity of fluorescence in scanning points of a fragment on the gel. Sizes of fragments (numbers in boxes rounded to the nearest integer) were labelled using the program Genotyper 2.1. The program labelled only fragments higher than 50. Arrows indicate species-diagnostic fragments, except the fragment 214 bp long, which also was amplified in the specimen of *L. saccharina* ID 78 from the Faroe Islands.

Table 6. Average nucleotide diversity within the species estimated from AFLP data of 43 specimens of *Laminaria*, using the model of Innan *et al.* (1999)

Species	Samples	π_1	π_2
L. digitata	20	0.014	0.023
L. hyperborea	15	0.014	0.025
L. saccharina	5	0.015	0.032
L. faeroensis	3	0.019	0.047

π_1: nucleotide diversity calculated assuming that *Laminaria* species are diploid selfing organisms.
π_2: nucleotide diversity calculated assuming that populations of *Laminaria* species are in Hardy-Weinberg equilibrium.

Table 7. Average pairwise nucleotide divergence (substitutions per site) between the species estimated from AFLP data of 43 specimens of *Laminaria*. Numbers above and below the diagonal are based on the models of Mougel *et al.* (2002) and Innan *et al.* (1999), respectively.

Species		Samples	1	2	3	4
1	*L. digitata*	22	–	0.059	0.093	0.086
2	*L. hyperborea*	15	0.068	–	0.094	0.092
3	*L. saccharina*	6	0.109	0.112	–	0.041
4	*L. faeroensis*	3	0.101	0.113	0.038	–

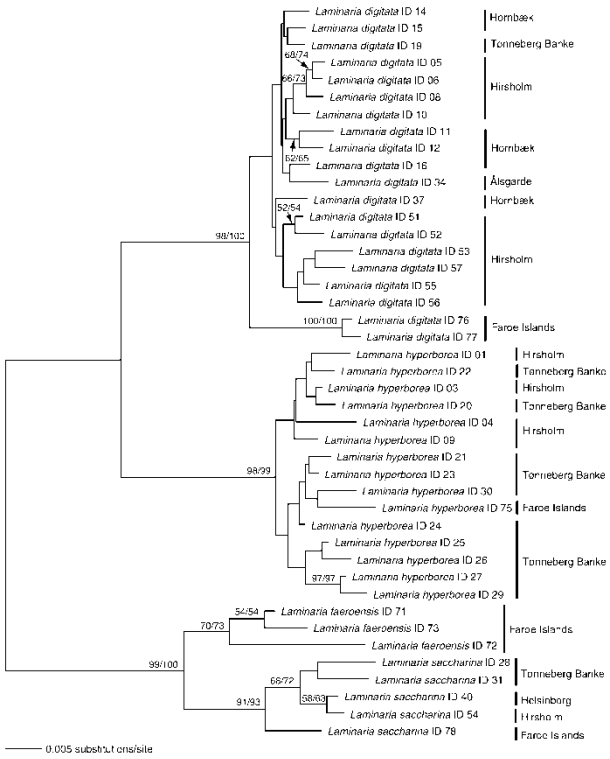

Fig. 14. An unrooted neighbour-joining tree based on AFLP data from 197 different fragment size categories, illustrating the nucleotide diversity within and between four *Laminaria* species. Nucleotide diversity was estimated using the model of Mougel *et al.* (2002) and corrected with the Jukes & Cantor (1969) substitution model. *Laminaria faeroensis* formed a monophyletic group in the *L. saccharina* clade. Bootstrap values are based on 1000 replications with 85% reduced resampling (Felsenstein, 1985; Mougel *et al.*, 2002) and with 100% resampling. Only values above 50% are given.

Discussion

Reproducibility of AFLP fragments

In order to test the reproducibility of the AFLP procedure, replicates of template DNA extracted from the same individuals were processed independently through the AFLP technique. The AFLP fingerprints for the same individual in this study ranged from 95% to 100% corresponding to a value of nucleotide diversity $\leqslant 0.3\%$. This can be considered an estimate of inaccuracy in our AFLP analysis and the results fall in the same range observed in other studies (e.g. Arens *et al.*, 1998; Winfield *et al.*, 1998). Differences in AFLP fingerprints from the same individual can perhaps be due to contamination of DNA from foreign organisms of bacterial, faunal or floral origin. Yet another possibility explaining the additional AFLP fragments is the occurrence of endophytic brown algae known in *L. saccharina*. Heesch & Peters (1999) have reported that the infection frequency in nature may be up to 100%. Contamination due to genomes from endophytic brown algae could be a problem in molecular studies such as AFLP analyses if some populations are more or less infected, and especially if the endophytes are different taxa or even the same taxon but one that is also genetically variable. However, as mentioned earlier individuals with extra AFLP fragments due to contamination with foreign DNA or with fewer unambiguous AFLP fragments due to a weak PCR amplification could still be identified to species level (e.g. *L. digitata* ID 48, Fig. 13).

Size homoplasy and consequence of insertions and deletions

Issues that should be considered are fragment type and fragment size homoplasy. The mathematical model by Innan *et al.* (1999) deals with both *Eco*RI-*Eco*RI and *Eco*RI-*Mse*I fragments and also takes size homoplasy into account. In the model developed by Mougel *et al.* (2002) only *Eco*RI-*Mse*I fragments are incorporated and size homoplasy is not taken into account. Instead they provide a correction factor for fragment dependence. In this study we have reduced the problem concerning *Eco*RI-*Eco*RI fragments by using *Eco*RI and *Mse*I primers with three selective

nucleotides. According to Innan *et al.* (1999) the expected ratio of numbers of *Eco*RI-*Eco*RI fragments to that of the *Eco*RI-*Mse*I fragments is $0.25^9/(2 \times 0.25^7) = 1/32 = 3.1\%$. Therefore it was mainly *Eco*RI-*Mse*I fragments that were amplified in the present work.

Another assumption necessary for the use of both mathematical models is that differences in homologous DNA sequences are caused only by substitutions. Differences caused by insertions and deletions are ignored in both models, which may lead to an overestimation of the nucleotide diversity because some of the AFLP fragments are homologous and only differ by an insertion or deletion of a few bp.

Fragment dependence

If a substitution causes a new *Eco*RI restriction site to appear inside an AFLP fragment and thereby results in two AFLP fragments, then these fragments are not independent. This seems not to be a major problem, as long as the selective amplification was performed with 3 selective nucleotides in both primers, because it is very unlikely that a 9 bp sequence, which has a probability of 0.25^9, exists inside an AFLP fragment consisting of only a few hundred bp. Correction for fragment dependence as described by Mougel *et al.* (2002) was not performed, as it requires comprehensive knowledge of the sequences in question. Instead, bootstrap analyses with reduced resampling (Felsenstein, 1985; Mougel *et al.*, 2002) were conducted to test the robustness of the inferred phylogeny.

Genetic diversity

Our AFLP data revealed no genetic differentiation between specimens of *L. digitata* sampled in region 1 and 2 (Fig. 1), separated by a distance of 200 km. This result is not in agreement with the findings in Billot *et al.* (2003), which found that continuous forests of *L. digitata* were genetically differentiated at distances greater than 10 km. However, they also observed that isolated habitats have reduced genetic variability, and this could explain the lack of population structure in *L. digitata* from Kattegat. This area is geographically relatively small, connected by south- and northward currents dependent on wind, and geologically young. The brackish Baltic Sea arose after the last glacial period. At ca. 8000 years ago, the sea level rose and marine water eroded the barrier, which separated marine water from the freshwater Ancylus Lake (Björck, 1995). All marine organisms present today from Kattegat to the Baltic Sea must have entered after this geological event. Therefore all specimens of *L. digitata* sampled from Kattegat probably belong to the same population, which has entered Kattegat recently. The small area and the short period of time explain the lack of genetic differentiation, and also the total lack of population structure in *L. hyperborea*.

In contrast, specimens of *L. digitata* from Danish coastal waters and specimens of *L. digitata* from the Faroe Islands were distinguishable, supported by bootstrap values of 100%. This result is only based on two specimens from the Faroe Islands and is only a weak indication that the populations of *L. digitata* from Danish coastal waters are genetically different from the populations of the Faroe Islands. Further studies are necessary to explore this in greater detail.

The AFLP technique distinguishes *L. faeroensis* from *L. saccharina* (Fig. 13) as shown in the phylogenetic tree (Fig. 14) where the *L. faeroensis* and *L. saccharina* clades were supported by bootstrap values of 73% and 93%, respectively. The clustering of *L. saccharina* ID 78 in Fig. 14 indicated that the grouping of *L. faeroensis* was not a result of geographic separation. These results indicate that *L. faeroensis* seems to be in the beginning of its speciation process and it seems reasonable to give *L. faeroensis* taxonomic status at the subspecies level, as *L. saccharina* subsp. *faeroensis*.

Phenotypic variability

Our data, showing no relationship between genetics and morphology, support earlier suggestions that phenotypic variability in *Laminaria* is to some extent environmentally induced (Sundene, 1962, 1964). The plants shown in Figs 4 and 10 resemble *L. saccharina* but have DNA fingerprints typical of *L. digitata* (see Fig. 4). This demonstrates how difficult it can be to distinguish between different forms of *L. digitata* and *L. saccharina* from Denmark (compare Figs 4 and 10 with 7 and 8), so that identification of specimens in the field requires caution. Furthermore, it is also evident that it is difficult to distinguish *L. hyperborea* from *L. digitata* based on the stipe character in the overlapping distribution area. We consider it likely that morphotypes of *L. hyperborea*, with the lamina divided into a few, but wide segments and considered by Rosenvinge & Lund (1947) to resemble *L. digitata* f. *cucullata* do not exist. The plant they observed is similar to the two specimens of *L. digitata* shown in Fig. 5.

The two principal morphotypes of *L. saccharina*, the *membranacea*-type and the *bullata*-type (Rosenvinge & Lund, 1947), represented by *L. saccharina* ID 40 and ID 54 (Figs 7 and 8), have a very

similar AFLP fingerprint (Fig. 13). Hence, dividing Danish specimens of *L. saccharina* into these types is not supported by our molecular data.

Concluding remarks

We hope to have demonstrated the usefulness of the AFLP technique to provide phylogenetic information. Data from rDNA sequences were generally in agreement with data from the AFLP fragments, but it was not possible to distinguish between *L. saccharina* and *L. faeroensis* by means of ITS or partial LSU rDNA sequences.

However, the phylogenetic analyses showed that the AFLP technique was able to delineate specimens not only at the species level but also at subspecies level. These results indicate that it seems reasonable to give *L. faeroensis* taxonomic status at the subspecies level within *L. saccharina*. Furthermore, our findings show the extensive phenotypic variability that specimens of *L. saccharina* including *L. faeroensis* exhibit. This work also demonstrated how difficult it can be to distinguish *L. digitata* from *L. hyperborea*, if samples are taken in the overlapping zone in the sublittoral where both species occur. Similarly it has been shown that morphotypes of *L. digitata* with an undivided lamina resemble *L. saccharina*. Identification of specimens in the field must therefore be done with some caution.

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