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Molecular evidence for an interfamilial laminarialean cross

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When generating kelp hybrid crosses *in vitro*, small blades are frequently seen in single-sex cultures of microscopic gametophytes as well as in putative hybrid crosses. We demonstrate a polymerase chain reaction (PCR)-based method that quickly determines the presence/absence of possible parental genomes in the observed blades. ITS1 nrDNA fragments that varied in length in a species-specific way were amplified. Amplification products from possible hybrid blades, apogamic blades and parental gametophytes were compared on agarose gels. Using these methods, we were able to identify a number of apogamic blades (i.e. formed directly from gametophytic cells) as well as a true hybrid sporophyte blade in crosses between *Alaria marginata* (Alariaceae) and *Lessoniopsis littoralis* (Lessoniaceae), members of two separate families of the Laminariales. To our knowledge this is the first genotypic confirmation of a hybrid kelp.

Key words: *Alaria marginata*, hybridization, ITS1, *Lessoniopsis littoralis*, nrDNA, PCR, rDNA

Introduction

Hybridization, the crossing of two individuals from different populations, species or genera, has been an important tool in understanding evolution and taxonomy. In practice, the ability to hybridize is often used as a judge of relatedness (Lewis, 1996*b*). Versions of the biological species concept (Dobzhansky, 1937; Mayr, 1963), or various corollaries (Mallet, 1995), often at least partly define separate species as those that cannot form viable hybrids. Certain groups of organisms, such as some tracheophytes (higher plants), seem easily to form natural hybrids among closely related taxa, whereas hybridization is much rarer in animals (Levin, 1979). In the red algae, hybrids of species in *Polysiphonia* and *Mastocarpus* (as *Gigartina*) have been described (Rueness, 1973; West *et al.*, 1978). However, cellular aspects of hybridization involve complex processes, with a myriad of causes of lack of development, sterility, or death of the hybrid (Lewis, 1996*b*).

Hybridization has been examined at a number of levels in the Laminariales. Interspecific hybrids seem quite common in some genera, such as *Laminaria* and *Macrocystis* (Lewis, 1996*b*). Intergeneric hybrids (e.g. between *Macrocystis* and *Pelagophycus*) also have been identified in the field or produced artificially by crossing (Sanbonsuga & Neushul, 1978; Coyer *et al.*, 1992; Lewis & Neushul, 1995). Even hybrids between members of different kelp families have been reported (Tokida *et al.*, 1958; Cosson & Olivari, 1982). The possibility that true hybridization might occur between members of different kelp higher taxa is supported by the observation that all members of the Laminariales studied by Müller *et al.* (1985) displayed sperm chemotaxis based on lamoxirene.

Kelps exhibit a complicated life history beginning with dioecious microscopic gametophytes, and syngamy giving rise to macroscopic diploid sporophytes. After a period of rapid size increase, the large sporophytes meiotically generate motile spores (meiospores) that give rise to gametophytes after recuitment.

Doubts concerning the possible crosses arise because a gametophytic or sporophytic morphology does not necessarily correspond to a given ploidy level (Nakahara & Nakamura, 1973; Le Gal *et al.*, 1996). Processes such as autodiploidization (a spontaneous doubling of chromosome number) and apospory (the generation of gametophytes without the production of spores) can give rise to diploid gametophytes. Apogamy (the generation of sporophytes without the production of gametes) and parthenogenesis/androgenesis (development of sporophytes from unfused gametes) can generate haploid sporophytes and other unusual ploidy levels (for review see Lewis, 1996*a*). Small blades are frequently observed in cultures of male and female gametophytes of different species. It is generally impossible to determine whether these result from hybridization, parthenogenesis or androgenesis, apogamy, or some developmentally arrested partial hybridization. Recently, Le Gal *et al.* (1996) have shown that unisexual gametophyte cultures as well as sporophytic or gametophytic growth forms can be of various ploidy levels in the Laminariales. A reliable genotypic test for the nature of the tissues in question is needed to distinguish true from putative hybids.

The Internal Transcribed Spacer 1 (ITS1) region of the nrDNA cistron was chosen for developing such a test. In

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most eukaryotes, the nrDNA cistron occurs as tandemly repeated units of usually several hundred copies approximately proportional in number to genome size (Li, 1983). Sequences for the highly conserved flanking 18S and 5.8S genes in kelps are currently available from GenBank. Only the ITS1 sequences show variation amongst the recognized kelp species and genera and can be used to investigate the various possible crosses within the Laminariales (Saunders, 1991).

We present here a method that at least partially addresses the difficulties encountered in assessing true hybrids by determining whether parental genomes are present in the hybrid offspring. We demonstrate the efficacy of this method on small blades grown in cultures containing attempted hybrid crosses between a member of the Lessoniaceae, *Lessoniopsis littoralis*, and a member of the Alariaceae, *Alaria marginata*.

Materials and methods

Cultures of *Alaria marginata* Postels & Ruprecht and *Lessoniopsis littoralis* (Tilden) Reinke used in this study (Table 1) were from our own collection. A culture identification scheme was developed for all taxa used for crossing experiments. Cultures were identified by a code consisting of the taxon (e.g. Am for *Alaria marginata*) followed by the number of the sporophyte from which the gametophyte was collected (e.g. 1), separated by a comma from a numeral to differentiate various gametophytes from the same sporophyte (e.g. 2), followed by the sex of the gametophyte (either male or female). Note that the actual *Lessoniopsis littoralis* culture codes in the Bamfield Marine Station collection begin with 'Ll', as opposed to 'L' which is utilized here for clarity.

Table 1. Clone, species, and general morphology of plants of *Alaria marginata* and *Lessoniopsis littoralis* from which DNA was extracted.

Species	Strain(s) used	Morphology
Clones		
Alaria marginata	Am1,1 female	Gametophyte
Alaria marginata	Am1,2 female	Gametophyte
Alaria marginata	Am1,3 female	Gametophyte
Alaria marginata	Am1,1 male	Gametophyte
Alaria marginata	Am1,2 male	Gametophyte
Alaria marginata	Am1,3 male	Gametophyte
Lessoniopsis littoralis	L1,1 female	Gametophyte
Lessoniopsis littoralis	L1,2 female	Gametophyte
Lessoniopsis littoralis	L1,3 female	Gametophyte
Lessoniopsis littoralis	L1,1 male	Gametophyte
Lessoniopsis littoralis	L1,2 male	Gametophyte
Lessoniopsis littoralis	L1,3 male	Gametophyte
Self-crosses		
Alaria marginata	Am1,1 female × Am1,1 male	Sporophyte
Alaria marginata	Am1,2 female × Am1,2 male	Sporophyte
Alaria marginata	Am1,3 female × Am1,3 male	Sporophyte
Lessoniopsis littoralis	L1,1 female × L1,1 male	Sporophyte
Lessoniopsis littoralis	L1,2 female × L1,2 male	Sporophyte
Lessoniopsis littoralis	L1,3 female × L1,3 male	Sporophyte
Attempted intergeneric crosses		
A. marginata × *L. littoralis*	Am1,1 female × L1,1 male	Sporophyte
A. marginata × *L. littoralis*	Am1,2 female × L1,2 male	Sporophyte
A. marginata × *L. littoralis*	Am1,3 female × L1,3 male	Sporophyte
A. marginata × *L. littoralis*	Am1,1 male × L1,1 female	Sporophyte
A. marginata × *L. littoralis*	Am1,2 male × L1,2 female	Sporophyte
A. marginata × *L. littoralis*	Am1,3 male × L1,3 female	Sporophyte

Both species were initially isolated from Barkley Sound, B.C., Canada on the 18th of January, 1990 and maintained in culture at the Bamfield Marine Station by L. Druehl. Note that the actual *Lessoniopsis littoralis* cultures in the Bamfield Marine Station collection begin with the two letter taxa designation 'Ll' (e.g. Ll1,1), as opposed to 'L' which is utilized here for readability.

Crosses

All equipment used in manipulating and storing gametophytes was initially soaked in 500 mM HCl overnight, rinsed thoroughly in distilled water, and then autoclaved for 30 min at 1·1 kg cm^{-2} and 121 °C. Three independently isolated male and female gametophytes of *A. marginata* and *L. littoralis* from previously generated single-sex, unialgal cultures were ground in cooled depression grinding plates with a glass stirring rod until no filaments were visible to the naked eye. The grinding plates were pre-cooled to −20 °C before use, then allowed to warm until the external frost melted, to maintain a temperature as close to 4–10 °C as possible during grinding. Approximately 95 % of the gametophyte was ground up, the other 5 % being allowed to propagate vegetatively under red light conditions. Subsequently 200 μl of 4 °C f/2 medium was added to each depression and then mixed with the ground-up gametophyte. About half of the resulting dilution was added to 2 ml 4 °C f/2 medium in a 35 mm × 10 mm sterile Petri dish (Falcon #1008). The same procedure was repeated for the individual of the opposite sex or other taxon used in the cross, and the ground-up alga was added to the same Petri dish. Sporophyte cultures and crosses were kept under 150 μmol m^{-2} s^{-1} cool-white fluorescent light (16:8 photoperiod) at 13 °C for about six weeks. GeO_2 was added to a concentration of 500 μg l^{-1} in cultures showing signs of diatom contamination (Chapman, 1973).

In total, seven reciprocal crosses were attempted for *A. marginata* and *L. littoralis* gametophytes (Table 2). Reciprocal crosses between strains Am1,1 and L1,1 were replicated three times, as were reciprocal crosses between Am1,2 and L1,2. Reciprocal crosses between Am1,3 and L1,3 were not replicated. Seven self-crosses, involving three males and three females, were attempted for *A. marginata*, and six self-crosses, also involving three males

Table 2. *Alaria marginata* and *Lessoniopsis littoralis* gametophyte crossing attempts and resulting sporophyte morphologies

Cultures used	No. of replicates	No. of crosses or cultures with sporophyte morphology
Female gametophytes		
Am1,1	3	3
Am1,2	3	0
Am1,1	1	0
L1,1	3	0
L1,2	3	3
L1,3	1	1
Male gametophytes		
Am1,1	3	0
Am1,2	3	0
Am1,3	1	0
L1,1	3	2
L1,2	3	0
L1,3	1	1
Self-crosses		
Am1,1 female × Am1,1 male	3	1
Am1,2 female × Am1,2 male	3	0
Am1,3 female × Am1,3 male	1	0
L1,1 female × L1,1 male	2	1
L1,2 female × L1,2 male	3	1
L1,3 female × L1,3 male	1	1
Intergeneric crosses		
Am1,1 female × L1,1 male	3	1
Am1,2 female × L1,2 male	3	0
Am1,3 female × L1,3 male	1	1
Am1,1 male × L1,1 female	3	2
Am1,2 male × L1,2 female	3	0
Am1,3 male × L1,3 female	1	1

Cultures listed with only parental culture code were set up as uniclonal cultures of individual gametophytes. The second column denotes the total number of replica crosses or uniclonal cultures started. The third column lists the number of replica crosses or uniclonal cultures with observed sporophyte morphologies.

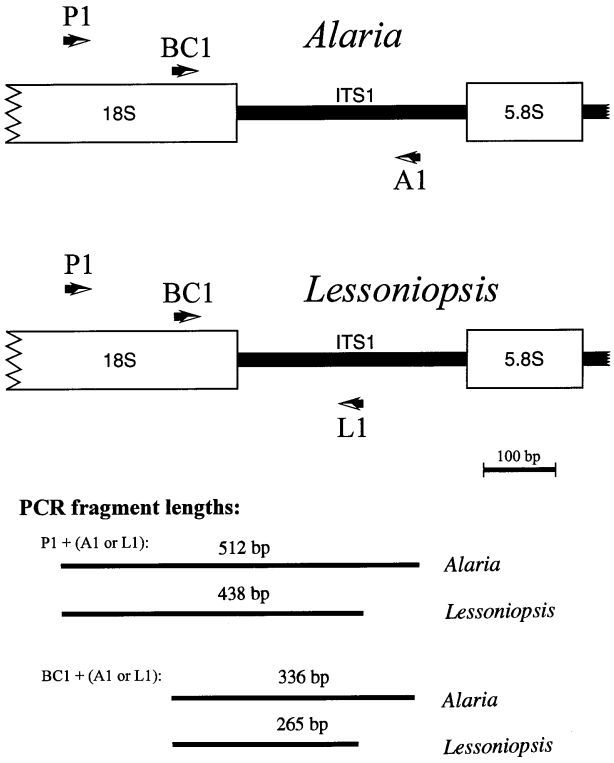

Fig. 1. Location of PCR primers and resulting fragment lengths for *Alaria* and *Lessoniopsis* rDNA. The 3′ end of the 18S subunit and the entire ITS1 and 5.8S subunits are shown. All possible fragment lengths from PCR using either of the forward primers (P1 or BC1) with each species-specific reverse primer (A1 OR L1) are also noted. Lengths are to scale except for the PCR primers, which are enlarged for clarity.

and three females, for *L. littoralis*. Single male and female unisexual cultures were also established as controls for each of the seven crossing attempts.

Cultures were checked on a weekly basis under × 100 magnification on an inverted microscope for the presence of blades and for any signs of diatom contamination. Individual blades were carefully dissected away from the gametophyte and placed in separate 1.8 ml micro-centrifuge tubes for DNA extraction.

DNA extraction

The gametophyte DNA extraction method of Mayes *et al.* (1992) was utilized with the following modifications. Proteinase K digestions in 300 μl of proteinase K buffer (50 mM EDTA, 100 mM Tris pH 8·5, 200 mM NaCl and 1% SDS) were performed at 20 °C for 2 h to reduce possible melting of algal polysaccharides. Organic extractions were carried out first with 0·5 volumes of phenol (TE-saturated and pH 7·6 Tris-equilibrated) (Sambrook *et al.*, 1989), without centrifugation or removal of the aqueous phase, and then with 0·5 volumes of chloroform/isoamyl alcohol (IAA) (24:1) followed by subsequent vortexing and centrifugation. One final extraction was done with chloroform/IAA (24:1) as described in Mayes *et al.* (1992).

Polymerase Chain Reactions (PCR)

Two separate taxon-specific reverse primers of nearly equal hypothetical melting temperature (T_m) were constructed from sequence data in Saunders (1991) for the ITS1 region of the ribosomal cistron. These were designed to preferentially amplify DNA from only one of the two species. The primer designed for *A. marginata*, A1, was 5′-GAGCCGCGCCCGGTAAAG-3′, and that for *L. littoralis*, L1, was 5′-GCGCTTTGATTCGAGAGACC-3′. These reverse primers were used in PCR reactions (Kleppe *et al.*, 1971; Saiki *et al.*, 1988) with one of two forward primers: P1, 5′-TAATCTGTTGAACGTGCATCG-3′, or BC1, 5′-GATTCCGGACTGTGGCTCGCG-3′, which anneal to sequences in the 18S subunit common to all known kelp (Saunders, 1991). BC1 anneals beginning 126 bp upstream of the 5′ end of the ITS1 region while P1 anneals further upstream, beginning 302 bp from the 5′ end of ITS1 (Fig. 1). For *A. marginata* the expected band sizes were 512 or 336 bp, and for *L. littoralis* 438 or 265 bp, depending upon which forward primer was used.

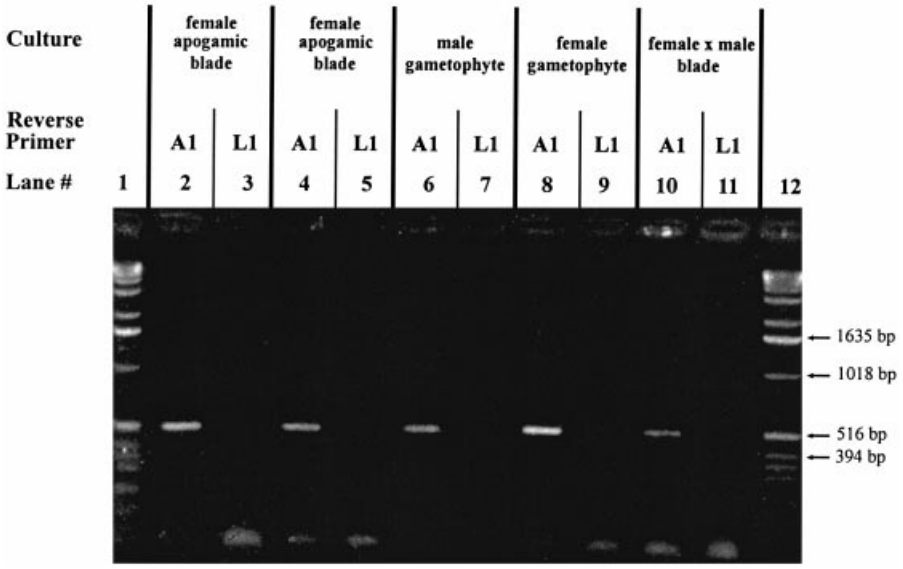

Fig. 2. Agarose gel from PCRs of DNA extracted from *Alaria marginata* tissues. Lanes 1 and 12 are 500 ng 1 kb DNA marker. The lanes are in pairs in which the first is from a PCR with the forward primer (P1) and the *Alaria* reverse primer (A1), and the other is from a PCR with the forward primer (P1) and the *Lessoniopsis* reverse primer (L1). Tissues used were as follows. Lanes 2, 3: blade in female gametophyte culture; lanes 4, 5: blade in the same female culture; lanes 6, 7: male gametophyte; lanes 8, 9: female gametophyte; lanes 10, 11: blade in self-cross. In all cases only the *Alaria* reverse primer (A1) when paired with the forward primer P1 produced a band. All bands were of the expected size of 512 bp.

Twenty-five microlitre reactions were used with 10.25 μl sterile ddH_2O, 2·5 μl 10 × PCR buffer (500 mM KCl, 100 mM Tris-Cl pH 8·3 and 0·1% gelatin), 2.5 μl 25 mM $MgCl_2$, 1·25 μl P1 or BC1 primer (20 μM stock), 1.25 μl of each reverse primer from 20 μM stocks (L1 or A1), 5 μl dNTPs (1.25 mM each stock), 2·0 μl template, and 0·25 μl Taq polymerase (Perkin Elmer-Cetus). A modified 'hot start' procedure (Erlich *et al.*, 1991) was used wherein the polymerase was added after pausing the thermocycler during the 67 °C step of the melting cycle. PCR conditions were one initial melting cycle (95 °C for 10 min, 67 °C for 30 s, and then 72 °C for 45 s), followed by 30 amplification cycles (94 °C for 30 s, 66 °C for 30 s, and then 72 °C for 45 s), and finally an extra extension step (72 °C for 5 min). All PCRs from a hybrid cross were done concurrently using a master mix to eliminate yield variations based on differing component ratios. PCR products were run on 15 cm 0·9% agarose/TAE gels (Sambrook *et al.*, 1989), post-stained for 15 min in a 250 ng ml^{-1} ethidium bromide/TAE bath, and then destained in ddH_2O for 10 min.

PCR amplifications of both male and female gametophyte tissue, sporophyte-like blades in single-sex cultures (if seen), possible hybrid blades and self-crosses (where successful) used a forward primer and each species-specific reverse primer pair in blind tests. In addition, two pseudo-hybrids corresponding to the two observed hybrids were constructed by extracting both parental types from unialgal cultures in one tube. These pseudo-hybrids were then PCR-amplified under the same conditions as the putative hybrid blades.

PCR amplification was also carried out on DNA of blades from self-crosses of the two taxa (A1,1 and L1,1) and a hybrid cross (Am1,1 male × L1,1 female) using both reverse primers (A1 and L1) with the forward primer, P1, in the same reaction. All PCRs were performed at the same time in a parallel fashion using the same master mix (cocktail) and conditions as above, including a no-template and a positive PCR control.

Subsequent PCR amplification of blades seen in actual crosses was performed once the predictive power of the tests proved reliable. PCRs of blades in the actual hybrid crosses were run with the two forward primers and each species-specific primer pair separately.

Results

Sporophyte morphologies developed in 5 of the 14 attempted hybrid crosses (Table 2). Sporophyte morphologies were also seen in 4 female cultures and 3 male cultures of the 28 single-sex cultures as well as in 4 of the 13 self-crosses (Table 2). Blades identified in uniclonal cultures of gametophytes were not noticeably different from self-cross sporophytic blades at the small sizes examined (approximately 50–300 cells).

Alaria marginata (strain Am1,1) genomic DNA from putative sporophyte blades, female apogamic or parthenogenic blades as well as individual male and female gametophytes gave bands of the expected size (512 bp) only in PCR reactions using the *A. marginata*-specific primer Am1 and P1, but not when using the *L. littoralis*-specific primer L1 and P1 (Fig. 2).

DNA from *Lessoniopsis littoralis* (strain L1,1) male gametophytes and female gametophytes gave bands of the expected size (438 bp) using primer pair L1 and P1 (Fig. 3). The apogamic male blade (also from L1,1) gave no

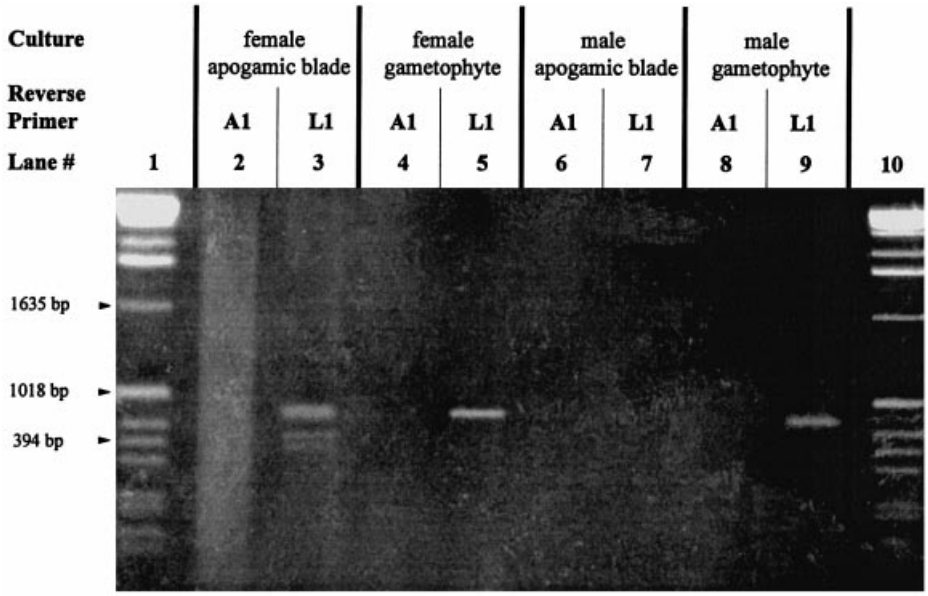

Fig. 3. Agarose gel from PCRs of DNA extracted from *Lessoniopsis littoralis* tissues. Lanes 1 and 10 are 500 ng 1 kb DNA marker. The lanes are in pairs in which the first is from a PCR with the forward primer (P1) and the *Alaria* reverse primer (A1), and the other is from a PCR with the forward primer (P1) and the *Lessoniopsis* reverse primer (L1). Tissues used were as follows. Lanes, 2, 3: blade in female gametophyte culture: lanes 4, 5: female gametophyte; lanes 6, 7: blade in male gametophyte culture; lanes 8, 9: male gametophyte. Lanes 2 and 3 were from a blade seen in a diatom-encrusted culture. Lane 2 has a smear representing many DNA sizes with no visible bands, probably due to too much genomic DNA. Lane 3, from the same diatom-encrusted culture, has three bands, the highest of which is the expected size (438 bp). Lanes 6 and 7, from a small apogamic blade in a male gametophyte culture, did not show any products. Lanes 4, 5, 8, and 9 only show bands (all of the expected size, 438 bp) in PCR reactions containing the *Lessoniopsis* reverse primer.

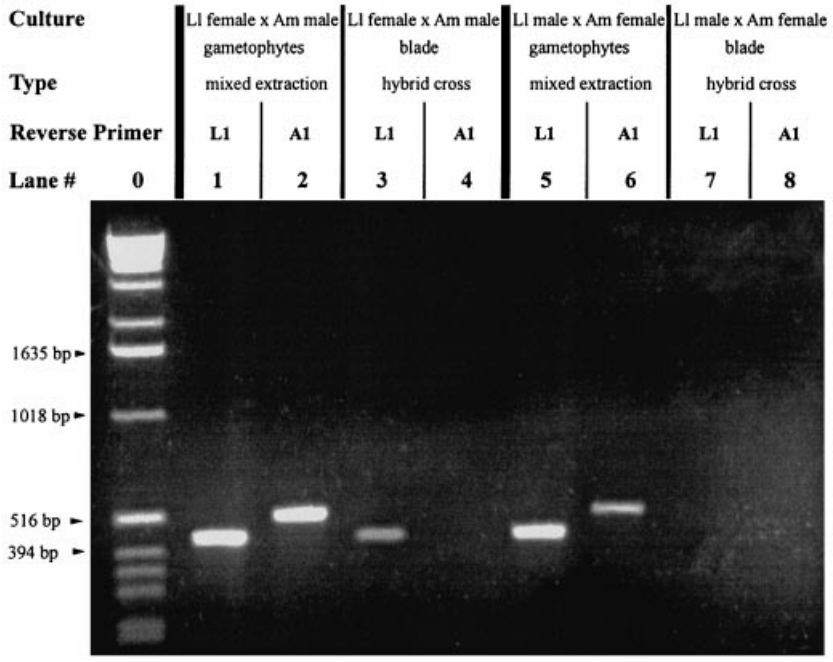

Fig. 4. Agarose gel from PCRs of DNA extracted from kelp gametophyte crosses and pseudo-crosses. Lane 0 is 500 ng 1 kb DNA marker. The lanes are in pairs in which the first is from a PCR with the forward primer (P1) and the *Alaria* reverse primer (A1), and the other is from a PCR with the forward primer (P1) and the *Lessoniopsis* reverse primer (L1). Pseudo-crosses were mixed extractions containing tissues from both gametophytic parents used in the adjacent true cross. Lanes 2–4 were from a cross or pseudo-cross of a *Lessoniopsis littoralis* female and an *Alaria marginata* male. Lanes 5–8 were from a cross or pseudo-cross of a *Lessoniopsis littoralis* male and an *Alaria marginata* female. The pseudo-crosses show both expected band sizes (438 bp for *Lessoniopsis* and 512 bp for *Alaria*. The 60-celled blade seen in a hybrid cross between a *Lessoniopsis littoralis* female and an *Alaria marginata* male (lanes 3 and 4) only had a band in the PCR with the *Lessoniopsis* reverse primer (L1) and the forward primer.

results, probably due to insufficient genomic DNA in the extraction. One L1,1 female culture contained only a few small diatom-covered blades and showed a band on the PCR of the expected size along with some faint smaller bands (though no bands at the *A. marginata* size of 512 bp) (Fig. 3).

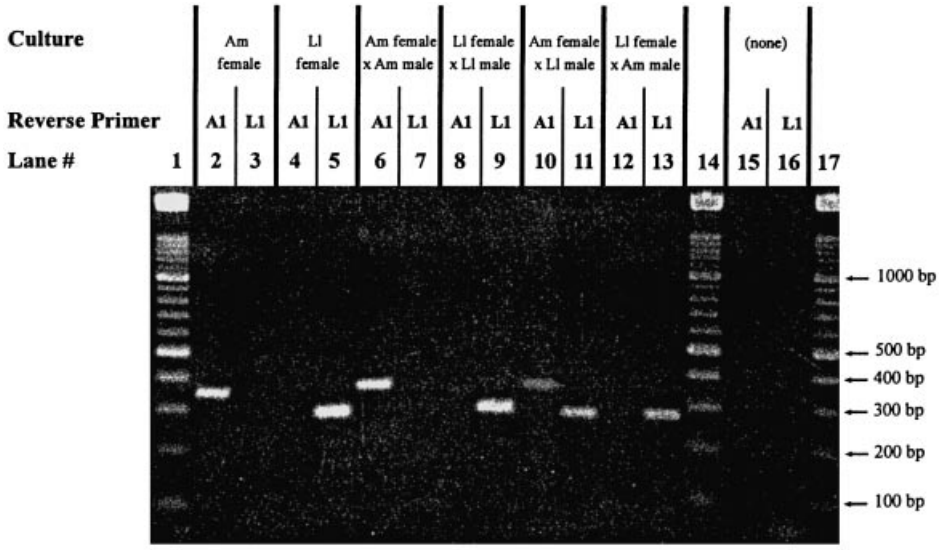

Fig. 5. Agarose gel from PCRs of DNA extracted from kelp gametophyte cultures, self-crosses and blades from two hybrid crosses. Lanes 1, 14 and 17 are 500 ng 100 bp molecular marker. The lanes are in pairs in which the first is from a PCR with the forward primer (BC1) and the *Alaria* reverse primer (A1), and the other is from a PCR with the forward primer (BC1) and the *Lessoniopsis* reverse primer (L1). Lanes 15 and 16 are negative controls containing PCRs without any template DNA. Lanes 2–5 are positive controls from individual female gametophytes and show bands of 336 bp for *Alaria* and 265 bp for *Lessoniopsis* as expected. Lanes 6–9 contain PCRs from each self-cross and also show the expected sizes of bands. The presence of both *Alaria*-specific and *Lessoniopsis*-specific bands in lanes 10 and 11 confirmed the hybrid nature of one blade examined from a cross of an *Alaria* female and a *Lessoniopsis* male. However, the presence of only the *Lessoniopsis*-specific band in lanes 12 and 13 suggests the other blade examined from the reciprocal cross of a *Lessoniopsis* female with an *Alaria* male was not a true hybrid.

Genomic DNAs from two possible putative hybrid blades resulting from a *L. littoralis* female × *A. marginata* male cross from strains A1,1 and L1,1 and an *A. marginata* female × *L. littoralis* male cross from strains A1,3 and L1,3 were individually subjected to PCR with each separate primer pair (A1 & P1 or L1 & P1) (Fig. 4). One of the possible hybrid blades (*c.* 20-celled blade) from a *L. littoralis* male × *A. marginata* female cross gave no results (Fig. 4, lanes 7 and 8), the other small blade (c. 60-celled) from a *L. littoralis* female × *A. marginata* male cross resulted only in the appearance of the *L. littoralis* band (Fig. 4, lanes 3 and 4), suggesting parthenogenesis or apogamy. The pseudo-crosses, performed by taking individual gametophytes from the parental type strains and coextracting them in the same tube followed by PCRs as above, produced both bands as expected (Fig. 4, lanes 1, 2, 5 and 6).

Blades seen in a hybrid cross of *A. marginata* strain Am1,3 with *L. littoralis* strain L1,3 were also amplified utilizing each species-specific reverse primer (Am1 or L1) individually along with the forward primer BC1 (Fig. 5). Bands were of the size expected for apogamic blades as seen in the single-sex controls (Fig. 5, lanes 2–5). A putative hybrid blade seen in a culture containing *L. littoralis* female and *A. marginata* male only showed evidence for the *A. marginata* male parental genotype (Fig. 5 lanes 12 and 13). However, a putative hybrid blade seen in a culture of *A. marginata* female and *L. littoralis* male showed bands from both parental types (Fig. 5, lanes 10 and 11). No evidence of any incorrect bands was observed.

PCR was also attempted using three primers (P1, L1 and Am1) concurrently on templates from two possible hybrid blades and both parental gametophytes (data not shown). Bands in the parental gametophyte PCRs were of the expected size but sometimes showed faint evidence for the other band. Using all three primers only resulted in bands for six of seven replicates of PCRs from two putative hybrids and often the *L. littoralis* band was brighter than the *A. marginata* band.

Discussion

All types of gametophyte cultures and crosses of *Alaria marginata* and *Lessoniopsis littoralis*, except isolated *A. marginata* males, produced plants having sporophyte morphology. The production of blades from unisexual female cultures is common (Lewis, 1996*a*). However, there are few reports of blades arising in unisexual male cultures (Nakahara & Nakamura, 1973; Nakahara, 1984; Lewis, 1996*a*).

The species-specific primers were created based upon known ITS1 sequences of one species in each genus and designed to anneal at different distances from either universal kelp primer (Fig. 1). By designing the species-specific primers to be similar in melting temperature (T_m), a universal kelp primer of approximately the same T_m could be used. The differences in the band sizes were purposely kept at > 50 basepairs (about 10% of the overall length) because smaller differences are difficult to see on agarose gels (Sambrook *et al.*, 1989).

PCR using species-specific primers singly paired with a universal kelp primer gave consistent results, but the PCRs

performed with both reverse primers in the same reaction were often missing bands or had occasional faint spurious and questionable bands. No such false bands were seen when only one reverse primer was used. Conceivably, some bands were missing when all three primers were used because of slight competitive polymerase rate advantages magnified during the geometric growth phase of PCR. If one PCR primer pair were slightly more advantageous at the temperatures used, or if the products were either shorter or formed less rigid secondary structure, then rates of product production could vary. Both these problems were avoided by using each primer pair separately.

The parentage study indicates that most of the blades seen in cultures with attempted crosses between gametophytes from *A. marginata* and *L. littoralis* were not true hybrids containing both parental genomes. These blades may be either partial hybrids, in which a full complement of chromosomes was not inherited from both parents, all of only one parental genotype, or apogamic/parthenogenetic/androgenetic blades (Lewis, 1996*a*). However, one blade was shown to contain nrDNA ITS1 representatives of both genomes. Although growing this blade to maturity was not attempted, the method did allow for the determination of parentage in blades as small as 60 cells.

Our study is the first investigation using differentially sized PCR products from ITS1 regions as the diagnostic feature directly to determine the genetic origin of a putative hybrid brown alga relative to its supposed parents. Earlier investigations have relied on indirect evidence of chromosome number, PCR amplification of anonymous regions and/or sporophyte morphology to define successful hybridizations. It has been assumed that a doubling of chromosomes in blades arising from attempted crosses is indicative of successful sexual fusion. However, there is a possibility that autodiploidization may have occurred (Müller, 1967). Increases in ploidy levels have been documented through several generations of parthenogenetic *Laminaria japonica* (Lewis *et al*., 1993). Several studies have employed the general morphology of young blades as an indicator of their origin. Abnormal blades (mis-shapen, often consisting of irregular cell sizes and shapes, and cells having many nuclei) are often assumed to result from some non-sexual process, whereas blades having a normal morphology are usually considered the products of sexual fusion (Nakahara & Nakamura, 1973). It seems reasonable that abnormal blades may reflect unisexual or mismatched bisexual parental genomic contributions. However, it is not so clear that the origin of normal blades is restricted to successful sexual fusion (Sundene, 1958; Tom Diek, 1992). An apogamous haploid blade may undergo autodiploidization, resulting in a homozygous diploid sporophyte (Nakahara & Nakamura, 1973).

Only one blade arising from an *A. marginata* female × *L. littoralis* male cross shared both parental ITS1 nrDNA types. This suggests that a successful sexual fusion took place between representatives of two separate laminarialean families (Alariaceae and Lessoniaceae). Whether the confirmed hybrid blade could ever become fertile was not investigated. Although there were false negatives, wherein the identity of specific blades was not determined, the presence of both rDNA types in at least one example shows the power of PCR-based typing for hybrid studies.

Other putative interfamilial crosses within the Laminariales have been reported, including an apparent wild *Alaria* × *Laminaria* hybrid (Tokida *et al*., 1958) as well as *Saccorhiza* × *Laminaria* hybrids in culture (Cosson & Olivari, 1982). Interfamilial fertility is consistent with the opinion that laminarialean families, as defined on the basis of morphology, are more closely related than their taxonomic hierarchical position would indicate (Saunders, 1991; Druehl & Saunders, 1992; Druehl *et al*., 1997). The ability to form hybrids is usually associated with closely related taxa, and often is used to argue against the separation of the two taxa into distinct species (Mayr, 1963).

The findings reported here may support previous reports of hybridization between separate families of the Laminariales. The procedures developed would be useful for confirming parentage before ploidy determinations as well as other genetic or hybrid studies because of the need for only a small amount of tissue. If the inherent technical difficulties of rearing sporophytes to maturity are overcome, full-grown hybrids with parentage confirmed using the techniques described herein could be generated for further investigations.

References

Chapman, A.R.O. (1973). Methods for macroscopic algae. In *Handbook of Phycological Methods: Culture Methods and Growth Measurements*. (Stein, J. R., editor), 87–104. Cambridge University Press, Cambridge.

Cosson, J. & Olivari, R. (1982). Premiers résultats concernant les possibilités d'hybridization interspécifiques et intergénériques chez les Laminariales des côtes de la Manche. *C. R. Hebd. Séanc. Acad. Sci., Paris*, **295**: 381–384.

Coyer, J.A., Engle, J.M. & Zimmerman, R.C. (1992). Discovery of a fertile *Pelagophycus* × *Macrocystis* (Phaeophyta) putative hybrid and subsequent production of F_2 sporophytes in the laboratory. *J. Phycol*., **28**: 127–130.

Dobzhansky, T. (1937). *Genetics and the Origin of Species*. Columbia University Press, New York.

Druehl, L.D. & Saunders, G.W. (1992). Molecular explorations in kelp evolution. In *Progress in Phycological Research*, vol. 8 (Round, F. E. & Chapman, D. J., editors), 47–83. Biopress, Bristol.

Druehl, L.D., Mayes, C., Tan, I.H. & Saunders, G.W. (1997). Molecular and morphological phylogenies of kelp and associated brown algae. *Plant Syst. Evol*. (*Suppl*.), **11**: 221–235.

Erlich, H.A., Gelfand, D. & Sninsky, J.J. (1991). Recent advances in the Polymerase Chain Reaction. *Science*, **252**: 1643–1650.

Kleppe, K., Ohtsuka, E., Kleppe, R., Molineux, I. & Khorana, H.G. (1971). Repair replication of short synthetic DNAs as catalyzed by DNA polymerases. *J. Mol. Biol*., **56**: 341–361.

Le Gal, Y., Asensi, A., Marie, D. & Kloareg, B. (1996). Parthenogenesis and apospory in the Laminariales: a flow cytometry analysis. *Eur. J. Phycol*., **31**: 369–380.

Levin, D.A. (1979). *Hybridization: An Evolutionary Perspective*. Hutchinson & Ross, Stroudsburg, PA.

Lewis, R.J. (1996*a*). Chromosomes of the brown algae. *Phycologia*, **35**: 19–40.

Lewis, R.J. (1996*b*). Hybridization of brown algae: compatibility and speciation. In *Cytology, Genetics, and Molecular Biology of Algae* (Chaudhary, B.R. & Agrawal, S.B., editors). SPB Academic, Amsterdam.

Lewis, R.J. & Neushul, M. (1995). Intergeneric hybridization among five genera of the family Lessoniaceae (Phaeophyceae) and evidence for polyploidy in a fertile *Pelagophycus* × *Macrocystis* hybrid. *J. Phycol.*, **31**: 1012–1017.

Lewis, R.J., Jiang, B.Y., Neushul, M. & Fei, X.G. (1993). Haploid parthenogenetic sporophytes of *Laminaria japonica* (Phaeophyceae). *J. Phycol.*, **29**: 363–369.

Li, W.-H. (1983). Evolution of duplicate genes and pseudogenes. In *Evolution of Genes and Proteins* (Nei, M. & Koehn, R. K., editors), 14–37. Sinauer Associates, Sunderland, MA.

Lüning, K. & Dring, M.J. (1975). Reproduction, growth and photosynthesis of gametophytes of *Laminaria saccharina* grown in blue and red light. *Mar. Biol.* (*Berl.*), **29**: 195–200.

Mallet, J. (1995). A species definition for the modern synthesis. *Trends Ecol. Evol.* **10**: 294–299.

Mayes, C., Saunders, G.W., Tan, I.H. & Druehl, L.D. (1992). DNA extraction methods for kelp (Laminariales) tissue. *J. Phycol.*, **28**: 712–716.

Mayr, E. (1963). *Animal Species and Evolution*. Harvard University Press, Cambridge, MA.

Müller, D.G. (1967). Generationswechsel, Kernphasenwechsel, und Sexualität der Braunalge *Ectocarpus siliculosus* im Kulturversuch. *Planta*, **75**: 39–54.

Müller, D.G., Maier, I. & Gassmann, G. (1985). Survey on sexual pheromone specificity in Laminariales (Phaeophyceae). *Phycologia*, **24**: 475–477.

Nakahara, H. (1984). Alternation of generations of some brown algae in unialgal and axenic cultures. *Sci. Pap. Inst. Algol. Res., Fac. Sci., Hokkaido Univ.*, **7**: 77–292.

Nakahara, H. & Nakamura, Y. (1973). Parthenogenesis, apogamy and apospory in *Alaria crassifolia* (Laminariales). *Mar. Biol.* (*Berl.*), **18**: 327–332.

Rueness, J. (1973). Speciation in *Polysiphonia* (Rhodophyta, Ceramiales) in view of hybridization experiments: *P. hemisphaerica* and *P. boldii*. *Phycologia* **12**: 107–109.

Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, T.T., Mullis, K.B. & Erlich, H.A. (1988). Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science*, **239**: 487–491.

Sambrook, J., Fritsch, E.F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sanbonsuga, Y. & Neushul, M. (1978). Hybridization of *Macrocystis* with other float bearing kelps. *J. Phycol.*, **14**: 214–224.

Saunders, G.W. (1991). Molecular evolution and phylogeny of the Laminariales: analysis of the nuclear encoded ribosomal cistron. PhD thesis, Department of Biological Sciences, Simon Fraser University, Burnaby, British Columbia.

Sundene, O. (1958). Interfertility between forms of *Laminaria digitata*. *Nytt Mag. Bot.*, **6**: 121–128.

Tokida, J., Ohmi, H. & Imashima, M. (1958). A chimaera of *Alaria* and *Laminaria* found in nature. *Nature*, **181**: 924–925.

Tom Diek (Bartsch), I. (1992). North Pacific and North Atlantic digitate *Laminaria* species (Phaeophyta): hybridization experiments and temperature responses. *Phycologia*, **31**: 147–163.

West, J.A., Polanshek, A.R. & Shevlin, D.E. (1978). Field and culture studies on *Gigartina agardhii* (Rhodophyta). *J. Phycol.*, **14**: 416–426.