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FEATURE ARTICLE

# Nested PCR detection of microscopic life-stages of laminarian macroalgae and comparison with adult forms along intertidal height gradients

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ABSTRACT: Recruitment of kelp species (Laminariales) to rocky marine habitats relies on the prior establishment of microscopic life-stages (zoospores, gametophytes, gametes and microscopic sporophytes). Unlike macroscopic sporophyte stages of kelp, microscopic life-stages have proved difficult to detect and identify in their natural habitat. Using a species-specific PCR-based assay, we developed a method to detect putative microscopic life-stages of 2 northeast Pacific kelp species, *Nereocystis luetkeana* (Mertens) Postels et Ruprecht and *Hedophyllum sessile* (C. Agardh) Setchell, from rocky intertidal substrate samples. Species-specific primers were evaluated for specificity and sensitivity. Comparisons of nested and unnested PCR were performed for both species. Nested PCR was used to screen for target species present on rocks obtained along an intertidal height gradient (0.0 to 3.5 m Lowest Low Water [LLW]) from 2 sites at monthly intervals in summer 2005. Vertical distribution patterns of the putative microscopic life-stages and the macroscopic sporophyte form were compared at both sites. Putative microscopic life-stages of *N. luetkeana* were detected at both sites and displayed a widespread vertical distribution with no clear spatial or temporal relationship. In comparison, *H. sessile* putative microscopic life-stage distribution was much more spatially restricted, showing a similar distribution to the macroscopic sporophyte. This study demonstrates the sensitivity, specificity and validity of utilizing molecular techniques to describe the distribution of microscopic life-stages of certain kelp species.

KEY WORDS: Kelp · Nested PCR · Microscopic life-stages · ITS rDNA · Intertidal



Microscopic life-stages of kelp are difficult to identify in nature. A nested PCR assay was used to detect and monitor the distribution of microscopic life-stages of 2 intertidal Pacific kelp species, *Nereocystis luetkeana* and *Hedophyllum sessile*. This technique is important for assessing zonation patterns, persistence and recovery of kelp species on rocky substrates.
*Photos: Caroline H. Fox & Robert J. J. Davey*

## INTRODUCTION

Members of the order Laminariales, often dominant in rocky nearshore and intertidal zones of temperate and polar seas, undergo lifecycles that involve the alternation of heteromorphic generations (reviewed in Dayton 1985, Foster & Schiel 1985, Schiel & Foster 1986, Steneck et al. 2002). Development to the diploid macroscopic sporophyte stage relies on a number of microscopic life-stages (zoospores, gametophytes,

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gametes and microscopic sporophytes) and, while identification within the Laminariales is largely based upon morphological characters of the macroscopic sporophyte (Setchell & Gardner 1925), species identification of microscopic life-stages has proved difficult. Currently, there exists no taxonomy for Laminariales gametophytes (Garbary et al. 1999), embryonic sporophytes or settled zoospores that can be used to identify organisms to species, genus or even family levels, although species discrimination of planktonic kelp zoospores is possible (Graham 1999, Graham & Mitchell 1999). Consequently, very little is known about the ecology of post-settlement kelp microscopic life-stages, despite their purported influence.

Microscopic life-stages of marine algae may play crucial roles in determining population persistence, distribution, recovery and dynamics. Referred to as 'banks of microscopic forms' by Chapman (1986), microscopic life-stages are thought to function analogously to terrestrial seedbanks (Edwards 2000), i.e. they are capable of persisting in environmental conditions too stressful for the large macrophyte form (Anderson 1982, Santelices 1990, Hoffman & Santelices 1991, Kinlan et al. 2003). Experimental evidence suggests that microscopic life-stages of marine algae may enhance recruitment (Edwards 2000), persistence (Edwards 2000, Kinlan et al. 2003), contribute to recovery following disturbance (Santelices 1990, Santelices et al. 1995, Blanchette 1996, Edwards 2000, Kinlan et al. 2003) and determine the presence of the macroscopic form (Graham 1996, Ladah et al. 1999, Edwards 2000, Swanson & Druehl 2000). More specifically, studies indicate that sensitivities of microscopic life-stages of kelp are related to vertical zonation patterns of the macroscopic sporophyte form (Swanson & Druehl 2000, Wiencke et al. 2000, 2004, 2006, Roleda et al. 2005), although efforts to characterize natural microscopic life-stage assemblages along tidal height gradients have been hampered by identification constraints.

*In situ* research involving post-settlement microscopic life-stages of Laminariales and closely related orders generally involves out-planting laboratory cultured microscopic life-stages (e.g. Hsiao & Druehl 1973, Reed 1990, Graham 1996, Edwards 2000, Wiencke et al. 2006), monitoring macroscopic recruitment on natural, artificial or manipulated substrates to infer information regarding microscopic life-stages (e.g. Druehl & Hsiao 1977, Reed et al. 1988, Reed 1990, Graham 1996, Reed et al. 1997, Edwards 2000) and/or identification of microscopic life-stages to the lowest taxonomic group possible (e.g. Laminariales; Garbary et al. 1999). Recently, a conventional PCR-based approach was used to identify extrageneric epiphytic and endophytic kelp gametophytes inhabiting the thalli of *Lessoniopsis littoralis* (Lane & Saunders 2005). A wide array of PCR-based molecular tools have been used to detect, monitor and map the distribution of microorganisms in a range of natural and manipulated environments. Of these tools, nested PCR is a highly sensitive and specific technology, often used to detect one or more species in environmental samples. For example, arbuscular mycorrhizal fungi in plant roots (van Tuinen et al. 1998), pathenogenic fungi in plant tissues (Zhang et al. 2005), seastar larvae in ballast water (Deagle et al. 2003) and a toxic dinoflagellate in ballast and estuarine samples (Patil et al. 2005) were detected using nested PCR protocols. Using a similar approach, we developed 2 nested PCR protocols for the detection of *Hedophyllum sessile* (C. Agardh) Setchell and *Nereocystis luetkeana* (Mertens) Postels et Ruprecht from epilithic and endolithic biofilm communities inhabiting natural intertidal rock substrates.

Using a combination of our nested PCR detection protocol and intertidal quadrat surveys, we described and compared the spatial and temporal distribution of macroscopic and putative microscopic life-stages of *Nereocystis luetkeana* and *Hedophyllum sessile* along a vertical tidal height gradient at 2 locations in Barkley Sound, British Columbia, Canada, that differ in wave exposure and presence of macroscopic sporophytes. *N. luetkeana,* considered an annual but often surviving longer, is a common subtidal canopy-forming species that may grow to over 10 m in length. In contrast, perennial *H. sessile* sporophytes are small prostrate forms lacking a stipe and are generally found in the mid- and low intertidal. Based on the absence of macroscopic sporophytes at the relatively wave sheltered site, no microscopic life-stages were predicted to occur. In comparison, at the site of higher wave exposure, macroscopic forms of both species persisted, and we predicted that putative microscopic life-stages would be present on intertidal rock. However, predicting the distribution of putative microscopic life-stages along a tidal height gradient is impeded by a lack of prior knowledge regarding specific tolerances of microscopic life-stages to environmental parameters, necessary sampling intensity to capture the distributions of the microscopic forms and insight into the time required for DNA degradation upon microscopic life-stage cell death. Nonetheless, given the degree of wave action and turbulence at the exposed site and the observation of reproductive (i.e. presence of sori) sporophytes throughout the sampling period, we predicted that putative microscopic life-stages of both species would be detected throughout the tidal height gradient. To the best of our knowledge, this study represents the first attempt to directly detect and characterize the distribution of putative microscopic life-stages of kelp inhabiting natural intertidal substrates.

## MATERIALS AND METHODS

Two sites (Bluestone Point and Dixon Island) located in Barkley Sound, British Columbia, Canada, were selected. The maximum predicted tidal flux in this region is approximately 4.0 m. Both sites have a northwest aspect and host visibly different macroalgal communities. Bluestone Point (48° 49′ N, 125° 09′ W) is more proximal to the open ocean and experiences high wave action. Dixon Island (48° 51′ N, 125° 07′ W), located 4.6 km from Bluestone Point and further from the open ocean, has a small rock outcropping just offshore that buffers wave action. Rock substrate in this region is identified as Westcoast Complex from the Upper Paleozoic and/or Triassic or Jurassic (Muller 1982). At Dixon Island and Bluestone Point, the Westcoast Complex is characterized as intrusive igneous rock predominantly composed of granite, granodiorite, quartz diorite and diorite (Van der Flier-Keller & McMillan 1987, J. Fox pers. comm.).

Three permanent vertical transect lines, approximately 5 m apart, were established at both sites and stainless steel screws were affixed to the substrate at every 0.5 m tidal height (Lowest Low Water [LLW]) from 0.0 to 3.5 m. During each low tide series in summer 2005 (May 24 to 25, June 23 to 25, July 20 to 21 and August 19 to 20), 0.25 $m^2$ quadrat surveys were performed at each 0.5 m tidal height along all 3 transects in order to monitor distributions (presence/absence) of macroscopic sporophyte Laminariales species (n = 24). At each site, 3 rock samples were collected at 0.5 m tidal height intervals along the middle transect using 2 geological hammers. Rock samples were selected for their visible absence of any macroscopic Laminariales, other algal species, barnacles and mussels with one notable exception. In the lower intertidal, the occurrence of 'bare' rock is fairly uncommon, and encrusting coralline algae were frequently included in the rock substrate sample. Rock samples were placed in sterile bags and transported at ambient temperatures in the shade to the laboratory for ca. 1 h until frozen at −80°C.

**Environmental DNA isolation.** Epilithic and endolithic biofilm communities of the rock substrates were sampled by grinding the rock surfaces with a Durabuilt™ rotary tool with an attached carbide drill tip (Dremel™ 3.2 mm tungsten carbide cutter). Due to heat production by the drill bit and potential sample degradation at room temperatures, grinding was performed while the sample was immersed in liquid nitrogen. A small surface area (ca. 4.15 $cm^2$) was ground until bare rock was observed (i.e. grinding the epilithic biofilm); it was subsequently ground 5 additional times to sample similar depths of the endolithic biofilm and the resulting powder collected. All equipment was either flame sterilized or autoclaved between samples, with the exception of the rotary wand, which was rinsed repeatedly with ethanol and sterile water and then flame dried.

Powder from replicate (n = 3) samples was pooled for DNA extraction using the FastDNA® Spin kit for soil (Q-Biogene) with the optional 15 min extended spin included and the use of a vortexer (Fisher Scientific Mini Vortexer) set on 6 for 2 min instead of a bead-beater. DNA was eluted in 70 µl of purified water, vacuum-dried for shipping and stored at −20°C until resuspended in 50 µl TE ( 10 mM Tris-HCl pH 7.4, 1 mM EDTA) for use.

**Macroscopic sporophyte DNA isolation.** Sporophyte tissues were obtained from meristematic regions of the youngest available sporophytes collected in and around Barkley Sound and frozen at −80°C until use. All sporophyte tissues were extracted using a modified CTAB-Proteinase-K DNA extraction protocol (modified from Ausubel et al. 1987, Doyle & Doyle 1990, Antoine & Fleurence 2003). Sporophyte tissues were immersed in liquid nitrogen and ground with a mortar and pestle. Approximately 100 µl of ground tissue was incubated for 1 h at 65°C in CTAB extraction buffer (sample to buffer ratio (w/v) 1:4, 2% (w/v) CTAB, 1% (w/v) polyvinylpyrolidone, 1% (v/v) β-mercaptoethanol, 100 mM TrisCl pH 8.0, 20 mM EDTA pH 8.0 and 1.4 M NaCl). Proteinase K was added (final concentration 1 mg $ml^{-1}$) and the mixture was incubated at 50°C for 1 h. The solution was extracted with phenol/chloroform/isoamyl (25:24:1 v/v/v; PCI), centrifuged (1600 × *g*, 10 min, but occasionally longer if debris in the supernatant remained) and the aqueous layer recovered. The recovered aqueous layer was precipitated in ⅔ volume of cold isopropanol overnight at −20°C. Precipitated DNA was centrifuged until a loose pellet formed (700 × *g*). The recovered pellet was rinsed with wash buffer (76% (v/v) ethanol, 10 mM ammonium acetate), left at room temperature for 1 h, pelleted again (1600 × *g*, 10 min), air dried and dissolved in TE buffer. A final purification step involved a 2× dilution with TE and 7.5 M ammonium acetate added to a final concentration of 2.5 M. Cold ethanol (2.5 volumes) was added to precipitate DNA; this was then centrifuged (10 000 × *g*, 10 min) and the pellet recovered and resuspended in TE.

**Cultures.** Gametophyte cultures were obtained from reproductive adults collected near study sites in Barkley Sound. Briefly, sori were excised from the thalli, scrubbed with paper towels, rinsed in a 0.07% solution of iodine in sterile seawater, rinsed 5 additional times in sterile seawater, blotted dry and left at 12°C for 1 to 2 h while wrapped in paper towels. Zoospores were released in sterile seawater for approximately 1 h, strained through cheesecloth and quantified with a haemocytometer. Cultures with an initial concentration

of 3000 swimming zoospores $ml^{-1}$ were grown in aerated glass jars with enriched seawater medium (1 ml each A and B algal nutrients in 1 l sterile seawater; Fritz A and B f/2 Algae Food, Fritz Industries) under long day (18:6 h light:dark) light conditions for 60 d. Before harvesting, gametophytes were examined under a microscope to ensure that additional life-stages had not yet developed. Gametophytes were harvested by scraping jar sides and bottoms and then collected on sterile filter paper and dried at 37°C for 12 h prior to weighing. Dry gametophyte tissues were ground in a mortar and pestle and DNA was isolated from 10 mg of each species using the same protocol as that for the environmental rock substrate samples. DNA extracts were electrophoresed in 1.5% agarose gels stained with ethidium bromide (0.2 µg $ml^{-1}$) and quantified using a Lambda HindIII standard and TotalLab™ software (Nonlinear Dynamics).

**Primer design.** Sequences of rDNA ITS1, 5.8S and ITS2 regions from 28 kelp species sequences were aligned using ClustalW (European Bioinformatics Institute). Regions of species dissimilarity in the ITS1 and ITS2 regions were chosen as potential primer sites. Primers in unique regions of target species were designed to have low 3′ GC content, high species-specific affinity and melting temperatures within 5°C of the other primer. Inner primers were also designed to have melting temperatures that were significantly higher than the outer, first round primer pair, as a means of deactivating those primers from second round PCR activity.

**Specificity.** NCBI (National Center for Biotechnology Information) BLAST searches were performed on potential *Nereocystis luetkeana* and *Hedophyllum sessile* species primer pairs to ensure their specificity. To further increase confidence in their specificity, species-specific primers were tested against 25 non-target DNA samples isolated from algal sporophyte DNA (Phaeophyceae, Chlorophyceae and Bangiophyceae).

All reactions for the specificity assay were performed on a MyCycler Thermocycler (Bio-Rad Laboratories) using species-specific primer pairs (*Nereocystis luetkeana* F/R2 and *Hedophyllum sessile* F2/R2) (Table 1) with optimized thermocycler temperatures and settings (Table 2). PCR reactions (25 µl volume) contained 2 µl template DNA ($10^1$, $10^2$, and $10^3$ dilutions), 5 µl Genereleaser™ (Bioventures), 1× buffer (600 mM Tris-$SO_4$ [pH 8.9], 180 mM ammonium sulfate), 2 mM magnesium chloride, 200 µM each dNTP, 0.4 µM each primer, and 0.875 U Platinum High Fidelity *Taq* polymerase (Invitrogen) overlaid with mineral oil. A preliminary thermocycler run with Genereleaser™, template DNA and mineral oil was performed according to manufacturer's instructions. Negative and positive controls were included in each thermocycler run.

As a positive control for sporophyte-derived DNA susceptibility to amplification, all Laminariales species were amplified using KG4 (Druehl et al. 2005, modified from Tai et al. 2001) and modified ITSP1 (modified from Tai et al. 2001) primers using identical PCR reagents as described above. All non-Laminariales sporophyte tissues were amplified using universal eukaryotic primers that target the 18S gene region (van Hannen et al. 1998). Thermocycler conditions for the universal eukaryotic primers followed van Hannen et al. (1999).

Table 1. Primer sequence, target and source information for unnested and nested PCR detection of *Nereocystis luetkeana* and *Hedophyllum sessile*

| Primer name | Primer sequence 5′- 3′ | Target | Source |
|---|---|---|---|
| KG4 | CTTTTCCTCCGCTTAGTTATATG | 25S | Druehl et al. (2005) modified from Tai et al. (2001) |
| Modified ITSP1 | GGAAGGTGAAGTCGTAACAAGG | 18S | Modified from Tai et al. (2001) |
| *N. luetkeana* F | AGCTCAATCAAGCGCTCTCG | ITS1 | Druehl et al. (2005) |
| *N. luetkeana* R2 | GTGGTGTACGGATTCCAGCAG | ITS2 | Present study |
| *H. sessile* F2 | GGTTCCTGCGGCTCTGTCCG | ITS1 | Present study |
| *H. sessile* R2 | AATGAGGCAGGCGGGCCT | ITS2 | Present study |

Table 2. PCR thermocycler conditions for unnested and nested PCR detection of *Nereocystis luetkeana* and *Hedophyllum sessile*. See Table 1 for sources

| Primer pair | Initial denature | Cycle conditions | | | Final extension | Cycle no. |
|---|---|---|---|---|---|---|
| | | Denature | Anneal | Extension | | |
| Modified ITSP1/KG4 | 94°, 3 min | 94°, 50 s | 63.6°, 50 s | 72°, 50 s | 72°, 8 min | 38 |
| *N. luetkeana* F/R2 | 94°, 3 min | 94°, 50 s | 69.1°, 50 s | 72°, 50 s | 72°, 8 min | 35 |
| *H. sessile* F2/R2 | 94°, 3 min | 94°, 50 s | 71.1°, 50 s | 72°, 50 s | 72°, 8 min | 35 |

**Sensitivity.** Unnested and nested PCR were performed on pure gametophyte-derived *Nereocystis luetkeana* and *Hedophyllum sessile* DNA in order to determine sensitivity limits. In the unnested protocol, 1 µl of 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg and 1 fg gametophyte-derived DNA were amplified using the species-specific inner primer pairs (*N. luetkeana* F/R2 and *H. sessile* F2/R2) (Table 1). In the nested protocol, the first round of PCR utilized outer modified ITSP1 and KG4 primers (Table 1). Using 1 µl of the first-round PCR product as a template, the second round PCR reactions were performed using the species-specific primers following outlined thermocycler settings (Table 2). Nested sensitivity reactions were carried out with undiluted first round PCR product as template, with the exception of nested 10 and 1 ng *H. sessile* reactions, which were carried out using 1:50 product dilutions due to the formation of spurious PCR products in the final reaction. All reactions were performed in 25 µl volumes that contained 1 µl template DNA ($10^1$, $10^2$, and $10^3$ dilutions), 1× buffer (600 mM Tris-$SO_4$ [pH 8.9], 180 mM ammonium sulfate), 2 mM magnesium chloride, 200 µM each dNTP, 0.4 µM each primer, and 0.875 U Platinum High Fidelity *Taq* polymerase.

**Environmental assays.** Screening for the presence of *Nereocystis luetkeana* and *Hedophyllum sessile* in mixed species genomic DNA isolated from intertidal rocks was performed using nested PCR. Crude DNA extracts were subjected to amplification for the first round of PCR with the Laminariales-conserved, modified ITSP1 and KG4 primers (Table 1). PCR reactions using 2 µl of 4 dilutions ($10^0$, $10^1$, $10^2$ and $10^3$) of DNA extracts were performed with reaction components and thermocycler settings described for the sensitivity protocol. For the second round, all 4 dilutions were pooled and nested PCR performed using either *N. luetkeana* or *H. sessile* inner primer pairs (Table 1) and 1 µl of the pooled PCR product as template. PCR reaction components were identical to the first round, and all thermocycler runs contained negative and positive controls. Reaction products were visualized in 1.5% agarose gel stained with ethidium bromide (0.2 µg $ml^{-1}$). To validate the technique by confirming species detection, positive reactions from rock samples collected from Bluestone Point in May and June 2005 were purified using the QIAQuick PCR Purification Kit (Qiagen) and commercially sequenced (Cleveland Genomics) using forward primers. Recovered sequences were compared with in-lab and published sequences of *N. luetkeana* or *H. sessile*. All subsequent screening identity for both species was confirmed by PCR fragment length.

## RESULTS

*Nereocystis luetkeana* F/R2 and *Hedophyllum sessile* F2/R2 primer pairs exhibited specificity for their target species using NCBI BLAST searches and empirical tests with 25 different non-target algal species (Fig. 1). Both species primer pairs also amplified DNA from target species gametophyte- and sporophyte-derived DNA (Figs. 1 & 2). Fragment lengths obtained from inner primer pairs were 412 bp and approximately 575 bp for *H. sessile* and *N. luetkeana*, respectively.

The use of nested PCR resulted in 100 and 1000 times greater detection sensitivity than unnested PCR for

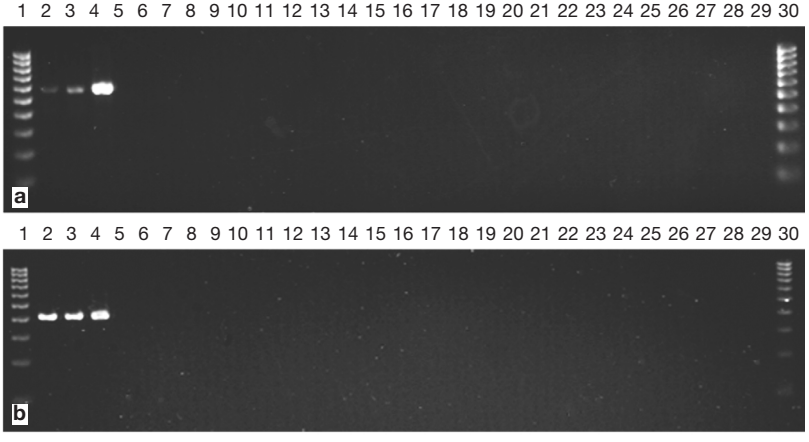


Fig. 1. Primer-pair specificity assay for (a) *Nereocystis luetkeana* F/R2 and (b) *Hedophyllum sessile* F2/R2. (a) Lanes 1 and 30: 100 bp marker; Lanes 2 to 4: *N. luetkeana*; Lane 5: *Agarum clathratum*; Lane 6: *Agarum fimbriatum*; Lane 7: *Alaria marginata*; Lane 8: *Alaria nana*; Lane 9: *Costaria costata*; Lane 10: *Egregia menziesii*; Lane 11*: Eisenia arborea*; Lane 12: *Hedophyllum sessile*; Lane 13: *Laminaria groenlandica*; Lane 14: *Laminaria saccharina*; Lane 15: *Laminaria setchellii*; Lane 16: *Laminaria sinclarii*; Lane 17: *Lessoniopsis littoralis*; Lane 18: *Macrocystis integrifolia*; Lane 19: *Pleurophycus gardneri*; Lane 20: *Postelsia palmaeformis*; Lane 21: *Pterygophora californica*; Lane 22: *Analipus japonicus*; Lane 23: *Colpomenia peregrina*; Lane 24: *Desmarestia aceuleata*; Lane 25: *Petalonia* spp.; Lane 26: *Sargassum muticum*; Lane 27: *Scytosiphon simplicissimus*; Lane 28: *Ulva* spp.; and Lane 29: *Porphyra* spp. (b) Lanes 1 and 30: 100 bp marker; Lanes 2 to 4: *H. sessile*; Lane 5: *A. clathratum*; Lane 6: *A. fimbriatum*; Lane 7: *A. marginata*; Lane 8: *A. nana*; Lane 9: *C. costata*; Lane 10: *E. menziesii*; Lane 11: *E. arborea*; Lane 12: *L. groenlandica*; Lane 13: *L. saccharina*; Lane 14: *L. setchellii*; Lane 15: *L. sinclarii*; Lane 16: *L. littoralis*; Lane 17: *M. integrifolia*; Lane 18: *N. luetkeana*; Lane 19: *P. gardneri*; Lane 20: *P.. palmaeformis*; Lane 21: *P. californica*; Lane 22: *A. japonicus*; Lane 23: *C. peregrina*; Lane 24: *D. aceuleata*; Lane 25: *Petalonia* spp.; Lane 26: *S. muticum*; Lane 27: *S. simplicissimus*; Lane 28: *Ulva* spp.; and Lane 29: *Porphyra* spp.

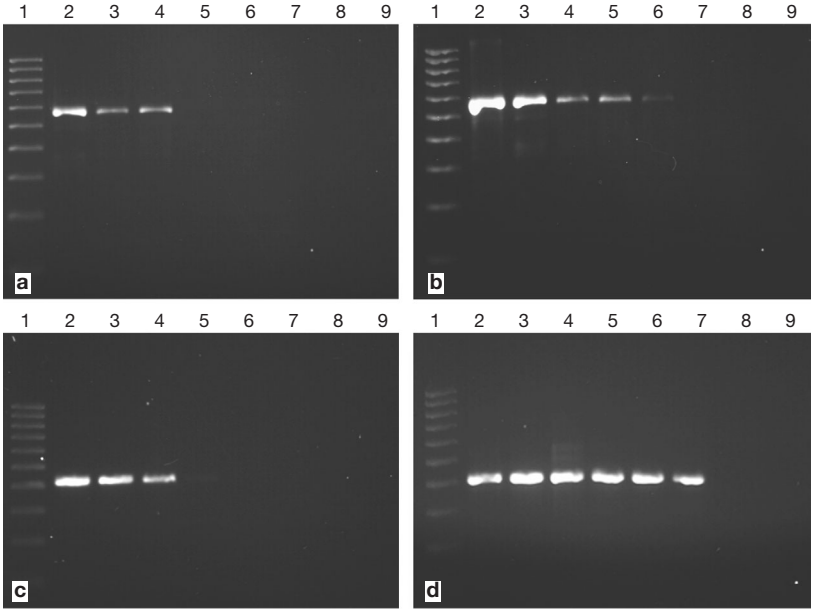


Fig. 2. *Nereocystis luetkeana* and *Hedophyllum sessile*. Detection sensitivity of gametophyte-derived *N. luetkeana* using (a) conventional unnested PCR and (b) nested PCR. Detection sensitivity of gametophyte-derived *H. sessile* using (c) conventional unnested PCR and (d) nested PCR. Lane 1: 100 bp ladder; Lane 2: 10 ng; Lane 3: 1 ng; Lane 4: 100 pg; Lane 5: 10 pg; Lane 6: 1 pg; Lane 7: 100 fg; Lane 8: 10 fg; Lane 9: 1 fg

*Nereocystis luetkeana* and *Hedophyllum sessile*, respectively (Fig. 2). Sensitivity of the nested PCR protocol for *N. luetkeana* was successful to 1 pg DNA, whereas sensitivity for *H. sessile* was successful to 100 fg DNA (Fig. 2). Using nested PCR, detection was possible to 1.08 ng dried *N. luetkeana* gametophyte and 139.9 pg dried *H. sessile* gametophyte.

Species detection of putative microscopic life-stages from environmental samples resulted in PCR products of expected size or a negative result (no PCR product) (Fig. 3). All sequences retrieved from positive environmental samples in May and June confirmed species identity using NCBI BLAST searches and alignment with published sequences. Retrieved *Nereocystis luetkeana* sequences exhibited some base pair variability (accession numbers EF043256, EF043257 and EF043258) and the sequencing reaction did not proceed far into the ITS2 region. Retrieved *Hedophyllum sessile* sequences were identical (accession numbers EF043259 and EF043260).

Detection of macroscopic and putative microscopic life-stages of the 2 kelp species at Dixon Island and Bluestone Point revealed biogeographies that appeared to either closely overlap or differ markedly (Table 3). At Bluestone Point, a large (>100 individuals), exclusively *Nereocystis luetkeana* kelp forest existed just offshore and many individuals were reproductive (i.e. presence of sori) over the course of the study period. No macroscopic sporophytes were recorded in the intertidal zone. However, putative microscopic life-stages of *N. luetkeana* were detected from 0.0 to 3.5 m at Bluestone Point, although no obvious temporal or spatial relationship was detected (Table 3). Dixon Island, located ca. 3.5 km from Bluestone Point, hosted no offshore or proximal *N. luetkeana* kelp forests, although dislodged floating sporophytes frequent the area and wash onshore, providing a potentially continual propagule source. Despite the relative absence of macroscopic sporophytes of *N. luetkeana* at Dixon Island, this species was detected along the tidal height gradient from 0.0 to 3.5 m (Table 3). Detection was less frequent than at Bluestone Point and, again, no apparent spatial or temporal pattern was found (Table 3).

Comparisons of *Hedophyllum sessile* macroscopic sporophytes and putative microscopic life-stages re-

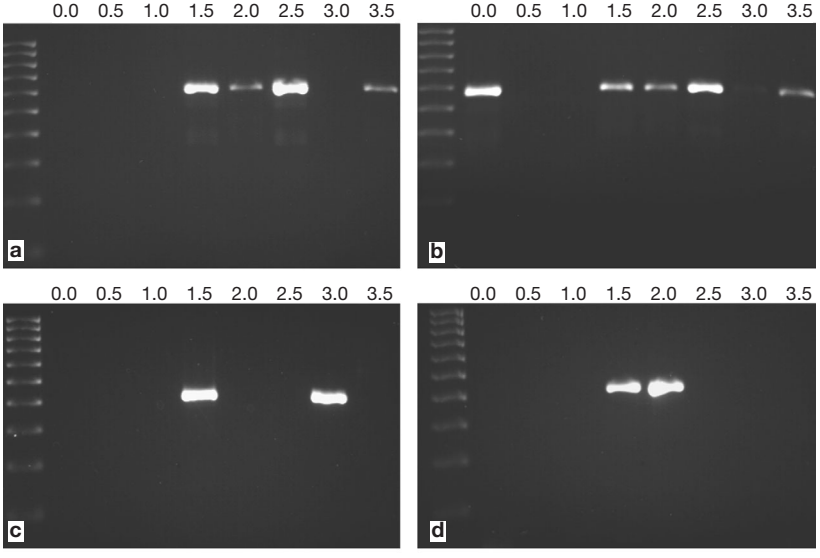


Fig. 3. *Nereocystis luetkeana* and *Hedophyllum sessile*. Detection of kelp from intertidal substrate samples collected from Bluestone Point using nested PCR. *N. luetkeana* detected in (a) May and (b) June. *H. sessile* detected in (c) May and (d) June. First lane: 100 bp ladder; numbers above other lanes indicate tidal height (0.0 to 3.5 m LLW) at which samples were retrieved

Table 3. *Nereocystis luetkeana* and *Hedophyllum sessile*. Distribution of kelp macroscopic sporophytes (S) and putative microscopic life-stages (m) along intertidal height transect (0.0 to 3.5 m LLW) at Bluestone Point and Dixon Island, Barkley Sound, Canada in May, June, July and August 2005. ND: not done

| Location | Bluestone Point | | | | | | | | Dixon Island | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | May | | June | | July | | August | | May | | June | | July | | August | |
| | S | m | S | m | S | m | S | m | S | m | S | m | S | m | S | m |
| *N. luetkeana* | | | | | | | | | | | | | | | | |
| Subtidal | • | ND | • | ND | • | ND | • | ND | | ND | | ND | | ND | | ND |
| 0.0 | | | | • | | • | | • | | | | | | • | | |
| 0.5 | | | | | | • | | • | | • | | | | • | | |
| 1.0 | | | | | | • | | | | | | | | • | | • |
| 1.5 | | • | | • | | • | | • | | | | | | | | |
| 2.0 | | • | | • | | • | | • | | • | | • | | | | |
| 2.5 | | • | | • | | • | | • | | • | | | | | | |
| 3.0 | | | | • | | • | | • | | • | | | | | | |
| 3.5 | | • | | • | | • | | | | • | | • | | | | • |
| *H. sessile* | | | | | | | | | | | | | | | | |
| 0.0 | | | | | | | | | | | | | | | | |
| 0.5 | | | • | | | | • | | | | | | | | | |
| 1.0 | • | | • | | • | | • | | | | | | | | | |
| 1.5 | • | • | • | • | • | • | • | • | | | | | | | | |
| 2.0 | | | | • | | • | | | | | | | | | | |
| 2.5 | | | | | | | | | | | | | | | | |
| 3.0 | | • | | | | | | | | | | | | | | |
| 3.5 | | | | | | | | | | | | | | | | |

vealed a markedly different pattern to that of *Nereocystis luetkeana*. No macroscopic sporophytes of *H. sessile* were recorded at Dixon Island and no putative microscopic life-stages were detected (Table 3), although *H. sessile* recruits nearby and macroscopic sporophytes also wash onshore. At Bluestone Point, a clearly demarcated *H. sessile* macroscopic sporophyte zone persisted and reproductive adults were observed at the site over the course of the study. Unlike *N. luetkeana*, *H. sessile* putative microscopic life-stages exhibited a spatial pattern that somewhat coincided with the spatial pattern of the macroscopic sporophytes (Table 3). Macroscopic sporophytes were recorded between 0.5 and 1.5 m tidal heights, whereas putative microscopic life-stages were detected between 1.0 and 2.0 m tidal heights, with one exception in May, where *H. sessile* was detected at 3.0 m (Table 3).

## DISCUSSION

Similar to other reports (Grote et al. 2002, Li & Hartman 2003, Zhang et al. 2005), our nested PCR protocol increased detection sensitivity by 100 to 1000 times over conventional, unnested PCR. However, sensitivity analyses were performed on single species gametophyte-derived DNA. The sensitivity of detection in environmental samples is likely lessened due to the presence of mixed species DNA and environmental PCR inhibitors such as humic acids (Godhe et al. 2001). An additional advantage of nested PCR is that the presence of PCR inhibitors in the first round of PCR that hamper amplification efficiency may be reduced for second round PCR (van Tuinen et al. 1998), allowing for fewer false negatives and more robust reactions. Also, nested PCR permits the user to work with first round PCR products instead of often limited amounts of crude DNA extracts, which in turn permits additional analyses.

Similar to studies of fungal diversity in soils where PCR-based molecular tools cannot discriminate between active, established fungi and inactive dormant forms (Bridge & Spooner 2001), this protocol cannot discriminate between recently settled zoospores that will not survive and gametophytes or microscopic sporophytes that have survived and established. Further, with large populations of macroscopic sporophytes in the study area, such as the large population of *Nereocystis luetkeana* at Bluestone Point, there is also potential for the detection of microscopic fragments of sporophyte tissues containing sufficiently intact DNA. In this study, detection of *N. luetkeana* at Dixon Island, where attached macroscopic sporophytes are absent, suggests that microscopic life-stages were likely responsible for positive results. Similarly, at the wave-exposed Bluestone Point site, fragments of the macroscopic sporophytes were likely dispersed throughout the intertidal, yet detected *Hedophyllum sessile* putative microscopic life-stages were somewhat spatially coupled to the limited distributions of the macroscopic sporophyte.

The distribution of putative microscopic life-stages of *Nereocystis luetkeana* at Bluestone Point and Dixon Island indicates that these life-stages are widespread in the intertidal, unlike the macroscopic sporophyte, which is almost exclusively subtidal. Such a distribution suggests that propagules are settling frequently throughout the intertidal and/or are able to survive in the low to high intertidal. Evidence suggests that it is unlikely that *N. luetkeana* microscopic life-stages would survive for long in the high intertidal, especially during summer months, given the relatively high UV exposure, desiccation and temperature stress. Previous research has established that *N. luetkeana* gametophytes and young sporophytes are sensitive to temperature (Vadas 1972). Further, a number of Laminariales microscopic life-stages have demonstrated sensitivities to UV exposure (Dring et al. 1996, Swanson & Druehl 2000, Wiencke et al. 2000, 2004, 2006) which increases with tidal height (Swanson 2000). One explanation for the observed widespread distribution of putative microscopic life-stages of *N. luetkeana* is that propagule dispersal occurred during the most recent tide and substrates were then sampled in early morning, before light, heat and desiccation stresses were maximal. Long distance transport of propagules is a potential explanation for the detection of putative microscopic life-stages at Dixon Island since dispersal and recruitment at distances of 500 m have been previously recorded for kelp (Reed et al. 1988). Zoospore release by reproductive rafting *N. luetkeana*, dislodged from large *N. luetkeana* kelp forests nearby, is another possible source of colonization. As wave action is a primary factor dislodging buoyant sporophytic *N. luetkeana*, the abundance of microscopic life-stages might be expected to decrease seasonally at the Dixon Island site, with the corresponding decrease in seasonal storm intensity and frequency; however, this pattern was not observed. Additionally, the detection of putative microscopic life-stages of *N. luetkeana* at Dixon Island may indicate the presence of accumulated microscopic life-stages, forming hypothesized banks of microscopic forms (e.g. Chapman 1986).

Unlike *Nereocystis luetkeana*, macroscopic and microscopic *Hedophyllum sessile* life-stages shared a similarly restricted distribution. With one exception, both macroscopic and putative microscopic life-stages generally occurred in the mid-intertidal. No microscopic life-stages of *H. sessile* were detected at Dixon Island, indicating that *H. sessile* zoospores may be more limited in their dispersal or survival ability, or occurred in abundances below the limits of detection. Of 6 kelp species examined, *H. sessile* was found to have the largest average zoospores and exhibited less inhibition by UV-B than deeper water kelp (Swanson & Druehl 2000). This relative tolerance to UV-B may contribute to the discerned upper limit of both macroscopic and putative microscopic *H. sessile* described in our study. Detection of putative microscopic life-stages in summer also advances the possibility that these forms may persist until environmental conditions improve, potentially many months later, given that *H. sessile* sporophyte recruitment generally occurs in late winter and spring (Milligan 1998).

Assays for detecting putative microscopic life-stages of 2 common kelp species, *Nereocystis luetkeana* and *Hedophyllum sessile*, on natural substrates were developed and utilized to compare vertical distributions of putative microscopic and macroscopic life-stages. This research is the first to directly compare putative microscopic life-stage distributions of kelp species with macroscopic sporophyte distributions, historically the primary focus of numerous kelp studies. The combined use of this molecular detection protocol with traditional macroscopic sporophyte surveys and controlled experiments to evaluate life-stage sensitivities to abiotic and biotic variables will allow for the testing of long-standing ecological hypotheses pertaining to Laminariales vertical distributions and mechanisms of persistence in variable environments. More complete investigations are required to assess the underlying ecologies of microscopic life-stages and their influences on kelp species and associated ecosystems.

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