GENETIC VARIATION AND DISPERSAL ECOLOGY OF THE LICHEN Erioderma pedicellatum IN NEWFOUNDLAND: RECOMMENDATIONS FOR MANAGEMENT OF A GLOBALLY RARE SPECIES

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Genetic variation and dispersal ecology of the lichen *Erioderma pedicellatum* in Newfoundland:

Recommendations for Management of a globally rare species.

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Abstract

Erioderma pedicellatum (Hue) P.M. Jørg is a globally rare and critically endangered lichen largely confined to the island portion of Newfoundland and Labrador. In 2002 the Boreal population was designated under the federal Species at Risk Act (SARA) as a species of "Special Concern", while the Atlantic population was listed as "Endangered". Under the provincial *Endangered Species Act* the lichen was designated as a "Vulnerable" species. This designation does not provide immediate protection for the lichen but requires the development of a management plan and periodic status assessment. Essential management baseline information on the species is lacking; especially on the ecology of the species as well as levels of genetic variability. This project attempts to answer key questions about the dispersal ecology of *E. pedicellatum* and identifies markers for analysis of genetic variation in Newfoundland and Labrador. Using the Internal Transcribed Spacer (ITS-1) of the ribosomal DNA (rDNA), two haplotypes were found among samples from Newfoundland and a single 62-year-old Swedish herbarium specimen. The common haplotype was found in both the Newfoundland samples and the Swedish sample. In addition through field research, this study describes for the first time the micro-ornamentation on the surface of *E. pedicellatum* spores and concludes through laboratory studies that the minute *E. pedicellatum* spores are actively discharged (<10 mm), can become trapped on the leg bristles or antennae of flying insects and may therefore be carried individually by these small animals. Given the high probability of an insect landing on an *E. pedicellatum* thallus and the shear abundance of insects in

the boreal forest, our hypothesis of insects as dispersal vectors of spores is possible. We predict the potential number of insects carrying *E. pedicellatum* spores to be in the range of 129-161 over a 15 year period, given the calculated probability of insects landing on thalli, 4000-5000 incidences. This has important implications for forest management in *E. pedicellatum* habitat and further research should build on these findings by testing maximum distances spores travel in the average gap sizes of the Avalon forests, the periodicity of dispersal, and implementing harvesting /cutting block sizes to test the dispersal limitation of the species. These findings should be compared to the genetic variation of the species. This is the first study documenting the genetic variation of this rare species and the results provide significant, important information on the global population. We conclude that given the low genetic variation and the lack of variation between North American and European populations, that the global population is one evolutionary unit. This supports the IUCN designation of a globally endangered population.

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General Introduction

Globally lichen populations are facing pressures associated with urban development (Young and Jarvis, 2001), logging (Peterson and McCune, 2001; Esseen and Renhorn, 1996; Rolstad et al., 2000; Pharo and Beattie, 1997; Hedenas and Ericson, 2000, 2003), global warming (Malcolm and Markam, 2000; van Herk et al., 2002), fire (Werth et al., 2006; Wolseley, 1991, 1994) and air pollution (Lambley and Wolseley, 2004; Maass, 1999; Richardson and Cameron, 2004; Richardson, 1992; Silberstein et al., 1996; Seaward, 1992). Wolseley (1995) summarizes the global anthropogenic and natural impacts on the lichen community as increasing and of concern for lichenologists. This is especially important for highly sensitive lichen species requiring stable environments to complete their life cycles. Many cyanolichens, or lichens containing cyanobacteria as the photobiont partner (lichens consist of a symbiotic relationship between an algae or cyanobacteria and a fungus) are particularly sensitive to environmental change and as they thrive mostly in old-growth habitats where the microclimatic conditions are most stable (Goward and Arsenault, 2000, 2001; Sillett et al., 2000).

Erioderma pedicellatum (Hue) P.M. Jorg. is classified as a cyanolichen as it contains the cyanobacterium *Scytonema* as an aggregate partner. Although not found in old-growth forests typical of west-coast Canada cyanolichens (Goward and Arsenault, 2000), it is found in mature (50 years +) stands of balsam fir (*Abies balsamea* (L.) Mill) forests or stands approaching the end of the forest life cycle in Newfoundland and Labrador (Maass and Yetman, 2002). Historically the lichen was

also found in Sweden and Norway in Europe, and in Nova Scotia and New Brunswick in Eastern Canada (Maass and Yetman, 2002). The only populations currently known are in Nova Scotia and Newfoundland with the latter province having 99% of the world's population. In 2003 *E. pedicellatum* was listed as critically endangered on the International Union for the Conservation of Nature and Natural Resources (IUCN) global Red-List due to declining population numbers and threats to local habitat (Scheidegger, 2003). In 2002 the Boreal population was designated under the Canadian Species at Risk Act (SARA) as a species of "Special Concern" and the Atlantic population was listed as "Endangered". Under the Newfoundland and Labrador *Endangered Species Act* the lichen was designated as a "Vulnerable" species. Designation as a vulnerable species in Newfoundland and Labrador requires a management plan for conservation of the species.

A management plan for *Erioderma pedicellatum* was drafted and released to the public by the Minister of Environment and Conservation, Government of Newfoundland and Labrador in July 2006, and lists seven main threats to *E. pedicellatum* populations, consisting of both anthropogenic and natural disturbances. Anthropogenic disturbances include land development, logging, air pollution, fire, pesticides and climate change, and natural stressors include wind, fire, insect outbreaks, stand senescence, and moose herbivory (Keeping, 2006). One of the main localities in Newfoundland and Labrador, Lockyer's Waters, has shown significant decline in population numbers since 1999 due to anthropogenic and natural disturbances (Conway, 2002). Lockyer's Waters is classified as a proposed

Ecological Reserve candidate under the NL Wilderness and Ecological Act (WER Act) giving it temporary protection. Lockyer's Waters, in the centre of the Avalon Peninsula, contains over half of the provinces population of the rare lichen (Maass and Yetman, 2002).

Rapid decline in localized populations of *Erioderma pedicellatum* due to a combination of anthropogenic and natural disturbances is a growing concern for sustainability of the world's population of this lichen. Populations that rapidly lose a high percentage of thalli may suffer from reduced genetic variation and a loss of rare alleles. Such a population bottleneck may eventually contribute to a reduction in the number of haplotypes in a population and reduce the evolutionary resilience of the species (Hartl & Clark, 1997). Further genetic variation may be lost as a result of random genetic drift. Populations with reduced genetic variation may be more susceptible to stochastic environmental events and eventually extinction (Frankham et al., 2002).

Mating systems may contribute to the lack of genetic variation of some lichens since self-compatible mating populations of sexually reproducing fungal partners provide virtual clones (Walser, 2004). Cloning (asexual) reproduction through thalli fragmentation or dispersal of diaspores may also reduce local genetic diversity (Cronberg, 2000). *Erioderma pedicellatum* only reproduces sexually and has never been known to reproduce asexually as other cyanolichens do (Maass and Yetman, 2002) and it is not known if *E. pedicellatum* has a self-mating system, in

which fungal strands of the lichen thallus self-fertilize. Other studies have shown that lichens are self-incompatible (Honegger et al., 2004; Scherrer et al., 2005) however, inferring sexual reproduction occurs in *Erioderma pedicellatum*.

Genetic variation of a lichen species depends greatly on dispersal capacity (Walser et al., 2001; Lindblom and Ekman, 2006) and the type of stand-level disturbance (Werth et al., 2006). Successful regeneration of lichens is complex and depends on a multitude of factors such as the mobility of lichen propagules (lichen propagules can be either sexual spores or asexual, often symbiotic, diaspores), the frequency of dispersal, the ability of a propagules to reach adjacent, suitable forest habitat, the ability of a fungal strain to find a genetically compatible photobiont partner (Seymour et al., 2005) and the ability of fungal strain in small populations to be sexually compatible (Vekemans et al., 1998). To date these factors and their contribution to reestablishment of *Erioderma pedicellatum* have not been investigated. The forest disturbance regime is complex on the Avalon Peninsula and is influenced by natural disturbance such as insect kill, wind throw and moose herbivory; and anthropogenic disturbance such as logging and land use. This, combined with a rapid 80 year natural succession makes for a complex forest system (Forest Ecosystem Strategy Document, 1997). Dispersal of lichen spores can be a limiting factor in species regeneration, especially in cyanolichens, if the gap size of the forest, which is determined in large part by the disturbance regime, exceeds the maximum dispersal distance of spores, preventing dispersal from older decaying

stands to younger regenerating stands (Dettki et al., 2000; Öckinger et al., 2005, Sillett et al.., 2000; Walser et al., 2001).

This study attempts to elucidate the specific aspects of the life history of *Erioderma pedicellatum* crucial to management of this lichen. Firstly, what are the intra- and inter-population level of genetic variation both among sites in Newfoundland and Labrador, and between historical amphi-Atlantic populations in Newfoundland and Europe? Secondly, how are *E. pedicellatum* spores dispersed; specifically what are the morphological characters of *E. pedicellatum* spores, and are there vectors within forest stands in Lockyers Waters that can successfully disperse the spores? By answering these two basic questions managers of *E. pedicellatum* populations can begin to understand the relationship between disturbance regimes in balsam fir forests on the Avalon Peninsula and the life history of the species. This will allow for the development of new scientific questions, but more importantly contribute to preliminary management processes for protecting the rare species. We make specific recommendations in this thesis on how to manage *E. pedicellatum* populations in Newfoundland and Labrador forests.

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Co-Authorship Statement

All manuscripts in this thesis were co-authored with Luise Hermanutz and Christoph Scheidegger. In all instances I was the principal contributor to the project design and proposal, implementation of the laboratory and field research components, analysis of the data and manuscript preparation.

CHAPTER 1

Comparison of the ITS-1 region of the critically endangered lichen *Erioderma pedicellatum* in Newfoundland and Sweden.

1.1 Introduction

An increase in human impact on global ecosystems including deforestation, pollution and urban development, has threatened global species diversity (Chapin et al., 2000; Sala, 2001). In particular several significant lichen communities are currently threatened (Wolseley, 1995). Of these lichens, epiphytic cyanolichens are most vulnerable to habitat change and disturbances, such as clear-cut logging (Goward 1994; Prestø and Holien, 2001) due to changes in microclimate (Renhorn et al., 1997; Rheault et al., 2003).

Fragmentation is common in the boreal forest as a result of anthropogenic and natural disturbance. Clear-cut logging and wind throw produce gaps in the continuous forest and separate adjacent forest stands. The prevalence of bog, fen or lakes and ponds also contribute to natural fragmentation. Habitat fragmentation may constitute a threat to boreal cyanolichens, as fragmentation produces island habitat and results in altered microclimate at fragment edges. In Newfoundland, the long history of land use (Forest Ecosystem Strategy Document 1997) and prevalence of lakes and bogs produce highly fragmented habitat across the landscape.

Small fragmented populations can result in reduced genetic diversity if genetic exchange between populations is limited, and may result in a greater likelihood of extinction (Frankham, 1995, 1997; Montgomery et al., 2000; O'Brien, 1985).

Templeton et al., (1990) suggest that if habitat loss and fragmentation results in complete genetically isolated habitat "islands", then each island becomes demographically independent and local extinction can occur. Scheidegger et al., (1998), for example, have shown that remote, isolated populations of *Lobaria pulmonaria* can become locally extinct due to genetic isolation. Such reduction in genetic diversity, or extinction of local populations, is not unique to lichens and has also been observed in mammals (Hale et al., 2001; O'Brien, 1983), birds (Westemeier et al., 1998) and plants (Young and Brown, 1996).

Erioderma pedicellatum (Hue) P.M. Jørg. (family Pannariaceae) is a rare amphi-Atlantic epiphytic cyanolichen found in mature boreal forest and is designated as critically endangered on the IUCN Global Red List (2003). In Europe, *E. pedicellatum* occurred in Norway and Sweden (Holien 1995), but these populations were extirpated in the early 1960's in Sweden and confined to a single specimen in Norway (Scheidegger 2003). Currently the species is known from Nova Scotia (32 thalli; Keeping, 2006) and Newfoundland (5060 thalli; Maass & Yetman 2002). Historically its distribution extended into New Brunswick; however the lichen has not been relocated since the early 20th century (Maass 1980). Over half of the current population is found on the Avalon Peninsula in eastern Newfoundland (2148 thalli). In 2003, the Committee on the Status of Endangered Wildlife in Canada (COSEWIC) designated the lichen as "Endangered" in Nova Scotia and New Brunswick and of "Special Concern" in Newfoundland (Maass and Yetman 2002); the species does not occur in Labrador.

Studies of genetic variation in lichens have mostly focussed within genera (Crespo et al., 1997; Martin et al., 2003; Thell 1999), families (Ivanova et al., 1999; Mattsson & Wedin, 1998; Myllys et al., 1999; 2005; Simon et al., 2005) and orders (Stenroos & Depriest, 1997, 1998). Several studies have investigated genetic variation within lichen species (Beard & DePriest, 1996; DePriest 1994; Dyer and Murtagh 2001; Groner and LaGreca 1997; Hogberg et al., 2002; Lindblom and Ekman, 2006; Prinzten and Ekman 2002; Zoller et al. 1999). The Internal Transcribed Spacer region (ITS) of the ribosomal DNA is widely used when studying genetic variation in lichenized ascomycetes (Bridge and Hawksworth 1998; Grube and Kroken 2000) however, Small Subunit (SSU), Large Subunit (LSU), Intergenic Spacer region (IGS) (Lindblom and Ekman, 2006), RBP2 (RNA Polymerase II), RAPDs (Heibel et al., 1999) and more recently microsatellites (Walser et al., 2003) have also been employed. The ITS-1 region was chosen for this study since it has been successfully used as a proxy of genetic variability in other rare lichen studies such as *Lobaria pulmonaria* (Zoller et al., 1999), *Cavernularia hultenii* (Prinzten and Ekman, 2002) and *Nephroma occultum* (Piercey-Normore et al., 2006), a COSEWIC designated "Species of Special Concern", in addition with the use of ITS-2. For this study only species-specific primers could be developed for ITS-1, and therefore it is the only rDNA region used. The ITS region has reported differences in levels of variation, ranging from low (Prinzten and Ekman, 2002; Zoller et al., 1999) to high (Lindblom and Ekman, 2006).

In this study was to genetic variability in the ITS1 region of the rDNA gene within and among isolated populations of *E. pedicellatum* in Newfoundland was investigated and compared to a single herbarium specimen from Sweden. Data on genetic diversity within this species are needed to optimise future conservation priorities, such as reintroductions, and management plans for this globally critically endangered species.

1.2 Methods

Lichen Samples

In Newfoundland a total of 91 samples were collected in 11 sites from four localities representing its island distribution (Table 1; Fig. 1). Only one sample was collected per host tree, 90% of which were balsam fir (*Abies balsamea*). All specimens were mature (i.e., > 10mm). Thalli were inspected for areas of necrosis, damage due to mite herbivory and parasitic fungal invasions. These areas were avoided during sampling and whenever possible only healthy portions of each thallus were taken. Thallus fragments, averaging $2mm^2$ were excised using a sterile surgical knife and stored in collecting envelopes. In rare cases where the thallus was loosely adhered to the tree trunk or branch or fallen to the forest floor, the entire thallus was taken. Samples were air dried at room temperature for 48 hours and then stored at $5^{o}C$ until analysis.

Thallus Preparation

Prior to DNA extraction, thalli were rinsed twice in distilled water to remove other attached lichens, bryophytes and liverworts, and then placed in preweighed, sterile 2ml eppendorf tubes with glass beads. Tubes containing thallus fragments were reweighed and wet thallus weights recorded for comparison to PCR products.

DNA Extraction

DNA extraction was carried out using three different methods to optimise amplification of DNA. For samples screened in 2000, DNA extraction was carried out using a protocol modified for lichens by Zoller et al., (1999), and the CTAB method (Velegraki et al., 1999). However these methods produced less PCR product compared with the GenElute plant genomic DNA kit (SIGMA™, St. Louis, MO, USA).

In 2001, DNA extraction was carried out using the GenElute™ kit (SIGMA™, St. Louis, MO, USA). After samples were cleaned and weighed (wet) thallus fragments (225 mg) were placed in 2 ml vials and frozen in liquid nitrogen. The samples were then dehydrated in an Alcatel 2004 vacuum dehydrator for at least 9 hours prior to DNA extraction. To extract genomic DNA lichen cells were first disrupted by thorough agitation for 5 minutes in a shaker mill using sterile glass beads placed in 2ul vials. Samples were then placed on ice. 350 ul of Lysis buffer A and 50 ul of Lysis buffer B were added to each tube and placed in a 65°C waterbath for 10 minutes. To precipitate the cellular debris, proteins and polysaccharides, 130ul of

precipitation solution was added, mixed well and then centrifuged at 14000 RPM for 5 minutes. The supernatant was pipetted onto a GenElute filtration column and centrifuged at 14000 RPM for 1 minute. To bind the DNA, 700 ul of binding solution was added to the flow through liquid and transferred to a GenElute Nucleic Acid binding column. Bound DNA was washed twice with 500 ul of Washing Solution and centrifuged at maximum speed for one minute. DNA was then eluted in 100 ul of warmed (65°C) TE buffer pH 8.4 and incubated at room temperature for 5 minutes. This final step was repeated twice yielding two 100ul aliquots of purified DNA. One aliquot was placed in –80°C for long-term storage. Following extraction DNA concentrations were measured using the Hoefer DyNA Quant 200 DNA quantifier (Hoefer, Pharmacia Bio- tech, San Francisco, CA).

PCR Amplification

Primers

For initial amplification the fungal specific primer ITS1-F (Gardes and Bruns, 1993) and the general primer ITS4 (White et al., 1990) were used. Other primer sets were used separately and in combination with ITS 1F and ITS4 (Table 2). Because each of the primer sets either yielded low concentrations of PCR products or multiple products due to the presence of fungal contaminants, new primer sets were designed using the computer programs PRIMER (Lincoln et al.. 1991) and OLIGO 4.0 (Primer Analysis Software, National BioSciences). This included a series of forward (F) and reverse (R) species-specific primers in the 5.8 conserved and ITS regions (Ep 73F, Ep 394R and ITS 1-135 F, ITS Erio). All primers were used in combination in an attempt

to increase efficiency (yield) and improve specificity. The primer set Ep 73F and Ep 394R were selected as they produced the highest yield and greatest specificity and are described here for the first time (Table 3). Only after species-specific primers were developed (Carbone and Kohn, 1999) were we able to produce PCR products that proved to be *E. pedicellatum* rDNA.

PCR

Polymerase chain reaction (PCR) was carried out in a 51ul reaction volume (32.5ul H_2O, 5ul 10x PCR buffer, 5ul 50mM $MgCl_2$, 2ul 1 mM dNTP`s, 2ul of selected primers, 0.5ul TAQ Polymerase and 2ul of DNA extract) using a standard cycling protocol: denaturing at 94°C for 2 minutes, 30 cycles of denaturating at 94°C for 1 minute, 1 minute annealing at 55°C, extension at 72°C for 1 minute, and a final extension at 74°C for 8 minutes. PCR products were purified prior to cycle sequencing reactions GenElute Plant Genomic DNA Purification Kit (SIGMA™, St. Louis, MO, USA).

DNA Sequencing

DNA sequencing was carried out using both the ABIPRISM 310 and ABIPRISM 377 MJ BaseStation™ (MJ Research, Waltham, MA, USA) automated sequencers. Because of the presence of a small poly-A microsatellite in the ITS1 region of the *Erioderma pedicellatum* rDNA (Fig. 2), TAQ polymerase slippage occurred immediately downstream from the microsatellite on the forward strand and immediately upstream relative to the microsatellite on the reverse strand. As a result,

the clarity of the sequences was sub-optimal in these regions but of high quality in the regions before and after the microsatellite on the forward and reverse strands respectively. Therefore sequences did not require editing in a computer sequence-assembling program. Single stranded forward and reverse electropherograms were visualized using the computer program Sequence Navigator PPC Alias and sequence alignments were consequently constructed manually.

Swedish Herbarium Specimen: Special Protocol

We expected highly degraded genomic DNA in the 62-year-old lichen specimen from Sweden. Low amounts of starting material and low amounts of genomic DNA translate into low amplification products in single PCR runs. In this study PCR products were not visible on 2% agarose gels. To remedy this problem multiple PCR's (50 ul each) were combined, precipitated in ethanol and re-eluted in 10ul. Only when 24 individual PCR products were combined was there sufficient DNA for sequencing.

Phylogenetic Analysis

Phylogenetic analysis was performed using the computer program PAUP (Phylogenetic Analysis Using Parsimony) (Smithsonian Institution, USA). Bootstrap values were determined using 1000 replicates, producing a bootstrap consensus tree. *Lobaria pulmonaria* was chosen as the outgroup because the DNA was available for analysis (from previous Walser and Zoller studies; see references) and it represented a

lichen of a different family (Lobariaceae). *Pannaria* sequences were downloaded from NCBI's BLAST search (Altschul et al., 1997). *Degelia plumbea* was sequenced in the study along with *Erioderma pedicellatum* and *E. sorediatum*, using the same protocol as *E. pedicellatum*, and the primers ITS 1F and ITS 4.

1.3 Results

Two haplotypes, A and B, were found in the analysis of ITS1 region in 91 samples collected from Newfoundland. These haplotypes were not exclusive to a specific geographic location (Fig. 1; Table 1). Haplotype A was more common occurring in 87 (95.5%) of the 91 samples investigated, with haplotype B occurring in just four samples (4.5%), one from Lockyers Waters and three from Bay D'Espoir. Haplotypes A and B are distinguished by 11 changes, 6 substitutions and 5 insertion/deletions (Fig. 2).

Subjecting Haplotype A to a BLAST search revealed a sequence of approximately 345 bp in length. The sequence contains a portion of the ITS-1 and the 5.8S regions, with none of the 18S region included in the sequence. The species-specific primer designed in this study was nested within the ITS-1 region; therefore the entire ITS-1 was not sequenced, leaving approximately 100 bp to investigate variation.

Haplotypes A and B were confirmed in two ways. First by constructing a bootstrap consensus tree of Pannariaceae species, using the Lobariaceae as an outgroup (Fig. 3), and second by subjecting the sequences to Blast search (www.ncbi.nlm.nih.gov/BLAST/). Following current taxonomy, the *Erioderma* group

paired more closely to its own family group (Pannariaceae) as compared to the outgroup. When comparing haplotype A with *E. sorediatum* (Fig. 4) there are 17 substitutions and 5 insertion/deletions exceeding the differences between the two haplotypes. There are 27 substitutions and 22 insertion/deletions between *E. sorediatum* and haplotype B (Fig. 5). The pairing of *E. pedicellatum* with *E. sorediatum*, *Pannaria* and *Degelia* is supported by Ekman and Jorgenson (2002) in a study of the Pannariaceae. Second, by determining the e-score, or dissimilarity score, with other sequences; a value of (5e-11 to 9e-09) showed a significant value of dissimilarity with other sequences.

Sequencing the single herbarium Swedish sample revealed an identical match to haplotype A, both from Lockyers Waters and Bay D'Espoir in Newfoundland.

1.4 Discussion

Two haplotypes, denoted here as A and B, were found in the analysis of the ITS1 region in 91 samples of *Erioderma pedicellatum* collected in Newfoundland, Canada. No genetic variation was found between the Newfoundland samples (haplotype A) and the specimen from Sweden.

There were difficulties encountered in successfully amplifying and sequencing specimens of *E. pedicellatum*. Sequencing of PCR products revealed the possible presence of fungal contaminants. The duplicate bands could also have been the result of mismatches between the *E. pedicellatum* template and the primer pair. Ekman (1999) describes this as a common phenomenon in short sequences lacking intron-like insertions. Few options produce clear, distinct PCR products, although Ekman recommends the use of species specific primers. The primer pair, Ep 73 F and Ep 394 R, described here for the first time, was used here to amplify *E. pedicellatum* rDNA, resulting in a clear product with a fragment length of 345 bp.

Using the ITS regions, other lichen studies have shown a low number of infraspecific polymorphisms (1 - 4) and a high number of haplotypes (Dyer and Murtagh, 2001; Groner and LeGreca, 1997; Lindblom and Ekman, 2006; Zoller et al., 1999) but the literature varies greatly. For example, Hogberg et. al (2002) compared the genetic variation of *Letharia vulpina* in Europe and North America using eight different loci and found almost no genetic variation in European populations. Conversely, Lindblom and Ekman (2006) found exceptionally high genetic variation within populations of *Xanthoria parietina* in Norway (up to 16 haplotypes). In

contrast this study showed a lower number of haplotypes (2) and a higher number of differences (11) between haplotype sequences. Prinzten and Ekman (2002) show similar findings of low number of haplotypes in *Cavernularia hultenii* in Newfoundland but a higher number of differences between the sequences. Our findings and the results of other studies, are consistent with Bridge and Hawksworth (1998) who concluded that the level of variation within the ITS regions of the rDNA gene may vary with different species and depend on the life history of that species. However a larger sample of European specimens would be needed to confirm conclusions regarding phylogeography. Grube and Kroken, 2000 illustrate the complexities of using ITS in determining variation by concluding that the investigation of a single locus may not accurately reflect the extent of the variation and the separation of a species, especially across continents.

Habitat fragmentation can reduce genetic variation in instances where gap sizes exceed dispersal capacity of species (Högberg et al., 2002; Templeton et al., 1990; Wallace 2002). Werth et al., (2006) have shown that different types of disturbance regimes (fire, logging) influence the genetic variation of rare lichen species differently, and local stand-level disturbances, depending on the size of the disturbance, may not reduce genetic variation in the short term. In Newfoundland, the relationship between forest fragmentation and the life cycle of *E. pedicellatum* is not completely understood (Maass and Yetman 2002) but could result in creation of genetically isolated habitat "islands" depending on dispersal capacity. Walser et. al (2005) investigated the relationship between genetic variation and local landscape

disturbance in British Columbia and Switzerland and found that lichen diversity in fragmented habitat can be high, depending on the gene flow and the geographic isolation of alleles. Studies to identify the key factors driving patterns of genetic variation such as reproductive and dispersal biology of *E. pedicellatum* are necessary. Initial findings suggest insects can disperse spores, with a high probability (Yetman, Chapter 2), therefore potential genetic exchange between isolated habitat "islands" would be possible.

The presence of haplotypes A/B, the rarity of haplotype B and sharing of haplotype A between North America and the single Swedish herbarium sample pose interesting questions about the evolution of the species. Jørgensen (1990) suggests that the species is a primitive member of a genus derived from Gondwanan stock. The species may have arrived in Europe along the Tethyan Sea and subsequently became isolated from the rest of the genus. Jørgensen believes that the species reached North America before the Quaternary glaciations and both the European and North American populations fluctuated with changes in the conifer forests as the glaciation proceeded. The island of Newfoundland had a complex deglaciation pattern, with the possibility of glacial refugia; however no forest survived within these refugia (Anderson and Macpherson 1994), therefore the populations may have established from a reinvasion of southern populations, or from trans-Atlantic dispersal. More sequences of historic European samples taken from herbarium specimens are needed to confirm the haplotypic frequency and the variability between European and North American samples. This study provides preliminary evidence that there is low

variation between trans-Atlantic populations, possibly caused by slow genetic drift or recent long distance dispersal.

Next Steps for Future Conservation

Results of this study are an important first step in assessing the level of genetic variation in *Erioderma pedicellatum* populations. Using a portion of ITS1, the presence of low genetic variation in *E. pedicellatum* populations in Newfoundland and the lack of variation between North America and the Swedish herbarium specimen have implications for long-term management of *E. pedicellatum*. Firstly, managers should consider additional genetic research to reveal with more certainty, the lack of variation within and among amphi-Atlantic populations, especially prior to any transplantation efforts. Secondly, preliminary results on genetic variation support the IUCN designation of a single amphi-Atlantic population of *E. pedicellatum*; however a larger proportion of the genome should be analysed to confirm our findings.

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Table 1.1 Portions of thalli or full thalli of *Erioderma pedicellatum* collected within Newfoundland for genetic analysis.

Locality	**# of Thalli/Parts Collected**	**Population** Size (# thalli)
Ripple Pond	20	121
Lockyers Waters (S-7)	20	211
Lockyers Waters (S-9)	20	174
Lockyers Waters (S-1)	1	30
Lockyers Waters (S-2)	1	46
Lockyers Waters (S-3.1)	1	249
Lockyers Waters (S-3.2)	1	Included in S-3.1)
Lockyers Waters (S-4)	1	135
Bay D'Espoir (S-1)	13	204
Bay D'Espoir (S-2)	12	115
Salmonier Nature Park	1	4

GPS Coordinates and population sizes for Lockyers Waters taken from McHugh (1998). Symbols S-1 to S-9 represent the Newfoundland locality in which samples were collected for genetic analysis and are in accordance with Maass and Yetman (2002).

Table 1.2: Primer sets used to amplify *Erioderma* rDNA. Successful amplification, including suitable concentrations and single fragments, are highlighted with an asterix.

Primer	Position	Direction	Sequence (5'-3')	Source
ITS-1F	SSU	F	cttggtcatttagaggaagtaa	Gardes and Bruns, 1993
ITS-4	LSU	R	tcctccgcttattgatatgc	Gardes and Bruns, 1993
ITS 1	SSU	F	tccgtaggtgaacctgcgg	White et al.., 1990
LR 15	LSU	R	taaattacaactcggac	Zoller et al.., 1999
LR 22	LSU	R	cctcacggtacttgttcgct	Zoller et al.., 1999
5.8S 304F	5.8S	F	catcgaatctttgaacgc	This Study
5.8S 261F	5.8S	F	agcgaaatgcgataagtaat	This Study
5.8S 280R	5.8S	R	attacttatcgcatttcgct	This Study
5.8S 326R	5.8S	R	aatgtgcgttcaaagattc	This Study
ITS 1-135F	ITS-1	F	tccgcatcccgtgggaccgt	This Study
ITS-Erio	ITS-1	F	tccgcatcccgtgggac	This Study
Ep 73F	ITS-1	F	cgagagaaacggcaacagg	This Study *
Ep 394R	ITS-2	R	gacgcagacccaacaccaa	This Study *

Note: Under *Position* ITS = Internal Transcribed Spacer Region, 5.8S = subunit of the ribosome gene, LSU = large subunit of the ribosome gene, SSU = small subunit of the ribosome gene. Under *Direction* F and R stand for forward and reverse respectively.

Table 1.3: Primer specifications for species-specific primer pair Ep 73F and Ep 394R used to amplify *Erioderma pedicellatum*.

Annealing Temp.	%GC Content	Tm Difference	Tm
56.5	52.1	0.8	79.3

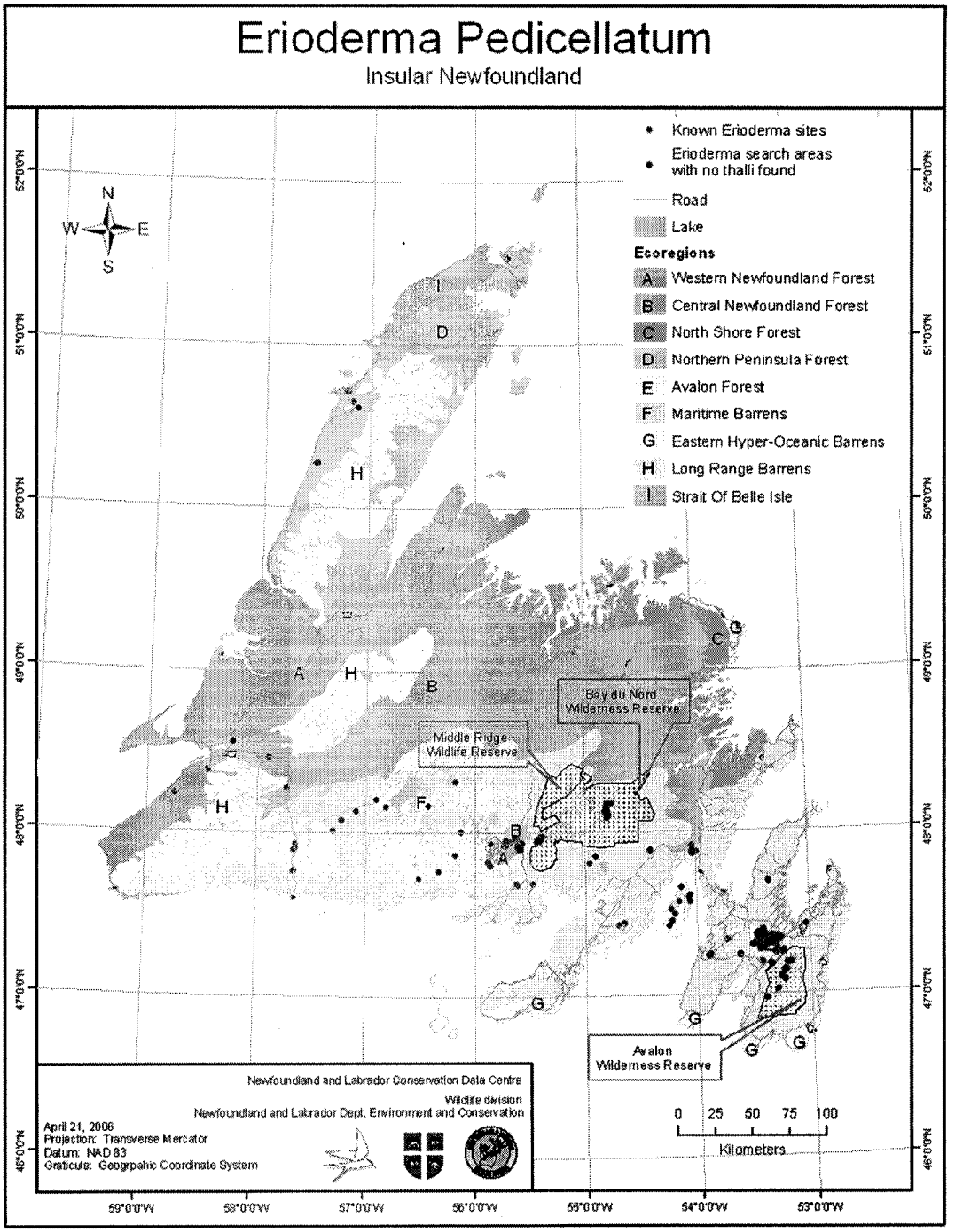

Figure 1.1 Known range of *Erioderma pedicellatum* sites in Newfoundland and Labrador. Of note are sites E (Lockyer's Waters) and B (Bay d'Espoir). Map used with permission from the Department of Environment and Conservation, Government of Newfoundland and Labrador. Haplotype B (1) was found in Lockyer's Waters and Bay d'Espoir (3).

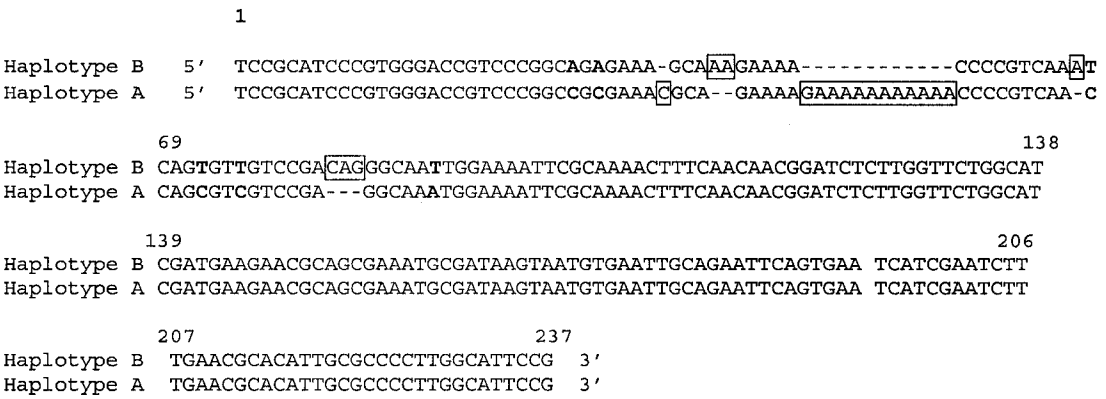

Figure 1.2 Sequence variation between two haplotypes (A and B) found in populations of *Erioderma pedicellatum* in Newfoundland. Of note is the ITS1 region (positions 1 through 102) and the 5.8S conserved region (positons 103 through to 237). Also of note is the PolyA microsatelite in upstream position 45. Point mutations are signified by in bold and insertions/deletions by frames.

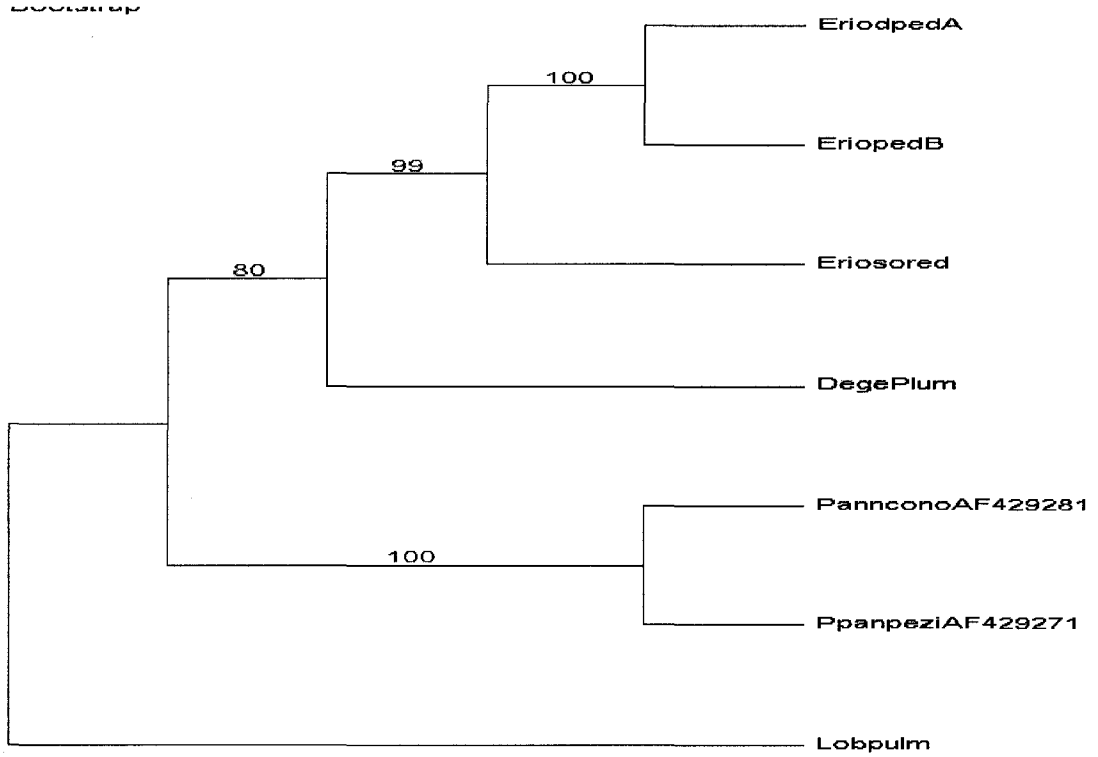

Figure 1.3 Bootstrap consensus tree (Bootstrap values shown) showing the relationship of Haplotype A and Haplotype B (Erioped A and B) with *Erioderma sorediatum* (Eriosored) and other family species (*Degelia*) collected in Madagascar using *Lobaria pulmonaria* as an outgroup. Specimens prepared and sequenced by the author: *Erioderma pedicellatum* (EriopedA and EriopedB), *Erioderma sorediatum* (Eriosored), Lobpulm = *Lobaria pulmonaria*, Degeplum=*Degelia plumbea.* Specimen sequences taken from NCBI genbank: PpanpeziA = *Pannaria pezizoides*, PannconoAF = *Pannaria conoplea.*

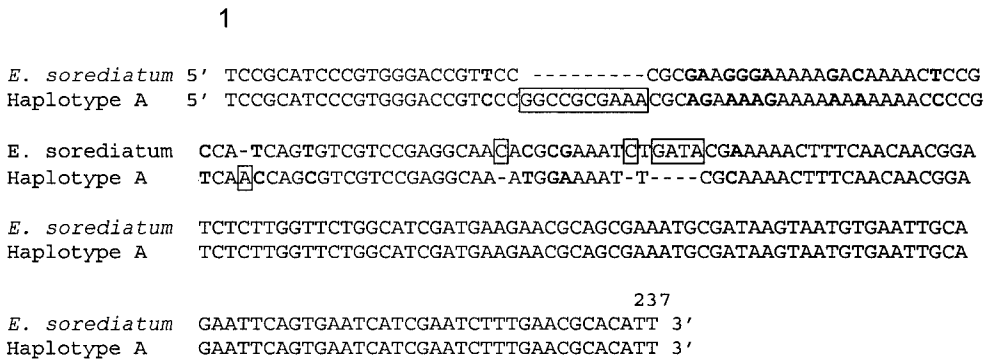

Figure 1.4 Comparison of the ITS1 region between *Erioderma sorediatum*, collected in Madagascar, and haplotype A of *Erioderma pedicellatum* from Newfoundland.

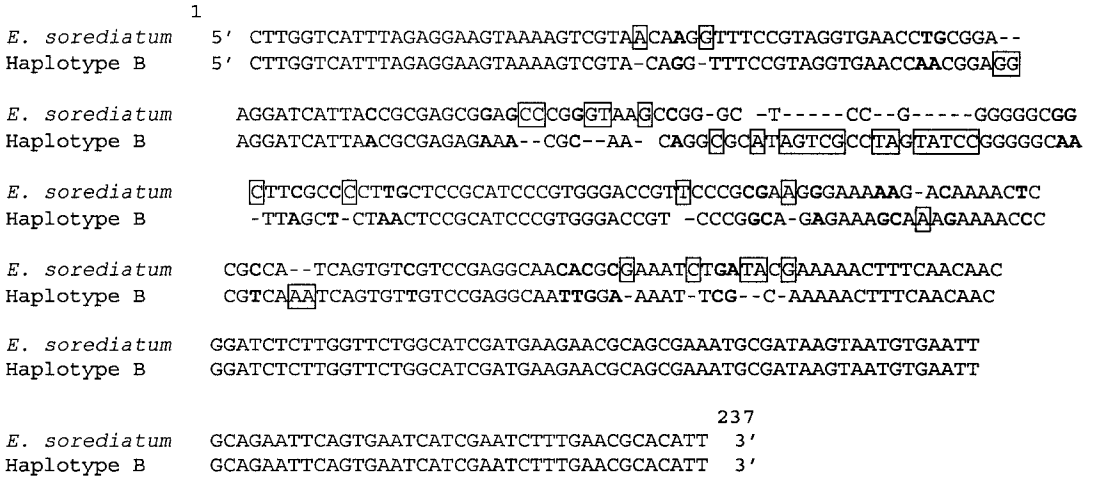

Figure 1.5 Comparison of the ITS1 region between *Erioderma sorediatum*, collected in Madagascar, and haplotype B of *Erioderma pedicellatum* from Newfoundland. Block = insertion/deletion; bold = substitution.

CHAPTER 2

Dispersal Ecology of the Critically Endangered Boreal Lichen, *Erioderma pedicellatum.*

2.1 Introduction

Arboreal lichens are key elements of forest biodiversity in Eastern Boreal Forests (Desponts et. al, 2004; McCune, 2000), yet little is known as to how arboreal lichens colonize new hosts when their host trees degenerate as a result of change associated with natural forest succession. There are many factors that limit the colonization of individual lichen thalli from an aging host tree to a younger, more stable recipient host (Sillett et al., 2000; Richardson, 2002; Kalwij et al., 2005). These factors may include challenges during the complicated lichenization process (Richardson, 1999), by the complex spatial dynamics of the forest landscape (Sillett and Goslin, 1999; Snall et al., 2003) or limited by spore production (Pyatt, 1969; Bailey, 1976), periodicity (Yamamoto et al., 1998; Clayden, 1997) or projection (Dettki et al., 2000; Walser et al., 2001). It is becoming increasingly important to understand the complexities of dispersal ecology in order to manage long-term persistence of lichen species (Keon, 2001). This is especially true for rarer species restricted to mature or old growth forests subjected to intense anthropogenic disturbances such as logging or forest management regimes (Neitlich, 1993; Hilmo et al., 2005; Sillett et al., 2000) and especially true given the global crisis in biodiversity extinction (Hoekstra et al., 2005).

Few studies have investigated the patch-dynamics of forests in relationship to the persistence of lichens (Wei-Dong et al., 2001). Depending on the type of dispersal model used; (i.e., metapopulation, source-sink or remnant species model), one may predict the persistence of lichens over time and space (Snall et al., 2003). Reduced dispersal between satellite populations over time can reduce genetic variation in species (Walser et al., 2003) and increase vulnerability to environmental change. Increased or decreased gap sizes or gap dynamics effectively change the dispersal models over time by altering the amount of genetic exchange between remote populations. Both natural disturbance such as wind throw and insect kill, and anthropogenic disturbance such as clear-cut logging contribute to gap dynamics in forests. An anthropogenic disturbance more often affects larger portions of forest compared with natural disturbance, creating larger forest gaps and therefore greater distances between neighbouring populations. Wherever the gap size exceeds the lichens maximum distance for spore projection (in the absence of dispersal vectors), the result is a dispersal limitation (i.e., lichen spores not reaching adjacent forest stands), even though there has been little investigation of factors. Depending on the metapopulation model used, dispersal limitation of lichen populations within forest stands can be explained by spatial aggregation and gap dynamics (Lobel et al., 2005). In other words the dispersal capacity of a species (the ability of a species to produce x number of ascospores and then disperse them) is largely due to the gap cycle of the forest stand and the spatial arrangement of the sub-populations. In this study we suggest that epiphytic lichens, similar to pollen dispersal syndromes found in vascular

plants (Degen and Roubik, 2004) have evolved mechanisms to aid in spore dispersal in such sites where natural and anthropogenic disturbance have created large gaps that exceed maximum dispersal capacity, and may result in lower abundance of cyanolichens (Benson and Coxson, 2002). It is predicted that many arboreal forest lichens that produce sexual spores have evolved mechanisms to aid in long distance spore dispersal.

Knowledge of the life history of a lichen is important in understanding how it persists in its surrounding environment. Most lichenologists have a conceptual understanding of how lichens disperse their minute spores in patchy environments but there have been few studies documenting this important aspect of life history. It is generally accepted that asexual and sexual lichen propagules are dispersed by several methods including wind, water and animals (Hale, 1969). The majority of studies have focused on wind dispersal (Bannister and Blanchon, 2003; Brodie, 1953; Bailey and Garrett, 1968; Heinken, 1999; Marshall, 1996; McCartney, 1997) and/or water dispersal (Armstrong, 1994; Bailey, 1966; 1968; Eldridge, 1996; Gilbert, 1996); few studies have investigated animal-lichen dispersal (Bailey, 1966; 1976; Lucking, 2000; McCarthy and Healy, 2000; Richards and Young, 1977).

Few studies have investigated the specific relationship between insects and the dispersal of spores in lichens (Peake and James, 1967; Tibell, 1994). Several studies show a secondary dispersal relationship of lichen spores through ingestion, by animals such as slugs and mites, and eventual defecation of spores (Miura and Mastumoto, 1997; Meier et al., 2002) and insects are well recognized as spore

dispersal vectors (Artbauerova and Janitor, 1988; Gaudet and Schulz, 1984; Levieux et al., 1991; Russin et al., 1984). For example Fox et al. (1991) showed the adherence of fungal spores (*Fusarium subglutinans*) to the carapace of the Engraver beetle (Scolytidae: *Ips* species) using electron microscopy. However none of the lichen or fungal studies has shown a co-evolutionary relationship between spore production and dispersal vectors, as has been extensively documented for vascular plants (Dafni et al., 2000).

Erioderma pedicellatum is found exclusively in the Eastern Boreal Forest of Atlantic Canada (Maass and Yetman, 2002) and is listed as globally critically endangered by IUCN (Scheidegger, 2003). Historically the lichen was also found in Sweden where the lichen population was eradicated in the 1950's (Holien, 1995) and Norway where last reports indicate the extirpation of the remaining European thallus (Maass and Yetman, 2002). *Erioderma pedicellatum* is now restricted to the Eastern Boreal forests of Nova Scotia (approximately 15 thalli) and especially Newfoundland where population numbers are estimated at approximately 5060 (Keeping, 2006; Maass and Yetman, 2002), with half of the world's population on the Avalon Peninsula.

The Eastern Boreal habitat on the Avalon Peninsula in Newfoundland has predictable, short lived forest life cycles (70-80 years). Dominated by balsam fir (*Abies balsamea*) these stands are susceptible to both natural and anthropogenic disturbance (Forest Ecosystem Strategy Document, 2003). This combined with natural fragmentation (barrens, bog, lakes, etc.) has created a continuum of gaps in

Erioderma habitat. As a result of this short turnover time and the patchy nature of the forest, *E. pedicellatum* must reproduce in these short lived mature fir stands and disperse spores to adjacent emerging forest stands before the eventual collapse of the host stand. To date there have been no studies of the dispersal ecology of this rare lichen, but Maass and Yetman (2001) speculate that dispersal is a limiting factor in the life cycle of the species. In this study, it was hypothesized that *E. pedicellatum* has evolved specific morphological characters and expulsion methods that increase the likelihood of dispersal in forested areas where gap size exceeds maximum dispersal distance.

This study documented various characteristics of the dispersal ecology of *E. pedicellatum* using both field and laboratory experiments. Sexual spore morphology is described. We then document timing of release and the quantity of spores released into its natural environment using in situ spore traps. Finally we investigate whether dispersal vectors of any kind contribute to dispersal of *E. pedicellatum* spores. Information on dispersal ecology is an important first step in understanding the life cycle and persistence of this rare species, and in assisting forest managers in implementing long-term conservation strategies for management plans.

2.2 Methods

Study Site

Research was carried out in Lockyers Waters (N 52 ° 46.419'; E 32 ° 81.86'), located in the central Avalon Ecoregion (Damman, 1981), on the island of Newfoundland,

Canada (Fig. 1), approximately70 kilometres east of the capital city, St. John's. This site contains the second largest population (953 thalli) of *Erioderma pedicellatum* known to the world (Maass and Yetman, 2002). Similar to other sites in Newfoundland the population is found in a balsam fir – feathermoss forest (Meades and Moore, 1989) near the base of a watershed, lying on a slope with a northwest exposure.

The forest stand is characterized by the dominant balsam fir with subdominant black spruce (*Picea mariana*) and paper birch (*Betula papyrifera*). Patches of sphagnum moss (*Sphagnum* spp.) are intermixed with feathermosses (*Hylocomium, Pleurozium*) on the forest floor; these mosses are important component as they maintain high moisture levels throughout the forest stand (Maass, 1980). Generally the stand is fragmented due to a recent history of logging, wind disturbance and insect kill (Forest Ecosystem Strategy Document, 2003). The average gap size in the forest site for this study is 5.7 metres. Trees are characterized as small to medium size (DBH = 39 cm $\pm$ 5 cm), with an average height of 7.1 m $\pm$ 0.16 m and with an average age of 61.1 years $\pm$ 1.3). The forest stand can be considered mature based on the average age and the percentage of dead standing trees in the site (42%).

Spore Morphology

To characterize spore morphology two specimens of *Erioderma pedicellatum*, semi-detached from the tree trunk, were collected from Lockyers Waters. Specimens were transported to the laboratory in sterile petri-dishes and kept at room temperature. One

day after collection apothecia were sectioned and prepared with Gold Sputter for scanning electron microscopy (SEM) the same day.

Spore Discharge

Laboratory experiments and field collections were used to document the seasonality and abundance of spore discharge in *E. pedicellatum*.

Laboratory

Three mature thalli were collected in Lockyers Waters in the spring of 2001. To ensure minimal impact on the population, thalli poorly attached to the substrate, with apothecia, were collected since these are generally lost over the winter season (Maass and Yetman, 2002). Immediately after collection, thalli were placed in individual, sterile petri dishes for transport to the laboratory.

In the laboratory lichen thalli were transferred to clean petri dishes prepared for microscopic investigation. Microscope slides covered with double sided tape were attached under the lid of the petri dish, positioned approximately 0.5-1.0 centimetres, perpendicular, from the apothecia. Thalli were saturated with water and allowed to dry for 24 hours prior to microscopic investigation to investigate spore discharge. To visualize released spores, microscope slides were stained with methylene blue and viewed under a standard compound microscope (40 x magnifications) for the presence of spores.

Field

In the field, 6 spore traps (Fig. 2.2) were established 1 cm from healthy, mature apothecia. The spore traps were designed to be flexible in order to manoeuvre the end suction cup close to the mature apothecium. This design does not alter the microclimate around the lichen, and is therefore superior to the microscope slide design used in previous studies (Clayden, 1997). Spore traps were monitored and collected from May 2000 to October 2000 on a biweekly basis or after a period of high relative humidity or precipitation. Spore traps were collected and placed in sterile petri dishes for transport to the laboratory at Memorial University. Traps were gold coated in a Gold-Sputtering device and immediately viewed with a scanning electron microscope (Hitachi 5570), since they were non-transparent and could not be viewed under a light microscope.

Spore Ejection: Formulating a New Hypothesis

Ten spore traps (Fig. 2.2) were set up in Lockyer's Waters in the summer of 2000 and monitored every 72-96 hours between May-November. Traps were collected from the field and immediately transported in sterile containers to the laboratory and prepared for SEM. Between the months of May-November no spores were collected in the field suggesting that some vector other than wind must play a role in the dispersal of spores. As such we decided to investigate in 2001 using both field and laboratory experiments whether insects play a role in dispersal of *E. pedicellatum* spores.

Laboratory

In May 2001 laboratory experiments were set up to investigate whether discharged *Erioderma* spores could be carried by flying insects. *Drosophila melanogaster* was chosen as the representative insect vector for several reasons; first, its laboratory maintenance is well documented, second the genus is a common inhabitant of the boreal forest (Tanabe et al., 2001) and third the morphological characteristics of *Drosophila* including small body, and body and leg bristles provide increased surface area for the adherence of spores, and reflected the general morphology of many small flies found in the boreal forest.

To test whether insects can carry *E. pedicellatum* spores, *D. melanogaster* larvae were allowed to mature and roam in an experimental chamber containing a mature *E. pedicellatum* thallus for 48 hours. Allowing *D. melanogaster* larvae to mature in a sterile chamber ensured that adult fruit flies would only be exposed to *E. pedicellatum* spores. Following exposure the fruit flies were killed (-15°C for 15 minutes), immediately brought to the laboratory, placed on double sided tape on the specimen stub, prepared with gold coating in a Gold-Sputtering device for approximately 30 seconds, and viewed under a standard scanning electron microscope (Hitachi 5570).

Field

To investigate if insects actively transferred *E. pedicellatum* spores *in situ*, timed insect surveys were conducted on the lichen's host trees along a transitional

gradient in the forest site in Lockyers Waters. This gradient consisted of thalli along the forest edge (FE), intermediate canopy zone (ICZ) and complete forest interior (CFI), with two trees (main tree and replicate) in each zone (six in total). Timed insect surveys of 10 minutes in duration were carried out along each section of the gradient (FE, ICZ and CFI) for 6 trials per tree (3 hour durations). The number of insects landing on the target host tree (trunk in middle of tree was monitored 1 m above ground) and on the lichen thallus was recorded. To qualify as a tree-landing, insects had to be on the target host tree for a minimum of 3 seconds and to be considered a thallus-landing, insects had to land directly on the thallus or land on the tree and then walk over the thallus. A one-way analysis of variance was used to determine if there was a difference in the number of landings along the transitional gradient. It should be noted that because insects were collected using visual inspection, small flies and wasps (eg. Agromyzidae), and their parasites, mites may have been overlooked.

Insect taxa were identified by studying the types of boreal insects present in Newfoundland and separating them in the field based on gross morphological characters including size, colour and shape. To collect information on thallus/tree landings by insects, spot checks were carried out, as described above, for a 3 hour period during the week days between June-September, rotating from lichen to lichen every 10 minutes, 1.5 hours in the morning and 1.5 hours in the afternoon. Weather conditions were recorded in a log book, and spot checks were carried out in all weather conditions.

Insects landing on *Erioderma* thalli were collected in sterile insect killing jars; a new jar was used for each collection to avoid contamination at random. Insects were then placed in sterile 2ml vials to determine if they had picked up spores and transported to the laboratory for SEM preparation the same day. Field insects were prepared using the same methods employed for *Drosophila* in the laboratory experiments.

2.3 Results

Spore Morphology

Cross sections of mature apothecia of *E. pedicellatum* spores revealed that spores are ellipsoid in shape, ranging in length from 4-6 um, width 2-3um. The spores have a unique surface morphology characterized by regular micro-ornamentation <1um in length. These micro-ornaments are not a result of a developmental stage of the spore as the same surface morphology was identified for fully mature spores upon release from the ascus (Fig. 2.3).

Spore Discharge

Laboratory

E. pedicellatum spores were visible on double-sided tape in the petri dish experiments, when viewed under a standard light microscope (40x). Spores were present individually and in groups of 8 having discharged the entire ascus, no spores

were conglomerated or grouped. Out of the total number of discharged spores, individual spores were more common than entire groups of eight.

Field

Spore traps collected from May-October 2000 did not yield the presence of any *E. pedicellatum* spores. The absence of spores during the late spring, peak summer and early fall lead us to hypothesize that *E. pedicellatum* spores may be carried by some other agent other than wind, possibly discharged and remaining on the surface of the apothecia until a vector displaces the spore.

Spore Dispersal

Laboratory

Experiments utilizing *Drosophila melanogaster* as an insect vector indicate that *E. pedicellatum* spores do adhere to the bodies of fruit flies. SEM micrographs (Fig. 2.4) showed the attachment of a single *E. pedicellatum* spore to the hair of an adult fruit fly. Out of eight flies analyzed, only one carried a spore. From the SEM micrographs there was no evidence of grouping or conglomeration of spores. This is consistent with our previous laboratory findings of passive single spore discharge where we detected single spores and asci (8 spores).

Field

Over 2200 minutes (> 36 hours) of observations were conducted along the transitional gradient in Lockyers Waters. From these observations, a total of 467 landings were recorded on host trees (Fig. 2.5) in 24 families of insects. On average only 3% of insects landing on target trees actually landed on or directly walked over the target thalli. Typically insects landed on the thalli by accident or casually by already being present on the tree. There appeared to be no attraction to the lichen thalli by the insects and landings were more likely so the result of a random event. The expected probability of an insect landing on an *Erioderma pedicellatum* thallus is 4%; the surface area of the thallus divided by the surface area of the tree trunk, calcuated 1m above ground to 1m below the crown. Using Chi Square analysis the difference between the expected value and the actual number or landings is $\chi^2 = 1.50$, with a $\chi^2 = 5.99$ (df = 2; $\alpha = 0.05$); there is no significant difference between the expected and the observed values. Comparing the three forest zones in our site, there was no significant difference between FE (147 landings), ICZ (173 landings) and CFI (147 landings). Analysis of variance (one way ANOVA) shows no significant difference between tree landings along the transitional gradient ($p=0.07$, $\alpha = 0.05$) There was no significant difference between forest zones along the transitional gradient when comparing the frequency of thalli landings ($p=0.16$, $\alpha = 0.05$) (FE=3/91, ICZ=5/171, CZI=2/76).

In total there were 14 families of insects and 10 species of spiders recorded landing on target host trees or on thalli of *E. pedicellatum*, including 2 families of

aquatic species (*Tipulidae* and *Culicidae*). *Muscidae* was the most common insect family visiting host trees. There was no differentiation made at the species level since many tree landings were very short in duration and insects were difficult to trap. Without more detailed analysis in the laboratory it would be difficult to identify specimens to the species level. Of the 24 families/species of insects collected, five were investigated as possible carrier of spores since they are relatively common inhabitants of Newfoundland forests (*Muscidae*, *Tipulidae*, *Culicidae*, *Crydsops species*, and *Anapsis rubis*). Of nine specimens prepared for SEM two showed the presence of *Erioderma* spores, on the antennae of a small flying beetle *Anapsis rubis* (Fig. 2.6), and on the body of the orbatid mite (Family *Oribatida*), as characterized in other studies (Stubbs, 1994). Assuming there is a consistent number of insect landings each day for the peak period of the summer months (or an average during the peak period), there could conceivably be one landing every two minutes, over the peak summer period that could equal almost 2000 landings on host trees, equating to nearly 80 thalli landings. Over a 10-15 year period as the *E. pedicellatum* population is maturing this could equate to 20-30,000 tree landings and 4000-5000 thalli landings.

2.4 Discussion

Results from this study provide information on the dispersal mechanisms of *Erioderma pedicellatum* in its Eastern Boreal habitat in Newfoundland. Field and laboratory experiments confirm that spores of *E. pedicellatum* are either discharged at a different time in the year (winter or very early spring?) or at a shorter distance than 1 cm, since no spores were collected in the field traps during the period May-November. Through SEM micrographs *E. pedicellatum* spore have visible, sculptured surfaces with detailed micro-ornamentation, but no hooks or barbs that would facilitate attachment to putative insect dispersal agents. It is also possible that the newly designed traps did not work in the field, even though spores adhered to double sided tape in the laboratory. In the laboratory, we did not observe discharged spores in conglomerations, even though we did record instances of full asci (8 spores) discharged. Laboratory experiments confirm that insects can carry *E. pedicellatum* spores and that the spores can be trapped in the leg hairs of *Drosophila melanogaster*. In addition field results suggest that insects can act as vectors to disperse *Erioderma* spores in its natural habitat including vertical distribution (via Orbatid mite) and tree-to-tree distribution (via the small flying beetle, *Anapsis rubis*), or possibly forest stand-to-forest stand distribution, even though this needs to be proven. Given the gap dynamics of the Avalon Peninsula forests in Newfoundland and the short life cycle of fir forests, our study suggests that insects can carry spores. Further research needs to determine if the insects are important dispersal vectors for *Erioderma* spores to reach new host trees.

This study provides baseline information on the discharge and morphology of the spores. Other studies indicate that the spores of *Erioderma* are ejected more commonly as a group of eight, rather than individually (Maass, 1980; Maass and Yetman, 2002). Under laboratory conditions spores are discharged more commonly as individuals rather than in groups. When viewed under a scanning electron microscope the eight spores per ascus reveal distinct surface sculpturing and micro-ornamentation. Lichens, share a wide variety of spore sizes and shapes (Bailey and Garrett, 1968); however few report characters like those reported here as most spores have smooth surfaces. In fact, Pentecost (1981) reports that out of a survey of 605 lichen species most had spores with smooth and uniform walls, and micro-ornamentation on the surface of spores was extremely rare. Some species have specific micro-ornamentation characteristics such as *Buellia* spp. which is among the best identification characteristics at the species level for spore ornamentation (Scheidegger, 1987). Some lichens such as Rhizocarpaceae and gelatinous halos!

This study did not provide evidence for active discharge of spores as has been reported for other lichen epiphytes (Clayden, 1997; Christmas, 1980; Pyatt, 1974). On the contrary, no spores were collected in spore traps in a five month period between May to October. Typically spores discharge at an average distance of 3-13 mm from the apothecium (Pyatt, 1974). Here no spores were collected up to 10 mm from apothecia. It may be that *E. pedicellatum* discharges the bulk of its spores between November and April, however other studies have shown that lichen epiphytes discharge spores throughout the year with the bulk of spores released in early spring

(Clayden, 1997). Dension, 2003 has shown that *Lobaria oregana* and *Lobaria pulmonaria* also disperse ascospores throughout the entire year. We hypothesize that *Erioderma* spores may be actively discharged, but reaching an average distance less than 10mm. The difference between the laboratory and the field results may be due to deficiency of the field spore traps, the time of year, the environmental conditions, or the low number of field traps. Passive discharge of spores is not uncommon and such passive discharge has been demonstrated with other lichens including those of the family Caliciaceae (Tibell, 1994).

Balsam fir forest stands on the Avalon Peninsula have a shorter life cycle than fir stands elsewhere in Newfoundland (Forest Ecosystem Management Strategy, 2003). Generally Avalon stands cycle every 70-80 years reaching a decaying stage late in the succession where the trees are vulnerable to wind throw as a result of butt rot (Keeping, 2006) and insect infestation (spruce bud worm; *Choristoneura fumiferana* and hemlock looper ; *Nepytia phantasmaria*). Often entire stands are decimated by strong prevailing winds leaving large gaps on the landscape. Scheidegger (1996) reports that *E. pedicellatum* thalli reach sexual maturity in the 20-30 years of the overmature phase of the stand when the light regime is favourable for colonization and growth of the lichen, due to the breakup of the canopy. A microclimatic balance must exist between favourable light regimes and a suitable, consistent level of moisture to prevent desiccation of the lichen thalli (Scheidegger, 1995; Hazell and Gustafsson, 1999; Maass and Yetman, 2002). The prevalence of gaps of varying sizes in the fir forest stand threatens the survival of the lichen, as the

microclimate changes quickly in areas once suitable for colonization. As well the increase of gaps caused by anthropogenic and natural disturbances in a forest stand increases the likelihood that a lichen will be limited by dispersal (Ockinger et al., 2005), reducing genetic variation if there is lack of gene flow/exchange between subpopulations and overtime become locally extinct (Hanski, 1999).

The gap dynamics of fir forests on the Avalon Peninsula create conditions for potential dispersal limitation for the lichen. As the gap sizes increase in weakened areas of the stand, *E. pedicellatum* thalli, limited to sexual reproduction only, face the challenge of ejected spores reaching the opposite edge of the bordering gap. Within a ten to fifteen year period of the decaying phase of the forest stand the thalli must reproduce, discharge and disperse spores to new trees in favourable sites. As the gap size increases with further degradation of the stand, dispersal mechanisms become vital to the survivorship of the species. Dispersal agents are therefore intimately linked to the life cycle of the lichen. If the spore never reaches a suitable tree it will never have the opportunity to germinate, find a compatible *Scytonema* partner and lichenize in a closed environment such as in the watersacs of the hepatic liverwort *Frullania asagrayana* (Maass, 1980; Scheidegger, 1996; Maass and Yetman, 2002). We hypothesize with the limitations of wind and water dispersal, insects provide one possible mechanism of dispersal, and a possible link between the decline of one local population and the emergence of another in a suitable adjacent stand by carrying healthy spores across significant gaps to adjacent stands. Walser et al., (2001) illustrate the limitations of wind dispersal in *Lobaria pulmonaria*, where diaspores

emerged in highest concentration only 1m from the nearest source and at extremely low concentrations 50m from the nearest source (1.2 diaspores per m^2), in the direction of the prevailing wind, and at a maximum of 350m from a source tree (Walser, 2004). Given the fact that over a 10-15 year period (assuming constant conditions with climate, insect and lichen population stability, etc.) insects in the Avalon boreal forests could land on the *E. pedicellatum* thalli 4000-5000 times, insects have great potential to disperse spores within and between forest stands.

This study provides preliminary evidence that lichen spores can be carried by small flying insects in the Boreal forest of Eastern Canada. SEM micrographs clearly illustrate the sexual ascospores trapped in the appendage hairs of insects both in the field and laboratory. Insects common to the Eastern Boreal Forest can be present in multitudes depending on local climate conditions and canopy cover. On days of low cloud cover and moderate temperatures (15-20 ^{0}C) tree landings of 2.1 insects per second were recorded on a branch in the intermediate canopy zone (ICZ). This frequent event would likely result in insects picking up spores in a chance encounter, possibly depositing them to a potential host tree in the same stand or adjacent forest stand in the same location. The results show that the frequency of landings is not significantly different along the forest edge, intermediate-canopy zone or complete forest interior at alpha 0.5. More landings were recorded in the centre of the forest zone to the intermediate canopy zone compared to the forest edge. Further research is needed to identify the relationship between gap edges, distance spores are carried, whether spores are deposited to other trees, frequency of insect landings and the

frequency of spore-vector dispersal. For other rare lichens with spores/diaspores, commonly dispersed by wind, maximum distances of 100-150m are common (Walser et al., 2001). Insect dispersal, depending on the range of dispersal of the insect species, could conceivably cover hundreds of meters to kilometres in distance.

We suggest that forest managers consider the important role of insects in the life cycle of *Erioderma pedicellatum* in conservation and management planning. Other researchers have shown the importance of dispersal capacity of rare species in the colonization of new stands, and hence the long-term conservation of the species (Niemela, 1997; Hanski, 1999; Ockinger, 2005). Managers must understand the importance of multi-species interaction in *E. pedicellatum* habitat and how the lichen has evolved a complex life history, adapting to large gap sizes and a mosaic of multi-aged stands across the landscape, similar to other cyanolichens with complex life histories (Kalwij et al., 2005). We conclude that the dispersal dynamics of *E. pedicellatum* spores may have evolved in response to the disturbance regimes of forests. This co-evolution idea needs to be investigated more; specifically investigating the evolution of spore dispersal cued to the gap dynamics of the forest. Further research also needs to be conducted on the life cycle of the lichen and whether there is an evolved life cycle with rapid stand replacement of the fir forests on the Avalon Peninsula and changing gap dynamics, primarily driven by wind disturbance. It is possible that managers should mimic this stand replacement in their cutting regimes to enhance dispersal capacity and multi-species interactions as shown in other lichen studies (Wei-Dong Gu, 2001). In British Columbia forest managers

have begun to use partial cutting systems in areas with abundant arboreal lichens, using a mean harvesting size of 0.5 ha (Coxson et al., 2003) to mimic the natural gap dynamic of the forest cycle, leaving higher lichen abundance on remnant trees and preserving local habitat conditions (Stevenson and Coxson, 2004). Robertson (1998) concludes that harvesting blocks on the Avalon Peninsula should be restricted to 5 ha based on the average gap size in forest stands, some of which contain *E. pedicellatum*. Based on the average gap sizes recorded in this study and (7.1 m) and the conclusions on dispersal we conclude that this recommendation should be revisited, including further research on dispersal distances of *E. pedicellatum* spores. Empirical evidence on the maximum distance of spores should be an input when determining the size of harvesting blocks. Managers should also consider longer harvest rotation in sites (Kuusinen and Siitonen, 1998) with *E. pedicellatum* to preserve lichen abundance and ensure a continuum of old-growth forest-types on the landscape, thereby increasing biodiversity (Humphrey, 2005). Hilmo et al., (2005) investigated the impact of logging on the colonization of new lichen epiphytes and found that the successful colonization of new thalli following larger scale logging (2.25 hectare clear cuts) was species specific. In other words the colonization response to logging by a lichen depended on the life history of that lichen, including its capacity to disperse spores. It is recommended that managers consider a wide range of specific scientific information, such as dispersal capacity and vector transfer, when considering the size and location of cutting blocks for *E. pedicellatum* habitat.

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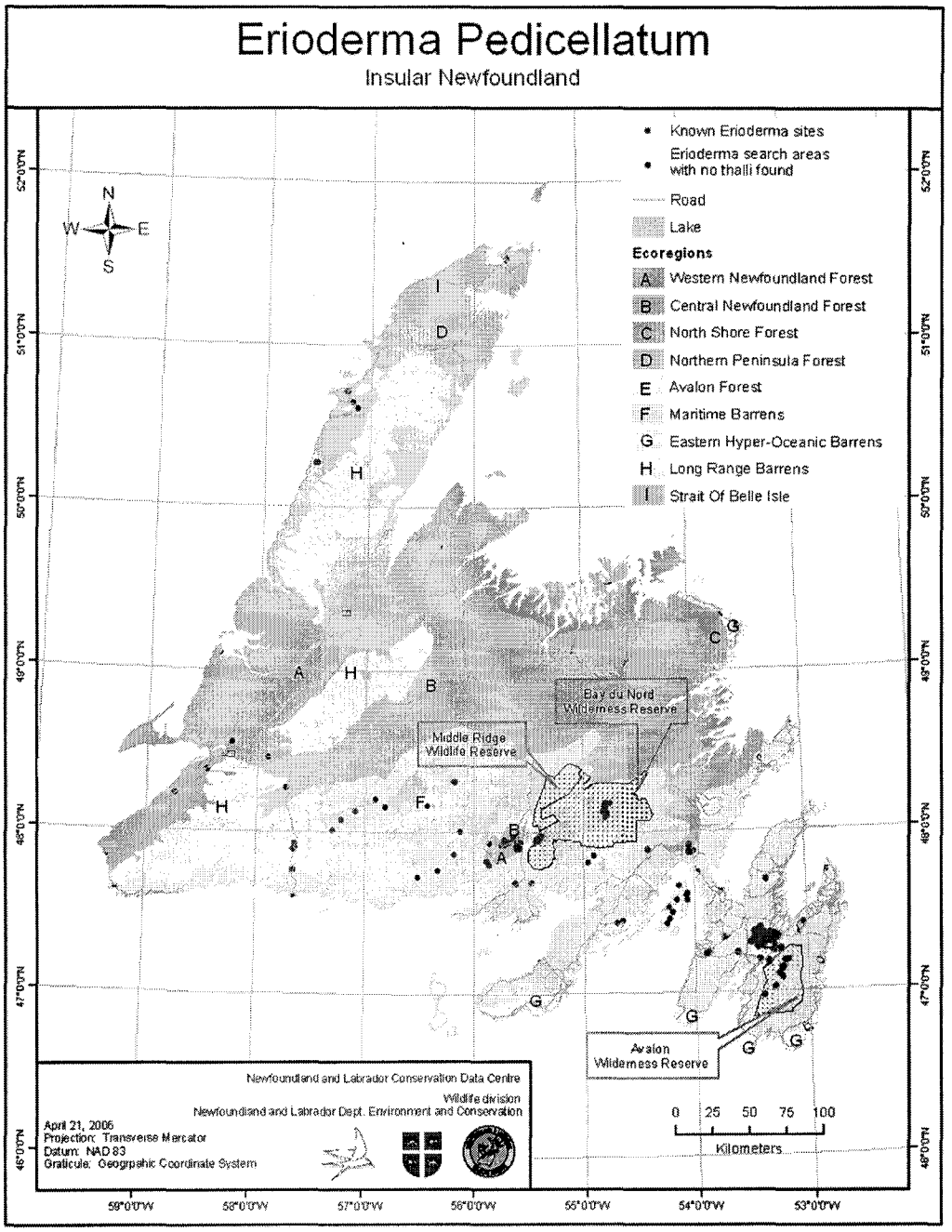

Figure 2.1 Known range of *Erioderma pedicellatum* sites in Newfoundland and Labrador. Of note are sites E (Lockyer's Waters) and B (Bay d'Espoir). Map used with permission from the Department of Environment and Conservation, Government of Newfoundland and Labrador.

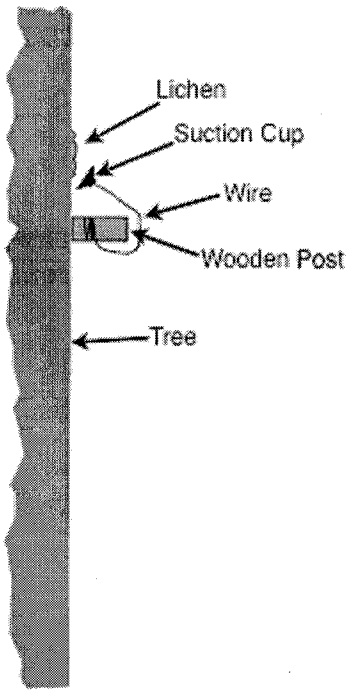

Figure 2.2: Schematic diagram of spore traps used to collect *Erioderma pedicellatum* spores *in situ.* A suction cup, lined with double sided tape, was used in the field to minimize surface area covering the lichen and risking potential alterations in microclimate.

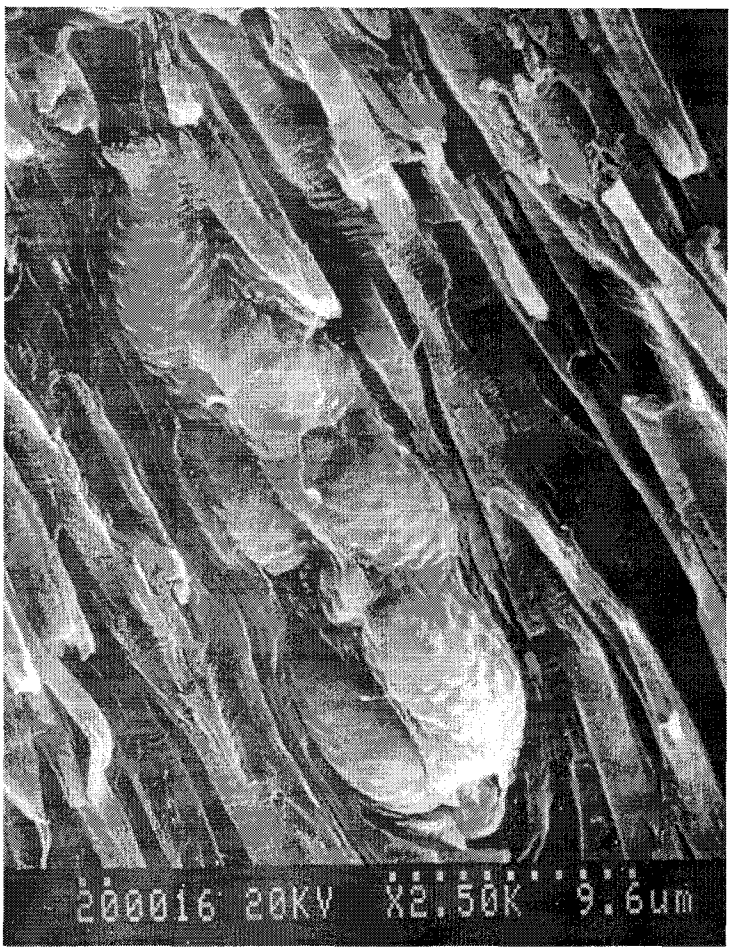

Figure 2.3: Cross section of an *Erioderma pedicellatum* apothecium showing one ascus, with six spores visible (but holding eight, evident from light microscopy). Ascus viewed at 2.50 K magnification.

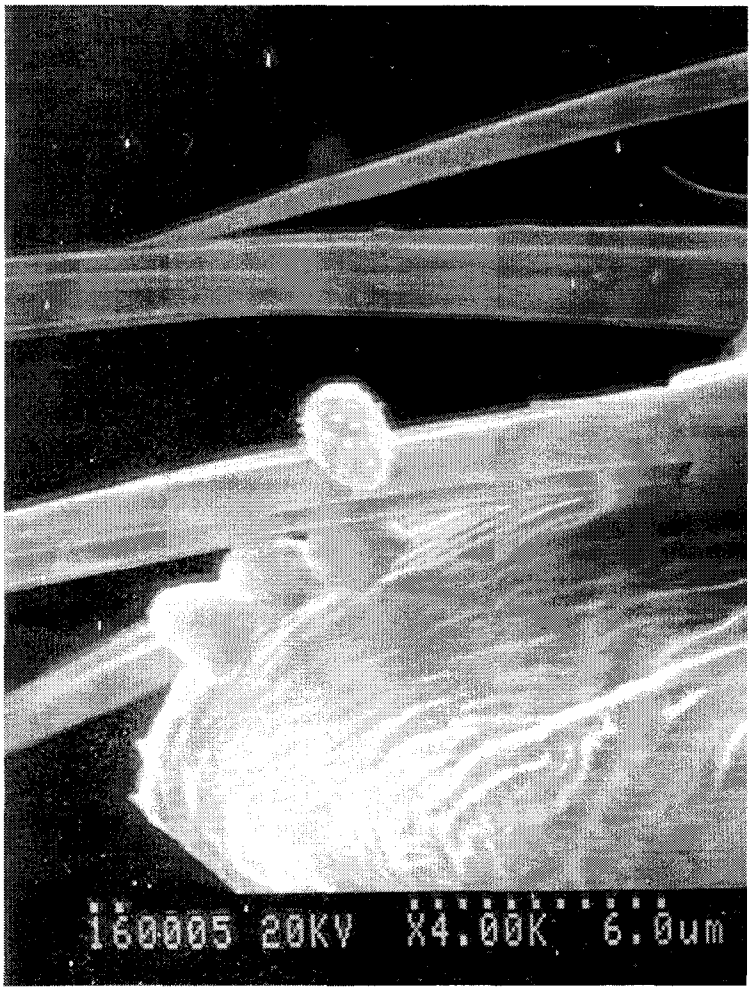

Figure 2.4: Spore of *Erioderma pedicellatum* adhered to leg bristle of *Drosophila melanogaster*, viewed under low-medium power (4.00 K) with a scanning electron microscope (Hitachi 5570).

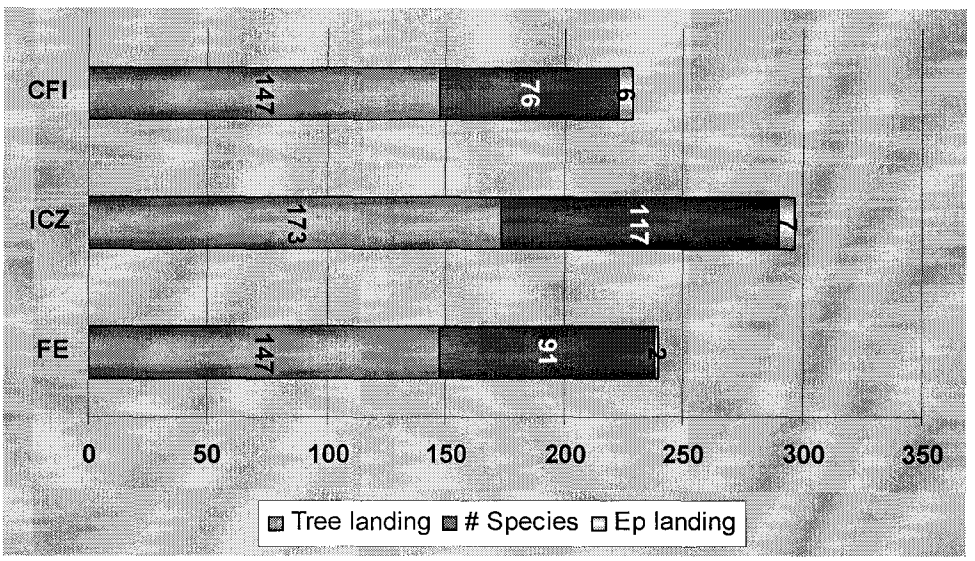

Figure 2.5: Frequency of insect landings on *Erioderma pedicellatum* thalli in Lockyers Waters in three forest zones, Complete Forest Interior (CFI), Intermediate Canopy Zone (ICZ), and Forest Edge (FE), showing number of insects landing on host trees (tree landing), number of insect species (# species) and number of insects landing on the thallus (Ep landing). ANOVA values for Ep landings ($p=0.07$) support no significant difference between the three forest zones. Landings were recorded over the entire time (10 minute intervals) at each forest zone.

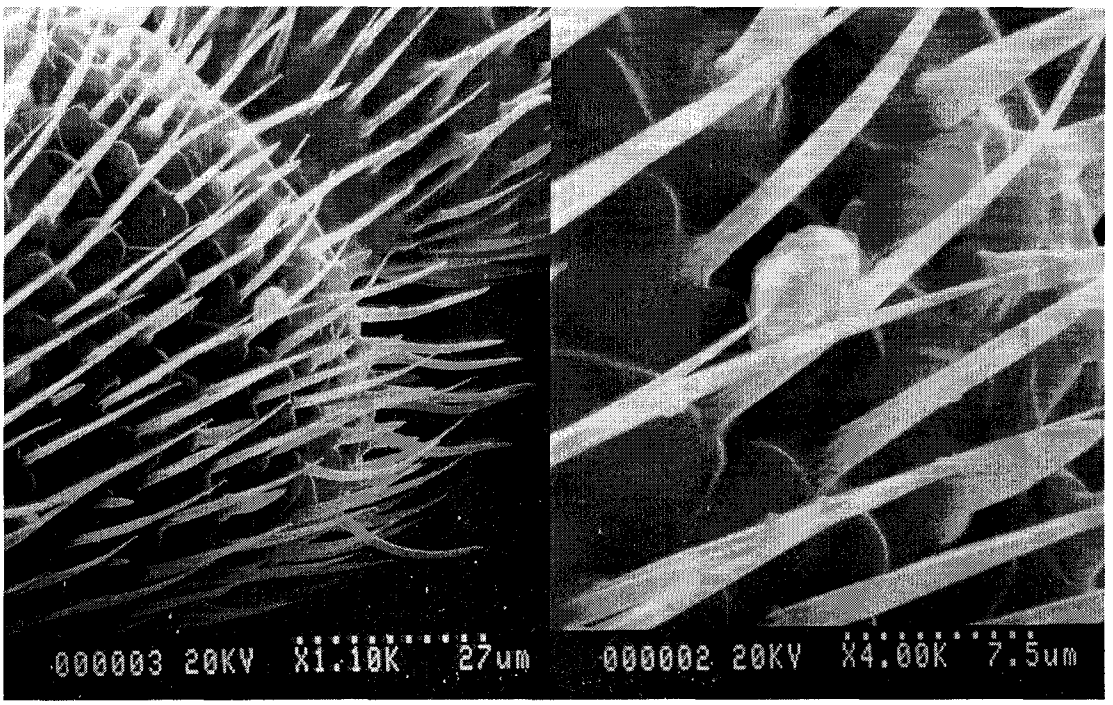

Figure 2.6: *Erioderma pedicellatum* spore on the antennae of *Anapsis rubis*, a small flying beetle, collected immediately following a landing on *E. pedicellatum* in Lockyers Waters. The second micrograph (right) is presented at a higher magnification (4.00 K = 4000 x).

Conclusion

In this thesis we investigated the genetic variation and the dispersal ecology of *Erioderma pedicellatum*. In Chapter 1 results indicated low genetic variation within subpopulations of *E. pedicellatum* in Newfoundland and Labrador. Two haplotypes were discovered in the Newfoundland populations but neither haplotype was specific to a geographic location. In addition Chapter 1 presents evidence of a lack of genetic differentiation between subpopulations in Newfoundland and a herbarium specimen in Sweden. Genetic evidence supports the designation of the global population as one evolutionary unit. This lack of variation between amphi-Atlantic populations may have important consequences in the future conservation of this species; however, because of the small size of the gene sequenced (number of base pairs) we must be careful in assuming low genetic variation between the continents. The methodology section outlines some of the problems encountered in sequencing the ITS region of the ribosomal DNA of *Erioderma* and has two important methodological findings. First, and perhaps most beneficial is the identification of species-specific primers for *E. pedicellatum*. Future genetic research should use these primers to build on the findings of genetic variability. Second a common methodology is presented for the extraction of nuclear DNA from older herbarium specimens (63 years old in this case). This procedure produced a clear PCR product and allowed for sequencing of the ITS region. Future genetic research should expand on these methods in comparing North American and historic European populations. Population comparisons of

historic and current populations in Nova Scotia and historic populations in New Brunswick should also be compared to Newfoundland and Europe.

The finding that the ITS 1 region of the single European sample was identical to haplotype A of the North American samples may have important management implications for conservation of the species. First, further genetic research should be investigated expanding on the current knowledge, investigating variation in other genes/loci including RBP II, ITS II, LSU, SSU and possibly microsatellites given the latest discovery in *Lobaria pulmonaria* (Walser et al., 2005). Second our findings support the IUCN designation of the global population as one evolutionary unit, classifying the global population as "Critically Endangered", even though we still must consider, only one European sample was sequenced. Before forest managers consider transplants of the lichen in Newfoundland and Labrador, within the Atlantic Canada region, or between North America and Europe more genetic research should be conducted.

In Chapter 2 we investigated several aspects of the dispersal biology of *Erioderma pedicellatum*. First we described the morphological description of the minute ascospores in the lichen. Contrary to the findings of other studies (Maass and Yetman, 2002), it was found that each ascus contains eight ascospores and these ascospores have distinct surface microornamentation. This microornamentation is not a result of a developmental stage of the lichen since we observed the exact same ornamentation on discharged spores. Second we demonstrated through laboratory experiments that the ascospores of *Erioderma* can be trapped in the legs of the

common fruit fly, *Drosophila melanogaster*. Spore traps placed in the field in Lockyer's Waters support that spores are possibly discharged from the asci at a distance of less than 10mm, since for a period six months we did not collect a single spore in the field traps, however there may be several other reasons for this negative result including lack of adhesiveness to the spore trap, environmental conditions, or the time of year. It is possible for example that the spores actively discharge during the winter at a distance of greater than 10mm, more work needs to be done here during seasons that were not monitored or using alternative spore traps. A series of field observations along a transitional gradient in the forest (forest edge to interior) illustrate the frequency and abundance of insect species that associate with the lichen. Results indicate that with the frequency of insect landings on the surface of the lichen thalli, insects can randomly disperse *E. pedicellatum* spores. Trapped insects in the field, viewed under scanning electron microscopy, confirm the presence of spores on the surface of some insects. Our results show a spore trapped in the antennae of *Anapsis rubis*, a small flying beetle and on the body of a common Boreal Orbatid mite. The flying beetle can possibly assist in long-distance dispersal and the Orbatid mite can possibly assist in vertical dispersal of spores on the same trees, however a determination of actual spore dispersal, and deposit on another tree needs to be investigated. For now we can conclude that spores can be carried by certain insects, although we are uncertain of distance, or the length of time a spore can stay on an insect body.

We conclude based on these field results and based on the morphological characteristics of the ascospore, that *E. pedicellatum* through random events, or through co-evolution, a principle that needs to be investigated further; these insects, depending on their own dispersal patterns could conceivably disperse these minute spores longer distances to adjacent forest stands. Given the documented evidence of dispersal limitations in rare cyanolichens or lichens dependent on old-growth, stable forest habitats (Peterson and McCune, 2001) our findings add important information for forest managers. The fact that *E. pedicellatum* only reproduces sexually and is likely dispersal limited, the identification of primary vectors is an important link in understanding the dispersal biology of the species. Insects may be a critical agent in ensuring viable spores reach suitable adjacent forest habitat.

Forest managers, in implementing conservation measures for *Erioderma*, should approach conservation from a habitat-scale rather than on a population or remnant tree scale. By conserving a mosaic of adjacent forest stands, or multi-aged sites, managers can preserve multi-species interactions of *E. pedicellatum*, for example interactions between insects and the lichen.

We have provided some initial findings on the life history of *Erioderma pedicellatum* including dispersal biology and genetic variation. Further research should build on the information presented in this thesis, however, in light of the anthropogenic and natural pressures on *E. pedicellatum* habitat (Keeping, 2006), these findings provide some initial conservation strategies for populations in Newfoundland and Labrador. New evidence is emerging on the effect of disturbances

on genetic variation in rare epiphytic lichens (Werth et al., 2006) in Europe. For the conservation of *E. pedicellatum* in Newfoundland, which hosts 99% of the world's entire population, it is critical to further investigate the dispersal biology of *Erioderma*, the gap dynamics of the balsam fir forests in Newfoundland, genetic variation and the evolutionary relationship between spore dispersal and the forest life cycle.

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