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Molecular investigation reveals epi/endophytic extrageneric kelp (Laminariales, Phaeophyceae) gametophytes colonizing *Lessoniopsis littoralis* thalli

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Abstract

A recent molecular investigation of kelp systematics revealed mitochondrial sequences that gave phylogenies inconsistent with those based on nuclear and chloroplast sequences for the species *Lessoniopsis littoralis*. Sequence from the mitochondrial *nad*6 region placed *L. littoralis* in the middle of a clade of *Alaria* species in our trees, whereas Rubisco and nuclear ribosomal DNA sequences resolved *L. littoralis* within the Alariaceae, but distinct from *Alaria*. To resolve these conflicting results, the *nad*6 region was sequenced from additional samples of *L. littoralis*. The resulting data variously placed *L. littoralis* with *Macrocystis integrifolia*, *Nereocystis luetkeana*, and an additional *Alaria* isolate. A series of hypotheses were devised and explored to effectively exclude introgression via hybridization as a viable explanation for our observations. Rather, molecular and microscopy data revealed that gametophytes of *Alaria*, *Macrocystis* and *Nereocystis* epi/endophytically, colonize the older portions of the thallus of *L. littoralis*. A substantial primer mismatch, unique to *L. littoralis*, was uncovered subsequently explaining why *nad*6 sequences from only *Alaria*, *Macrocystis* and *Nereocystis* were amplified from *L. littoralis* sporophyte samples, although the DNA from the gametophytes likely represented only a small percentage of the total DNA extracted.

Keywords: gametophyte; kelp; Laminariales; *Lessoniopsis littoralis*; mitochondria; *nad*6.

Introduction

The northeastern Pacific coastline is one of the richest areas of kelp taxonomic richness in the world, with 40 species recognized from Baja California to the Aleutian Islands, Alaska. Vancouver Island, located off the coast of British Columbia, Canada, is within this region and has the greatest number of kelp species (28) in any one area of this range (Druehl 1970). It is common to find more than 15 species living sympatrically in the inter- and subtidal zones along the western Vancouver Island coast. Members of the Laminariales make up the majority of the seaweed biomass in the intertidal zone of this region and their size and distinctive morphology have made them an extensively studied order of brown algae (Lane et al. in press).

Members of the Laminariales exhibit an alternation of heteromorphic generations of different ploidy levels. The macroscopic sporophytes are well characterized and the morphological classification within the Laminariales is based on features of this diploid generation (Setchell and Gardner 1925). Kelp gametophytes are haploid, dioecious and sexually dimorphic; male filaments are typically smaller in diameter and more branched than their female counterparts (McKay 1933, Hollenberg 1939). Our understanding of the microscopic, filamentous, gametophytes is poor compared with that of the sporophytes, and a comprehensive morphological survey of kelp gametophytes has never been completed, making identification to even genus impossible (Garbary et al. 1999a). In addition, little is known about the ecology, distribution and abundance of kelp gametophytes *in situ*.

Until recently, classification in the "derived" families of the Laminariales had undergone little change since circumscription by Setchell and Gardner (1925). Setchell and Gardner (op. cit.) used gross morphological characters of the sporophyte to separate genera into families nearly 80 years ago. In their classification scheme, species with sporophylls (blades specialized for reproduction) were placed in the Alariaceae, those with splitting between the stipe and blade in the Lessoniaceae, and the species with simple blades were included in the Laminariaceae. However, the morphological classification was not without problems. For instance, *Lessoniopsis littoralis* was discussed by Setchell and Gardner (1925) as an anomaly because it has both splitting and sporophylls, characteristics of two families. Setchell and Gardner decided to place *L. littoralis* in the Lessoniaceae, because its habit is closer to *Lessonia* than to *Alaria*.

Recent molecular phylogenies (Saunders and Druehl 1993, Druehl et al. 1997, Yoon and Boo 1999, Yoon et al. 2001, Lane et al. in press) have indicated that the morphological characters used by Setchell and Gardner for kelp classification result in an unnatural grouping of taxa. Substantial taxonomic revision of the Alariaceae-Laminariaceae-Lessoniaceae complex has led to a re-organization of these three families, as well as the description of a new family, the Costariaceae (Lane et al. in press). One consequence of the taxonomic changes in the Laminariales was the placement of *Lessoniopsis littoralis* in the Alariaceae (Lane et al. in press), in association with *Pterygophora californica* (Figure 1), rather than grouping in the Lessoniaceae, as proposed by Setchell and Gard-

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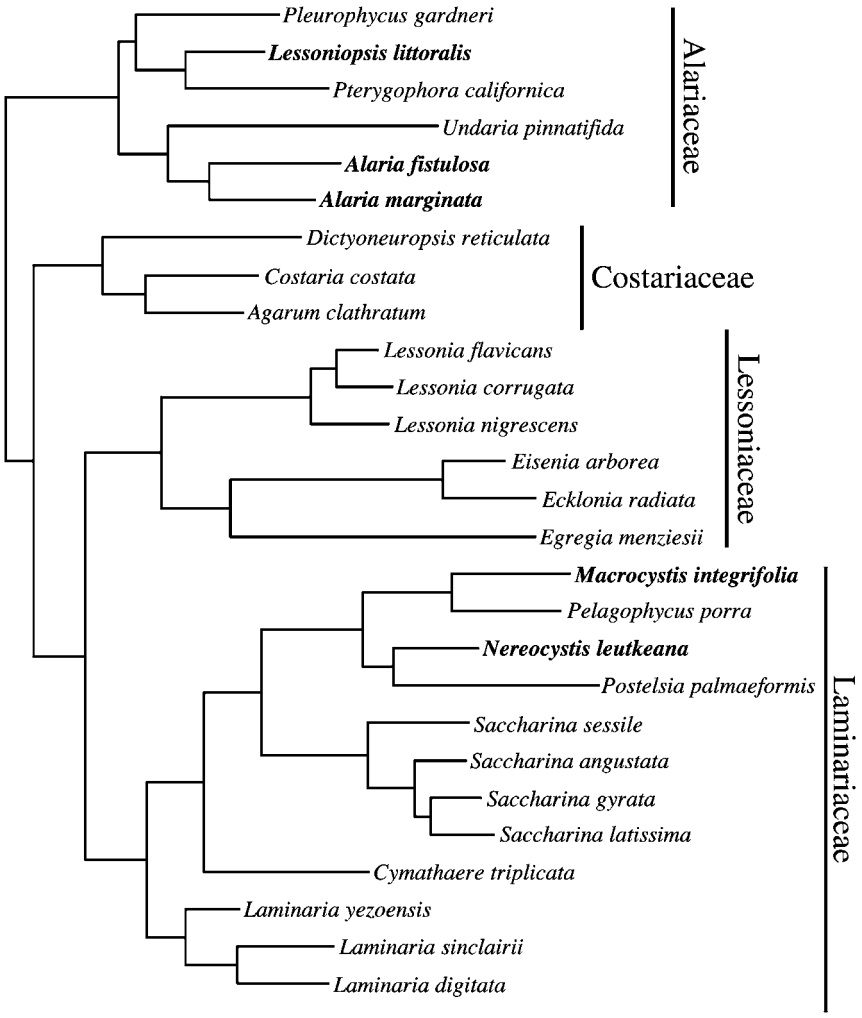


Figure 1 Illustration of the Laminariales phylogeny as proposed by Lane et al. (in press).
Both *Alaria* and *Lessoniopsis* are resolved in the Alariaceae, though not in close association. *Nereocystis* and *Macrocystis* are placed in the Laminariaceae, relatively distant from taxa in the Alariaceae. Taxa discussed here are in bold text.

ner (1925). This relationship was robustly supported by nuclear and chloroplast DNA sequences. However, when the mitochondrial NADH dehydrogenase subunit six gene (*nad*6) was sequenced initially, we discovered that the *nad*6 sequence from a sample of *L*. *littoralis* was nearly identical to that for a collection of *Alaria*. While both genera are members of the Alariaceae, sequence data from either nuclear or chloroplast regions did not support a particularly close association for these taxa. In addition, the sequence from the *nad*6 region is highly variable within the Laminariales (Lane et al. in press), making nearly identical sequences between genera unlikely.

Our sequence data indicate a possible transfer of the mitochondrial genome from *Alaria* to *Lessoniopsis* through a hybridization/introgression event (transfer of a maternally inherited organelle resulting from a hybridization and subsequent breeding of the offspring back to the paternal lineage). Intergeneric hybridization within the Laminariales has been reported frequently in the literature (Tokida et al. 1958, Neushul 1962, Sanbonsuga and Neushul 1978, Coyer and Zaugg-Haglund 1982, Migita 1984, Coyer et al. 1992, Lewis and Neushul 1995), but hybridization had not been assessed with molecular tools until recently (Kraan and Guiry 2000, Liptack and Druehl 2000, Druehl et al. 2005). Molecular evidence indicates that hybridization is very rare, and that parthenogenesis, apogamy and apospory are common in culture, resulting in either normal or abnormal morphological development, which has been misinterpreted as F1 hybrids (Druehl et al. 2005). The only intergeneric cross confirmed by molecular investigation was between *Alaria marginata* and *Lessoniopsis littoralis* (Liptack and Druehl 2000), which leaves open the possibility of an introgression event explaining the anomalous mitochondrial results for *L. littoralis*.

The mitochondrion is maternally inherited in the Fucales (Coyer et al. 2002), a related order of brown algae (Draisma et al. 2001, Rousseau et al. 2001). If a hybridization event occurred between a female *Alaria* and a male *Lessoniopsis littoralis*, followed by a female of the F1 generation mating with a male *L*. *littoralis*, the mitochondrial genome of *Alaria* could be transferred to *L*. *littoralis*. However, the work of Coyer et al. (2002) also indicated that the chloroplast is maternally inherited in the Fucales. Our mitochondrial data were aberrant compared with the chloroplast data sets, suggesting either a different inheritance pattern for the chloroplast and mitochondrion in the Laminariales, or that our incongruent data sets were not the result of mitochondrial introgression. Sequencing *nad*6 from additional samples of *L*. *lit-*

toralis only further confounded the problem because the new sequences allied with *Macrocystis integrifolia*, *Nereocystis luetkeana* and an additional *Alaria* isolate.

To explain the perplexing array of *nad*6 sequence data from four independent samples of *Lessoniopsis littoralis*, we devised and tested a series of hypotheses, ruling out introgression as a course, and establishing contamination, owing to cryptic gametophyte habitat, as the explanation.

Materials and methods

Sample collection

Samples, collected as indicated in Table 1, were transported back to the laboratory in seawater where the thalli were cleaned with cheesecloth to remove epiphytes. Subsequently, a portion of the thallus from each sample was individually dried for molecular analysis on silica gel. Blades from *Lessoniopsis littoralis* collected at Execution Rock were divided into tip, middle and base portions, and two pieces of the thallus were removed from each region. One piece of tissue from each portion was cleaned and dried while the other was left unclean and preserved in 4% formalin for microscopy. All dried material was ground with a mortar and pestle under liquid nitrogen and stored at -20°C for DNA extraction.

DNA extraction and sequencing

Extraction of DNA and routine PCR amplification and sequencing of the *nad*6 region were performed as described previously (Lane et al. in press). In addition, genus-specific primers were developed for the *nad*6

Table 1 Collection locations and GenBank accession numbers for species used in this study.

Classification	Collection location	Accession number	
Alariaceae		*nad*6	*nad*1
Alaria esculenta (Linnaeus) Greville	Prospect Point, Resolute Bay, N.W.T., Canada	**AY878857**	nd
Alaria marginata Postels *et* Ruprecht	Seal Rock, Oregon, USA	AY857907	nd
Alaria nana Schrader	Louis Druehl culture	**AY878859**	nd
Alaria taeniata Setchell	Louis Druehl culture	**AY878860**	**AY862412**
"Lessoniopsis littoralis (Farlow *et* Setchell) Reinke 1" (*Alaria* sp.)	Amphitrite Point, Ucuelet, B.C., Canada	**AY878861**[1]	**AY862406**
"*Lessoniopsis littoralis* 2" (*Alaria* sp.)	Frank Island, Ucuelet, B.C., Canada	**AY878862**[1]	**AY862407**
Lessoniopsis littoralis	Frank Island, Ucuelet, B.C., Canada	AY857909	**AY862407**
Lessoniopsis littoralis[2]	Execution Rock, Bamfield, B.C., Canada	nd	nd
Lessoniopsis littoralis[2]	Execution Rock, Bamfield, B.C., Canada	nd	nd
Lessoniopsis littoralis[2]	Execution Rock, Bamfield, B.C., Canada	nd	nd
Lessoniopsis littoralis[2]	Execution Rock, Bamfield, B.C., Canada	nd	nd
Lessoniopsis littoralis[2]	Execution Rock, Bamfield, B.C., Canada	nd	nd
"Lessoniopsis littoralis 3" (*Macrocystis integrifolia*)	Cape Beale, Bamfield, B.C., Canada	**AY878868**[1]	**AY862411**
"*Lessoniopsis littoralis* 4" (*Nereocystis luetkeana*)	Amphitrite Point, Ucuelet, B.C., Canada	**AY878870**[1]	**AY862408**
Undaria pinnatifida (Harvey) Suringar	l'Etang de Thau, France	AY857912	nd
Pleurophycus gardneri Setchell *et* Saunders	Pachena Beach, Bamfield, B.C., Canada	AY857911	nd
Pterygophora californica Ruprecht	Cape Beale, Bamfield, B.C., Canada	AY857910	nd
Laminariaceae			
Macrocystis integrefolia Bory	Cape Beale, Bamfield, B.C., Canada	AY857915	**AY862409**
Nereocystis leutkeana (Mertens) Postels *et* Ruprecht	Cape Beale, Bamfield, B.C., Canada	AY857914	**AY862410**
Pelagophycus porra (Leman) Setchell	San Diego, California, USA	AY857916	nd
Postelsia palmaeformis Ruprecht	Cape Beale, Bamfield, B.C., Canada	AY857913	nd

Sequences in bold are reported for the first time here, those in normal type are from Lane et al. (in press).
[1] indicates aberrant sequences (i.e., from donor genera in brackets), which were originally sequenced as *Lessoniopsis littoralis*, but were renamed later based on our findings. "nd" indicates that data were not determined.
[2] indicates isolates that were separated into lower, middle and tip portions for analysis. All *nad*1 sequences are coding strand only.

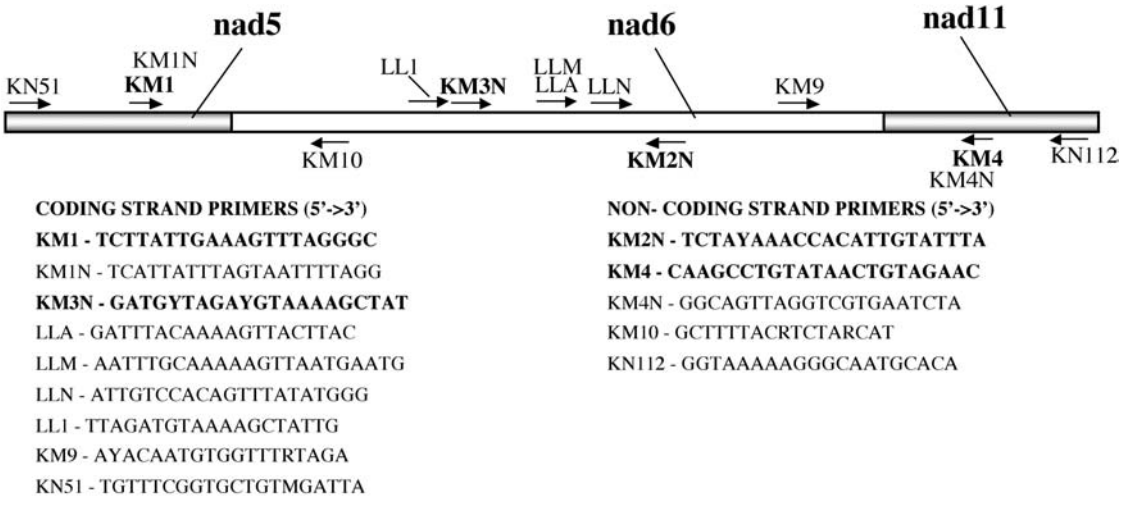


Figure 2 Oligonucleotide primers used to PCR amplify and sequence the *nad*6 region of kelp mitochondrial DNA. Primers in bold were typically used for PCR and sequencing in the Laminariales.
KM1 and KM4 were utilized for both PCR amplification and sequencing, whereas KM2N and KM3N were typically only sequencing primers. KM1N and KM4N were novel PCR primers designed for *Lessoniopsis littoralis*. Primer pairs KN51/KM10 and KM9/KN112 were used to amplify and sequence through the standard *nad*6 PCR primer regions in *L. littoralis*. All of the LL primers are genus-specific (LL1–*Lessoniopsis*, LLA–*Alaria*, LLM–*Macrocystis*, LLN–*Nereocystis*) and were used exclusively to amplify species specific fragments. Illustration not to scale.

region of *Alaria*, *Nereocystis*, *Macrocystis*, and, later, *Lessoniopsis* (Figure 2), based on known sequences. Specific primers were tested against all of the genera studied, using the same thermocycler conditions as our original *nad*6 primers, to ensure amplification of only the target species. Each of our samples of *Lessoniopsis littoralis* DNA (Table 1) was tested with all of the genus-specific primers to determine the identity of the mitochondrial variants within. Negative controls (without DNA), for each primer, were conducted simultaneously to ensure that contamination was not occurring during PCR. All oligonucleotide primers used for PCR and sequencing of *nad*6 are shown in Figure 2.

An additional set of primers was created to flank each of the original PCR primers of Lane et al. (in press) (KN51/KM10 around KM1, as well as KM9/KN112 around KM4) to check the sequence of *Lessoniopsis littoralis* at these sites (Figure 2). Primers for the mitochondrial *nad*1 gene [forward (LN11) 5′ TTATGGCMGGTATTCAAAG 3′ and reverse (LN14) 5′ TTAATTAGGMAYCCAATC 3′] were designed based on published mitochondrial sequences (Oudot-Le Secq et al. 2001, 2002) and were used under identical PCR and sequencing conditions as the *nad*6 primers (Lane et al. in press). Sequence data from the *nad*1 region were read only from the coding DNA strand, whereas *nad*6 sequence data were read from both the coding and non-coding strands. Species-specific primers for the internal transcribed spacer (ITS) of the nuclear ribosomal DNA for *Alaria marginata*, *Lessoniopsis littoralis*, *Macrocystis integrifolia* and *Nereocystis luetkeana* were used from Druehl et al. (2005) with amplification parameters described therein.

Sequence analysis

Complementary and overlapping sequences were edited and aligned using Sequencher™4.2 (Gene Codes Corporation, Ann Arbor, USA). Multiple sequence alignments were constructed for both the *nad*1 and *nad*6 data with MacClade 4.06 (Maddison and Maddison 2003). The seven-sequence *nad*1 alignment was explored using the unweighted P settings and UPGMA in Paup*4.0b10 (Swofford 2002). Phylogenetic trees for the 15-taxa *nad*6 alignment were created with 100 random sequence additions in unweighted parsimony. Neighbor-joining and maximum likelihood (10 random sequence additions) *nad*6 analyses were performed using model parameters (TIM+G) determined in Modeltest V3.5 (Posada and Crandall 1998). Bootstrap analyses were carried out with 1000 replicates for parsimony (10 random additions) and neighbor-joining. Bayesian inference was completed with default values in MrBayes V3.0b4 (Huelsenbeck and Ronquist 2003) using the GTR+I+G model with the model parameters and posterior probabilities estimated during the analysis. Trees were sampled every 100 generations for 4 million generations and a "burn-in" of 200,000 generations was determined by visual inspection of likelihood values. The first 400,000 generations were discarded to ensure stabilization, and the remaining trees were used to construct a consensus tree to estimate posterior probabilities.

Light microscopy

Light microscopy was used to investigate portions of the Execution Rock thalli for epi/endophytes. Samples used for microscopy were not cleaned before examination and epiphytes were visible under a dissecting microscope. Tissue was removed where epiphytes were observed and sectioned using a freezing cryostat (International Equipment Company, Needham Heights, USA). Material was either left unstained or stained with a 3% analine blue, 6.3% acetic acid solution, and permanently mounted in a 50% aqueous karo solution with 3% formalin. Observations were made on a Leica DM5000 B microscope (Wetzlar, Germany) and photomicrographs were taken

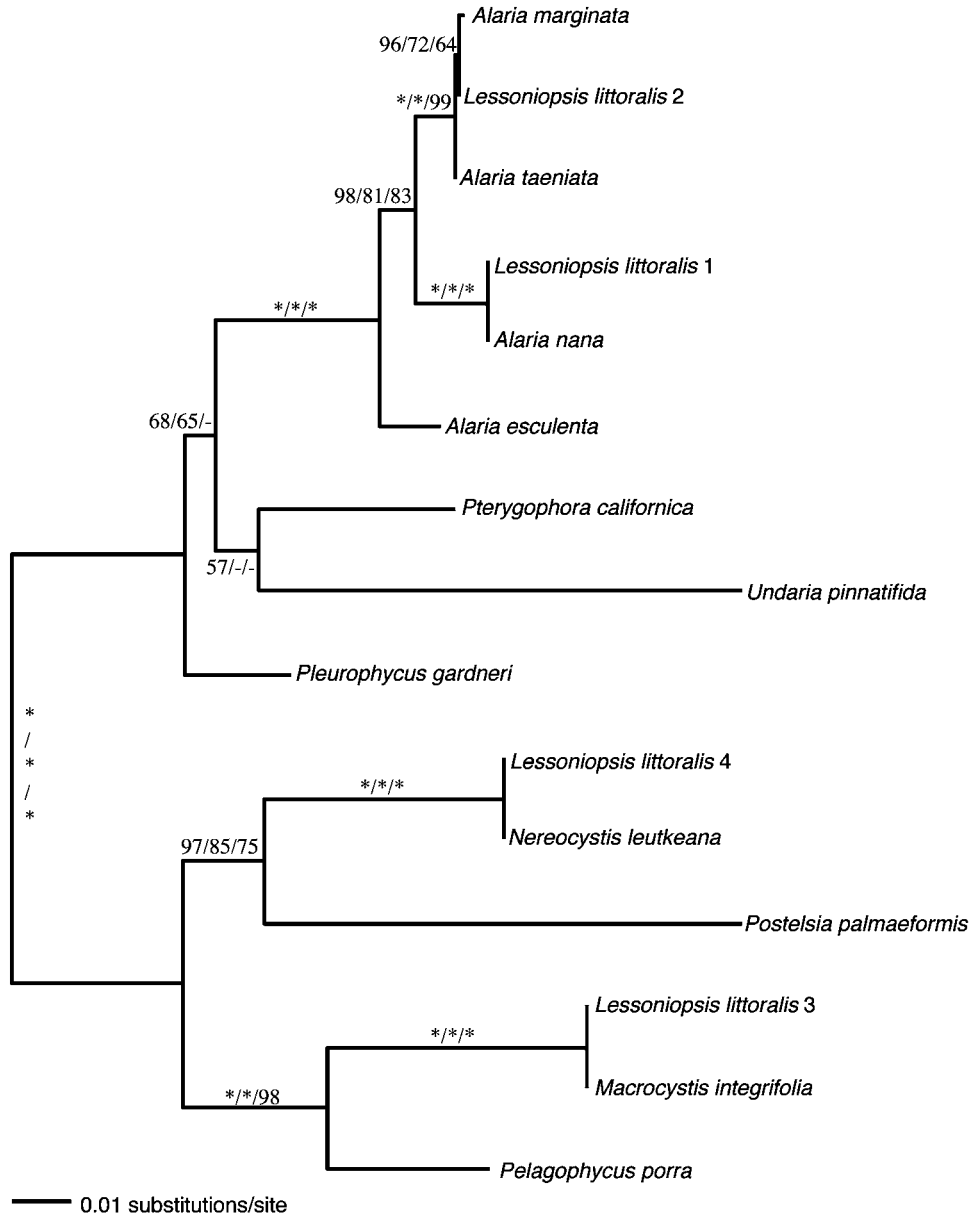


Figure 3 Bayesian consensus tree from mitochondrial *nad*6 data.
Only sequences obtained from samples of *Lessoniopsis* and the genera with which they group closely were included. Support values are presented as Bayesian posterior probabilities, NJ bootstrap and parsimony bootstrap, respectively. An "*" represents a value of 100, while "–" indicates <50% support in a particular analysis.

using a Leica DFC 480 digital camera. Photomicrographs were imported into Adobe Photoshop 8.0 (San Jose, USA) for editing.

Results and discussion

The first *nad*6 sequence isolated from *Lessoniopsis littoralis* was nested within a clade of *Alaria* species and on this basis we hypothesized that we had uncovered an example of mitochondrial introgression. *Alaria* and *Lessoniopsis* are relatively closely related genera (Lane et al. in press), and they have been shown to hybridize in the laboratory (Liptack and Druehl 2000). However, when we sequenced the *nad*6 from additional samples of *L. littoralis*, the resulting data were nearly identical to sequences from a second *Alaria* isolate, *Macrocystis integrifolia* and *Nereocystis luetkeana* (Figure 3). Several recent, independent, mitochondrial introgression events between genetically distant members of the Laminariales would be required to explain these results under a hybridization/introgression hypothesis. Whereas the literature is replete with reports of kelp hybridization, more recent molecular investigations suggest that intergeneric hybridization is rare. Rather, research indicates that parthenogenic, apogamous and aposporic sporophyte growth resulting in normal or aberrant morphologies (Nakahara and Nakamura 1973) is common in culture (Kraan and Guiry 2000, Druehl et al. 2005). If hybridization under ideal conditions in the laboratory is rare, it seemed highly improbable that multiple introgression events between distantly related taxa in the field could explain our results.

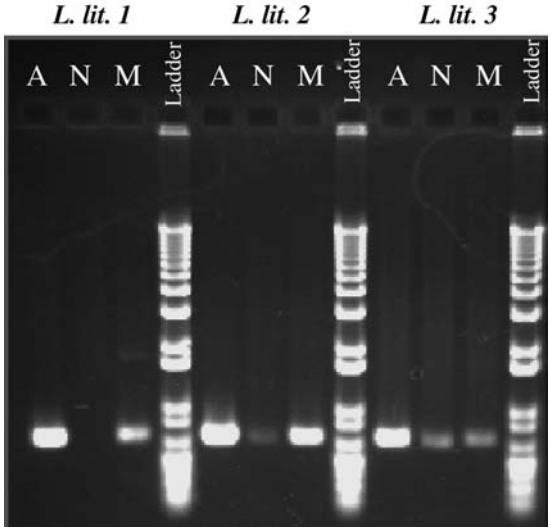


Figure 4 Results of a PCR screen using *Lessoniopsis littoralis* DNA, but genus specific primers for the *nad*6 region of *Alaria* (A), *Nereocystis* (N) and *Macrocystis* (M).
All three primer sets produced positive reactions in the samples collected from Ucuelet and Bamfield, British Columbia, Canada. Only the *Nereocystis* specific primer failed to amplify the *nad*6 in *L. littoralis* 1. Results from *L. littoralis* 4 (not shown) were identical to those for *L. littoralis* 2 and 3. All negative control lanes (not shown) were blank. *L. lit.*=*Lessoniopsis littoralis*.

To investigate the extent of the aberrant kelp DNA in our samples of *Lessoniopsis*, genus-specific PCR primers were constructed for the *nad*6 of *Alaria*, *Macrocystis* and *Nereocystis* and used as probes for foreign *nad*6 sequence in the samples tested previously. PCR produced positive results from at least two of the donor genera for every sample of DNA tested (Figure 4), indicating the presence of the *nad*6 gene from multiple species in each *L. littoralis* sample. This weakened our hypothesis of simple hybrid introgression.

We next attempted to determine if full complements of mitochondrial DNA from *Alaria*, *Macrocystis* and *Nereocystis* were present in our samples of *Lessoniopsis littoralis*, or if only partial regions of the donor genomes were present. Primers were designed to amplify and sequence the *nad*1 gene, because of its position on the opposite side of the circular mitochondrial genome, relative to *nad*6 (Oudot-Le Secq et al. 2002). The resulting *nad*1 sequences from our *L. littoralis* samples (Table 1) were nearly identical to one another, were genetically distant from any of the potential donor genera (Figure 5), and were presumed to be *bona fide L. littoralis* sequences. Although we failed to test the outlined hypothesis, we did provide the first evidence for a *L. littoralis* mitochondrion in our samples. Nonetheless, the use of *nad*6 genus-specific primers revealed mitochondrial DNA from the donor genera as well (Figure 4). One of our samples, *L. littoralis* 1, contained DNA from only *Alaria* and *Macrocystis*, whereas the other three, *L. littoralis* 2–4, contained mitochondrial DNA from all three donor genera (Figure 4). It appeared that *L. littoralis*, in addition to its own mitochondrial genome, contained a mosaic of full or partial mitochondrial DNA.

To establish that this phenomenon was specific to the mitochondrial genome of the donor genera, we used species-specific primers for the internal transcribed spacer (ITS) of the ribosomal DNA (Druehl et al. 2005) to probe for nuclear DNA from *Alaria marginata*, *Macrocystis integrifolia* and *Nereocystis luetkeana* in our samples of *Lessoniopsis*. PCR revealed results for the nuclear primers that were identical to our results for the genus-specific mitochondrial primers (Figure 6); *Alaria* and *Macrocystis* DNA was revealed in *L. littoralis* 1, and the remaining samples (*L. littoralis* 2–4) contained DNA from all three donor genera. Thus, it was clear that our samples of *L. littoralis* were contaminated with nuclear, as well as mitochondrial DNA from the donor genera.

The only plausible conclusion remaining was that our samples were contaminated. However, great care was taken in the laboratory to avoid contamination of samples, and we had not observed contamination in any of our 41 kelp DNA samples used previously (Lane et al. in press, and Table 1). To test this further, genus-specific primers were tested on four DNA samples from other genera in the Alariaceae, and six from the Laminariaceae. No contamination was found (data not shown). Therefore, the contamination was limited to, and affected all of our four samples of *Lessoniopsis littoralis*. This prompted two further questions: why did the *nad*6 of *Lessoniopsis* fail to PCR amplify; and why did this contamination occur only in our samples of *L. littoralis*?

During an earlier study on kelp hybridization (Druehl et al. 2005), the authors discovered that an equal mix of DNA from two species would occasionally result in only a single product, where two would be expected. In such cases, sequences of the products showed no evidence of a weak secondary signal, indicating a "competitive exclusion" of one DNA over the other. When the two DNA samples were amplified independently, both produced a clean product. Competitive PCR has been described in forensic science (e.g., Fregeau et al. 2003), but has not been introduced previously in the phylogenetic literature to our knowledge. Speculating that the *Lessoniopsis littoralis nad*6 was not amplifying because of exclusion by the donor DNA, a new combination of primers (KM1N–KM2N, KM3N–KM4N; Figure 2) from highly conserved regions across our alignment was used in an effort to amplify *bona fide nad*6 from *L. littoralis* as two overlapping fragments. The new primer combinations produced a *nad*6 sequence that was unique in our data set, grouped in the Alariaceae, but was distinct from other genera, and had a placement for *L. littoralis* consistent with previous nuclear and chloroplast analyses (Lane et al. in press). This provided the necessary *nad*6 sequence from *Lessoniopsis* for phylogenetic analysis, and was consistent with our competitive PCR hypothesis. However, a further experiment indicated that competition was not, strictly, the source of our PCR problem.

We were now able to design specific primers for the *nad*6 from *Lessoniopsis littoralis* and use these as a positive control in our genus-specific PCR reactions and establish that all of our *L. littoralis* samples (Table 1) contained a *bona fide L. littoralis nad*6 gene (data not shown). However, there were a few samples of *Lessoniopsis* (Figure 7; see below) for which donor DNA was not detectable with our various genus-specific primers, whereas our novel *Lessoniopsis* specific primer yielded a

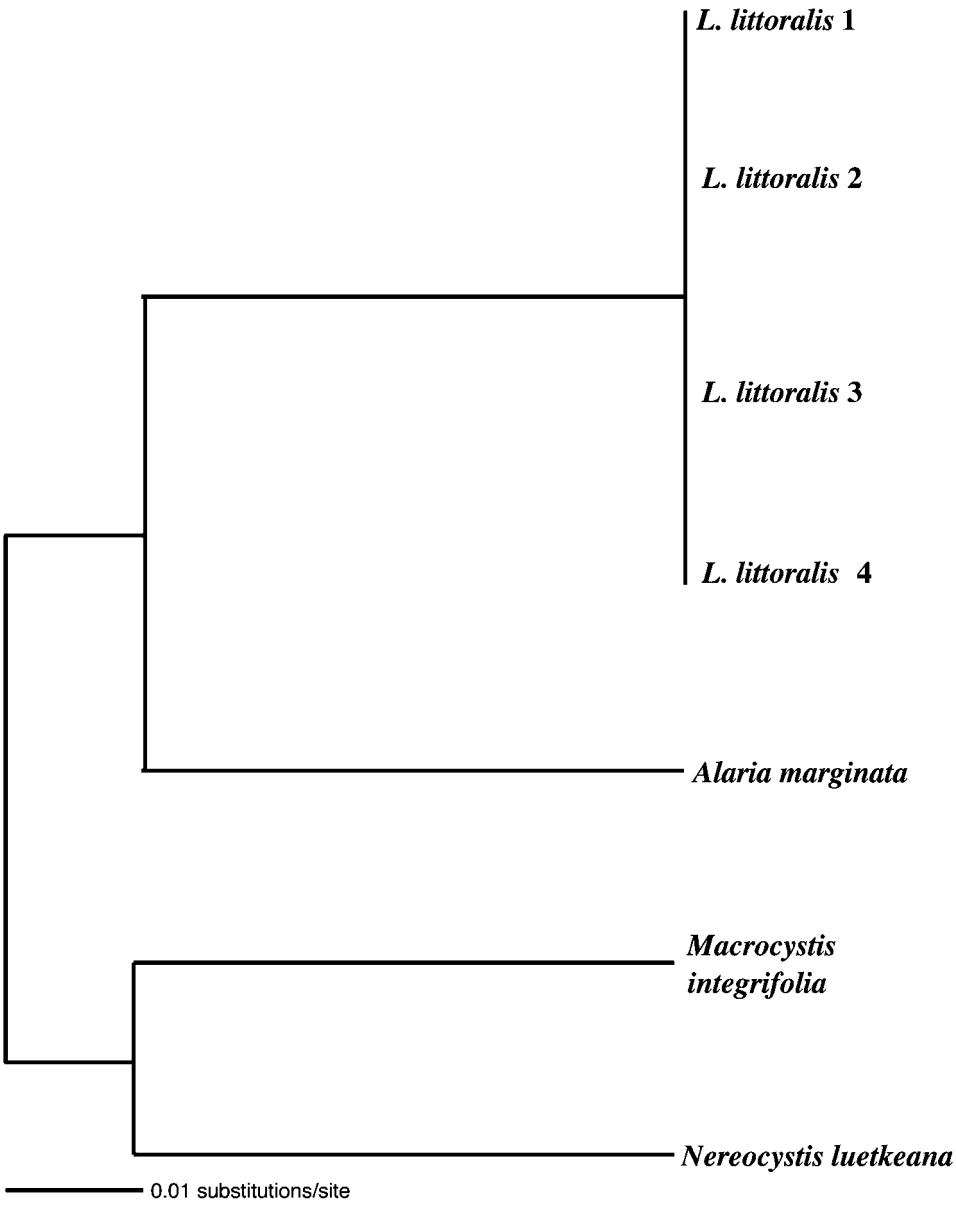


Figure 5 UPGMA tree from coding strand sequence for the *nad*1 region of *Lessoniopsis littoralis* and members of the three "donor genera".
All four samples of *L. littoralis* had nearly identical *nad*1 sequences and group independently from the other genera included.

positive result. Nonetheless, our initial *nad*6 amplification strategy still failed to amplify product from these samples. This was clear evidence that our initial *nad*6 primers were not functioning for *Lessoniopsis*, despite successfully amplifying this region from every other genus of kelp studied by Lane et al. (in press), and that we were not dealing with straightforward competitive PCR.

To assess the problem with our initial *nad*6 amplification strategy in *Lessoniopsis*, new PCR primers were designed to flank the original primers [KN51 and KM10 around KM1, as well as KM9 and KN112 around KM4 (Figure 2)] of Lane et al. (in press) to determine if primer mismatch could explain the lack of PCR amplification in this taxon. While there was only a single mismatch in the *L. littoralis* sequence at the site of KM4, KM1 contained five mismatches, with three among the seven nucleotides at the 3′ end of the primer. Clearly, these incongruencies contributed to the results we obtained. One final question remained, what was the source of the contamination that led to the aberrant results?

Light microscopy and molecular tools were used to determine if epi/endophytic gametophytes were responsible for contamination in the *Lessoniopsis littoralis* DNA samples. The blades of *Lessoniopsis littoralis*, like most kelp, grow from the intercalary meristem between the base of the blade and the stipe. Therefore, the oldest portion of the blade is at the tip. Tips of *L. littoralis* blades are typically frayed and worn from wave surge, providing an opening in the cortical layer of the thallus that may be exploited by invading gametophytes of other kelp genera to colonize the exposed medulla, possibly explaining the source of contamination in our study. We hypothesized that gametophyte infection should increase towards the tips, relative to the base of the blades. Five new samples of *L. littoralis* were collected (Execution Rock, Table 1), but DNA from distinct portions of the blade (base, mid-

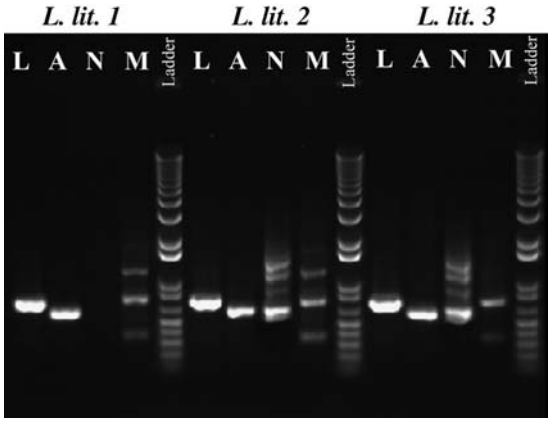


Figure 6 Image of a 0.8% agarose gel showing three samples of *Lessoniopsis littoralis* amplified with ITS primers specific to *Lessoniopsis* (L), *Alaria* (A), *Nereocystis* (N) and *Macrocystis* (M). The DNA samples used for this reaction were the same as those used in Figure 4. Results from *L. littoralis* 4 (not shown) were identical to those from *L. littoralis* 2 and 3. Primers for both *Macrocystis* and *Nereocystis* regularly produced multiple bands, but the band between 650 bp and 700 bp (sixth and seventh bands from the bottom of the ladder, respectively) was gel extracted and always produced the expected sequence product. All negative control lanes were blank. *L. lit.*=*Lessoniopsis littoralis*.

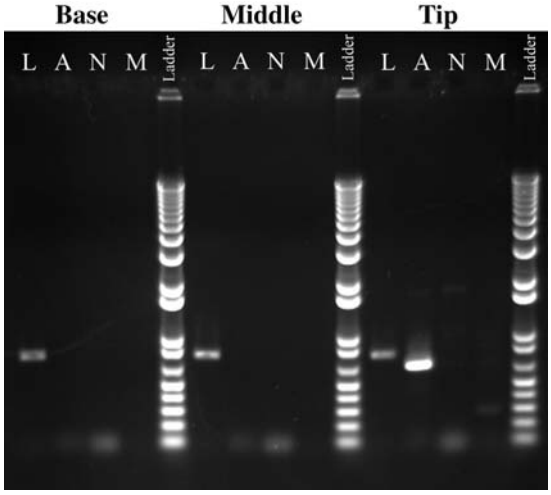


Figure 7 Results of a PCR reaction using *nad*6 primers specific to *Lessoniopsis* (L), *Alaria* (A), *Nereocystis* (N) and *Macrocystis* (M) to screen base, middle and tip portions of the blade of a *Lessoniopsis littoralis* sample from Execution Rock.
PCR primers specific to *Lessoniopsis* produced a DNA band in every segment of the blade. Of the other specific primers, only the *Alaria* primer amplified DNA, and only in the tip portion of the blade, indicating the presence of *Alaria* contamination at the tip of this *Lessoniopsis* sample. All negative control lanes were blank.

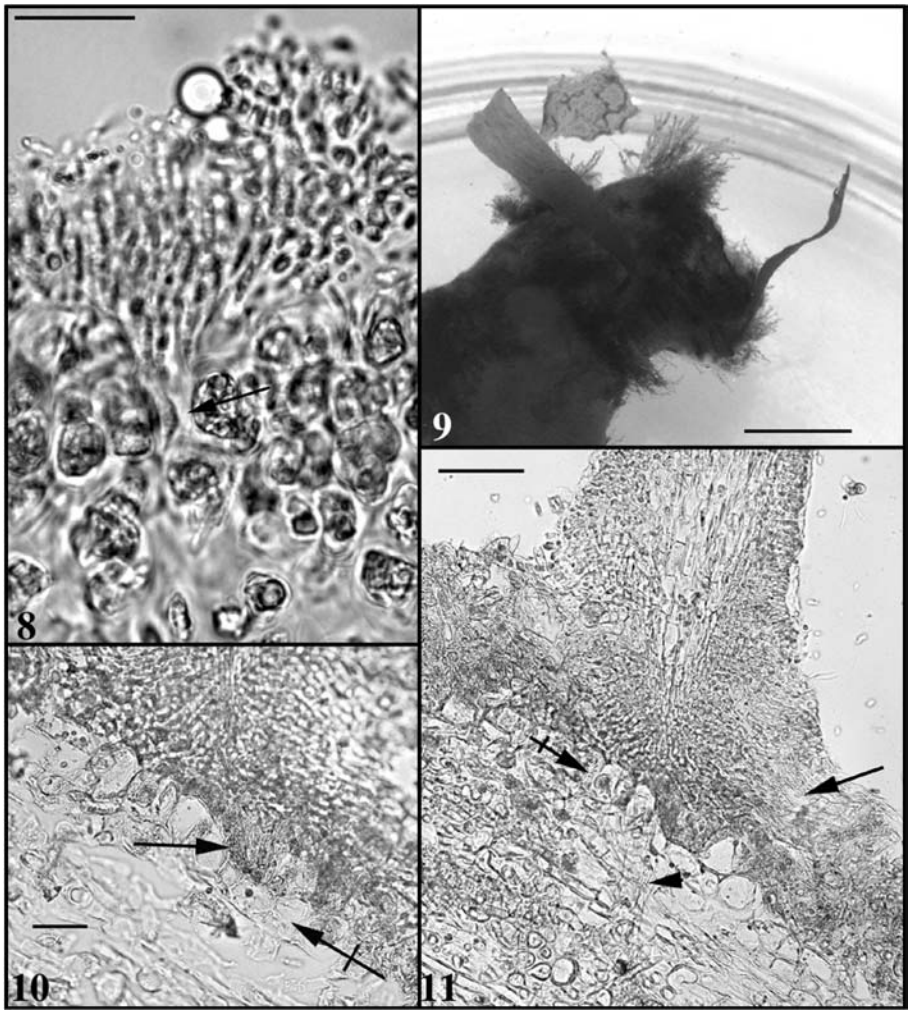
dle, tip) was PCR amplified separately allowing us to determine the distribution of contamination along the blade (Figure 7). We were able to amplify *L. littoralis nad*6 with its genus-specific primers from every portion of the blades tested, but only the tip portion of the blades revealed contamination from the various donor genera (e.g., Figure 7). Four of the five new *Lessoniopsis* samples were contaminated at the tip with DNA from at least one of the donor genera (two contaminated with *Macrocystis*, one with *Alaria* and one with both *Alaria* and *Nereocystis*), consistent with our hypothesis of donor gametophyte contamination.

Kelp gametophytes have been reported as endophytes of red algae by numerous authors (Garbary et al. 1999b, Garbary et al. 1999a, Sasaki et al. 2003, Hubbard et al. 2004, Kim et al. 2004). While kelp gametophytes cannot be identified to genus, Garbary et al. (1999a) suggested that likely candidate species were *Agarum fimbriatum* Harvey, *Alaria marginata*, *Costaria costata* (C. Agardh) Saunders, *Laminaria groenlandica* Rosenvinge, and *Nereocystis luetkeana* based on the dominant kelp in areas where infected algae were collected. Futhermore, one of us (GWS) has collected a single red algal blade that bore a juvenile sporophyte from each of the genera *Egregia*, *Macrocystis*, and *Nereocystis* providing indirect proof for gametophytes of these taxa living in red algae. A more recent study of cultures of filamentous red algae inoculated with spores from known kelp species has shown that gametophytes from both *Alaria esculenta* and *Nereocystis luetkeana* will readily become epi/endophytic in the presence of a red algal host (Hubbard et al. 2004). To our knowledge, kelp gametophytes have never been reported to be endophytic in a species of brown algae. In the case of the Laminariales, this could be an oversight based on the masking of the gametophyte filaments among the filamentous medulla of kelp sporophytes.

Examination of the tips of uncleaned *Lessoniopsis littoralis* samples under a dissecting microscope revealed brown epiphytes on the thallus. Interestingly, the majority of epiphytic clumps on the thallus were found on the edges of damaged portions of the blade or surrounding holes punctured through the thallus. These areas may be missed when the thallus is cleaned with cheesecloth because the cloth could pass over holes or small indentations on the edge of the blade without removing the epiphytes entirely, whereas no amount of surface cleaning will remove endophytes, contributing contaminating DNA to extracted samples.

Light microscopy of sections from damaged areas indicated the presence of an array of epiphytes including diatoms, and clumps of small filaments, which erupt to the surface from between cortical cells (Figure 8). Following the filamentous cells of these potential gametophytes into the filamentous medulla of the host was nearly impossible due to the similarity in cell structure. If these filamentous thalli are gametophytes growing epi/endophytically on *Lessoniopsis*, why are developing sporophytes absent on *Lessoniopsis* in the field (hours of observation over many trips and locations have failed to reveal this phenomenon), despite apparently high rates of colonization (eight of nine *L. littoralis* samples tested here were contaminated)?

Lessoniopsis littoralis thrives in the low intertidal zone of exposed areas of coastline and is subject to wave velocities as high as 14–16 m/s and acceleration in excess of 400 m/s^2 (Denny et al. 1985). Typical sporophytes have adapted to remain attached to the substratum and resist the forces of flow and drag in this habitat. For sporophytes attempting to grow on the surface of *L. littoralis* in high wave exposure, the smooth surface of the host thallus likely prevents the developing sporophyte from attaching as firmly as it would to rock. This is impor-



Figures 8–11 Light micrographs of *Lessoniopsis* and *Laminaria setchellii*.
(8) Small filaments of cells on the surface of the tip portion of a sample of *Lessoniopsis*. The arrow indicates the area between two cortical cells where the filaments appear to exit from the thallus surface. Scale bar=25 μm. (9) Immature sporophytes attached to a mature sporophyte of *Laminaria setchellii* found in the field. Scale bar=5 mm. (10) The haptera (arrow) fill in indentations but do not penetrate the thallus (crossed arrow). Scale bar=45 μm. (11) Attachment site of an immature kelp sporophyte to *Laminaria setchellii*. The immature haptera of the attached sporophyte (arrow) spread across the surface (crossed arrow) of the mature thallus. There appears to be a thick filament (arrowhead), which extends from the immature sporophyte into the medulla of the mature thallus. Scale bar=100 μm.

tant because an organism that is bent by flowing water, such as a developing sporophyte, has the greatest stress at its attachment site (Koehl 1984, Figure 4c). It is quite likely that developing sporophytes are quickly dislodged by wave energy or lost as the blade tip of the host is eroded. For this reason immature sporophytes attached to mature *L. littoralis* may be extremely rare in the field, despite rampant colonization by gametophytes.

Whereas no sporophytes were observed attached to *Lessoniopsis littoralis* sporophytes, we did find one example of immature sporophytes attached to a mature *Laminaria* sporophyte in the field (Figure 9). An individual blade of *Laminaria setchellii* P.C. Silva living in an area with moderate surf exposure was discovered with two attached sporophytes. The haptera of the developing sporophyte spread over the mature thallus without penetrating it (Figure 10). However, sections of the immature sporophyte attachment site revealed a filament extending from the developing stipe into the thallus of the host (Figure 11). Whether this is an indication of an endophytic origin of the female gametophyte that gave rise to this sporophyte is unclear.

It appears that epi/endophytic colonization of mature *Lessoniopsis littoralis* is a common phenomenon, but this can only be an ecologically significant habitat for kelp gametophytes in two ways. The first is if the widespread belief that the egg remains attached to the female gametophyte after fertilization (Bisalputra et al. 1971, van den Hoek et al. 1995) is untrue in high wave energy. If the egg or zygote is sheared from the gametophyte, and remains viable to settle on hard substratum, an epi/endophytic strategy could result in a genetic contribution to the next generation. Another possibility is if gametophyte filaments grow large enough to fractionate, or disperse with pieces of the host thallus as it erodes. Clonal cultures of gametophytes are commonly made by macerating an individual (Druehl et al. 2005) and using the fractionated filaments to begin new cultures. As the filaments grow on the host thallus, and it is eroded by wave energy, this may act to spread the gametophytes vege-

tatively to habitat more suitable for sporophyte development.

Conclusion

A combination of molecular techniques and microscopy has revealed a cryptic habitat for kelp gametophytes. Our results underscore the necessity of interpreting aberrant molecular results with caution. The taxonomy of *Lessoniopsis littoralis* has been unclear since Setchell and Gardner (1925) decided to place it in the Lessoniaceae, based on habit, rather than in the Alariaceae, with other sporophyll-bearing kelp. The phylogenetic position derived for *Lessoniopsis* based on our original *nad*6 sequences would have been very different depending on which sample we used, and incorrect, regardless of the sample included in the data set. Furthermore, what at first appeared to be an intergeneric hybridization/introgression event was, upon subsequent investigation, revealed to be an issue of cryptic contamination.

Whether kelp gametophytes invade damaged portions of the sporophytes of other kelp genera, remains unclear. Kelp sporophytes were found growing on *Laminaria setchellii* in the field, but developing sporophytes were never observed in the field attached to *Lessoniopsis littoralis* despite continued observations on our part. However, the potential for sporophyte growth on mature thalli of *L. littoralis* can be inferred from our results: eight out of nine *L. littoralis* sporophytes tested were contaminated by gametophytes. We suggest that wave action and blade erosion are likely reasons for the lack of sporophyte growth on *L. littoralis*.

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