

***PSEUDEVERNIA FURFURACEA* –
PATTERNS OF DIVERSITY
IN A SHRUBBY LICHEN**

MASTER THESIS IN BIOLOGY

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Summary

Pseudevernia furfuracea is a shrubby lichen commonly found around the Baltic Sea. The lichen appears to reproduce mainly by producing isidia, propagules of the lichen cortex. It is very morphologically and chemically diverse and can be found on different substrates, such as nutrient poor bark trees and rocks. The main objective of this study was to describe genetic variation in the ITS region and link this to variations in morphology, substrate ecology and secondary chemistry. In total, 36 specimens of *P. furfuracea* were collected from 21 sites in Sweden, Finland and Estonia. Seven haplotypes were distinguished in the ITS region. A statistically significant connection between haplotype and colouration of the lichen was found. Lighter coloured specimens of *P. furfuracea* are more likely to be of the ancestral haplotype. No other significant correlations between the different characteristics studied were found, suggesting that *P. furfuracea* should be regarded as a single species within the Baltic Sea area. This is also supported by the haplotype network.

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Introduction

Lichens are manifestations of symbiotic relationships between fungi (mycobiont) and algae or cyanobacteria (photobiont). The largest group of lichenized fungi belongs to the ascomycetes, a group of fungi that produces spores in a sac-like structure, known as ascus (Purvis, 2000). The photobionts most commonly found in lichens are green algae of the genus *Trebouxia* (Moberg & Holmåsén 1990; Purvis 2000).

Lichens are also known to produce a range of secondary substances, which are deposited in the medullary or cortical layer of the lichen (Culberson 1969). The exact functions of the chemicals are not yet fully understood, but different suggestions have been made: antiherbivoric function; antibiotic activity; protection against ultraviolet radiation or the substances may help the lichen in detoxifying toxic metals (Purvis 2000).

Lichen taxonomy

Taxonomical and population studies in lichenology have long utilized secondary chemistry as a complement to morphological characteristics. Other characteristics like biosequential schemes (i.e. molecular pathways of how precursors to secondary substances are formed) (Huovinen & Ahti 1982) and isozyme patterns (Mattsson 1994) were suggested as being of great value for lichen taxonomy. Today the use of genetic methods is by far the most widespread working tool. A striking example of this is the assembling of the fungal tree of life, based on genetic data from more than 10,000 fungal sequences (Lutzoni *et al.* 2004). This fungal tree reveals the evolution of both lichenized and non-lichenized fungi.

By sequencing the DNA of the fungal counterpart, inter- and intraspecific genetic variation of the lichen can be detected and rRNA genes are often used for this. As ribosomal RNA genes can be found in all organisms, variation in the genes is expected to be fairly low. In fungi, rRNA genes exist as multi-copy gene families, with one or more intergenetic spacer regions (IGS) creating repeated units (biology.duke.edu 1) (Fig. 1). In these repeated units, the internal transcribed spacer (ITS) region is perhaps the most commonly sequenced DNA region in fungi. This is because this region may contain a higher degree of variation than other RNA regions (biology.duke.edu 1).

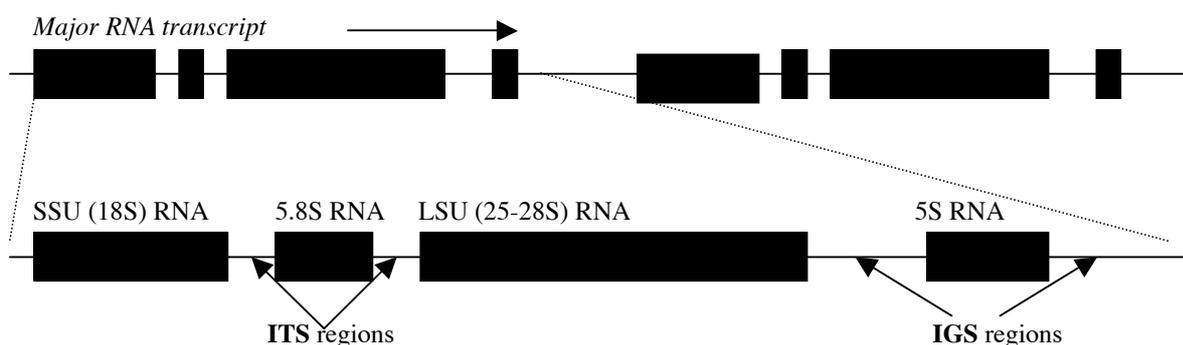


Figure 1. RNA genes with IGS and ITS regions.

Lichen reproduction and dispersal

Sexual reproduction in lichens involves production of fungal spores, which via dispersal can come in contact with suitable algal cells and form new lichen individuals. Sexually

reproducing lichens form the fungal spores in asci, which are located in fruiting bodies called *apothecia*. Sexual reproduction involves the recombination of genes, which increases the intraspecific genetic variation.

Asexual or vegetative reproduction includes production of soredia, isidia and thallus fragmentation. These vegetative diaspores contain both fungal and algal cells and may disperse via vectors or abiotic factors and form new lichen thalli where conditions are suitable. Lichens mainly relying on vegetative reproduction have earlier been presumed to have low genetic diversity due to the low number of recombination events. These organisms have sometimes been regarded as evolutionary dead ends, though several recent studies have showed that they are as genetically variable as sexually reproduced lichens (Mattsson 1994; Printzen & Ekman 2002; Printzen *et al.* 2003; Lindblom & Ekman 2006, 2007; Mattsson *et al. in prep*).

Predominantly asexual lichens are common throughout the Baltic Sea area. They are easily found in a wide range of habitats and on various substrates. This study focuses on one of these lichens, *Pseudevernia furfuracea*.

Pseudevernia furfuracea

Pseudevernia furfuracea is a shrubby lichen, common in Sweden and is also found throughout Europe (Rikkinen 1997). Its main substrates are nutrient poor bark trees such as birch, pine and spruce, but can also be found on old wood and siliceous rocks (Moberg & Holmåsen 1990; Hallingbäck 1995). Birch, pine and spruce are all classified as nutrient poor bark trees, which refers mainly to a low pH on the tree trunk. Lichen species commonly inhabiting poor bark tree species are for example *Hypogymnia physodes* and *Parmelia sulcata*, and the lichen flora on these trees are often referred to as poor bark societies (Du Rietz 1945).

The main reproductive strategy of *P. furfuracea* appears to be asexual, via the dispersal of isidia (Moberg & Holmåsen 1990; Rikkinen 1997). Apothecia producing specimens, i.e. reproducing sexually, are very rare.

The species displays a wide morphological plasticity, from very tiny light coloured specimens to larger dark individuals with broad branches. It is in addition chemically diverse, producing different secondary metabolites: olivetoric acid and physodic and oxy-physodic acids (Culberson 1969). In the few existing scientific studies of this lichen species in Scandinavia, morphotypes have been suggested as being a response to the physical environment (Rikkinen 1997). In a Norwegian study Halvorsen & Bendiksen (1982) further suggested that chemical variations should be treated as chemical strains within the same species, which also is supported by findings in a Swedish study from 1977 (Culberson *et al.*). The Norwegian study could not find any significant correlation between the distribution of chemical strains and substrate ecology. Altogether, previous studies suggest that *P. furfuracea* should be regarded as one single species. So far no studies on the intraspecific genetic variation of *Pseudevernia furfuracea* have been published to confirm or reject these suggestions.

Main objective

It is possible to use genetic evidence to support or reject indications made in earlier studies that *P. furfuracea* should be regarded as one species? The main objective of this thesis was to examine genetic variability in different populations of *Pseudevernia furfuracea*, and investigate whether these variations are correlated to variations in secondary chemistry, morphology and substrate ecology.

Methods

Sampling

Pseudevernia furfuracea was collected within the Baltic Sea project “Ecological and Societal Systems in Interaction”, from April to June 2005. Lichen individuals were randomly collected from tree trunks within a circle with a 10-meter radius from the plant *Daphne mezereum*. Up to four lichen specimens were collected on each site. One specimen of *P. furfuracea* was collected from each tree and one or two specimens were collected from each tree species.

Lichens were also collected within the biology courses “Bevarandekologi” and “Ekologisk Fältmetodik” during spring 2005. These lichen specimens were randomly collected on tree trunks on randomly selected sites in the Mid-South part of Sweden. Substrate of each specimen was recorded. Altogether, thirty-six specimens were collected from twenty-one sites: eleven in Sweden, three in Finland and seven in Estonia (Table 1).

When performing statistical analyses, the substrates were divided into two groups: (i) poor bark trees (birch, spruce, pine and juniper) and (ii) medium to rich bark trees (remaining tree species).

Table 1. Number of *P. furfuracea* thalli (n=36) collected in the Baltic Sea project “Ecological and Societal Systems in Interaction” and during biology courses “Bevarandekologi” and “Ekologisk Fältmetodik”.

Country	No of specimens	No of sites	Year and month of collection
Sweden	17	11	2005, April-July
Finland	6	3	2005, May
Estonia	13	7	2005, June-July

DNA extraction, PCR amplification and sequence alignment

Fresh lobe tips were selected and cleaned with a razor blade, removing visible contaminants such as parasitic fungi and mite faeces. DNA was extracted using Viogene’s Plant Genomic DNA/RNA Extraction Miniprep System (Viogene, USA) according to manufacturer’s instructions with slight modifications. DNA was eluted with distilled water to a final volume of 200 μ L. The complete internal transcribed spacer (ITS) region, ITS1-5.8S-ITS2, was amplified using primers ITS1F’5 and ITS4R’3 (White *et al.* 1990; Gardes & Bruns 1993).

PCR was performed in a total volume of 25 μ L, containing 5 μ L extracted DNA, 1 μ L of each primer and 18 μ L distilled water. In each PCR tube, one bead containing nucleotides and polymerase (Puretaq Ready To Go PCR Beads, Amersham Biosciences) was also added. The set of cycling parameters used, is fully described in Wedin *et al.* (2004). PCR products were purified using Viogene’s PCR-M Clean Up System (Viogene, USA), according to manufacturer’s instructions. Purified DNA products were sequenced by Uppsala Genome Center. Sequences were edited with BioEdit (mbio.ncsu.edu 2) and aligned using software programs EditSeq (dnastar.com 3) and MegAlign (dnastar.com 4). Alignments were checked by eye and unique haplotypes were double-checked. If a nucleotide sequence was ambiguous in the chromatogram, the sample was either re-sequenced or a potentially polymorphic site was treated as a non-polymorphic site. Identification of sequences was performed using BLAST search in GenBank.

Morphology

Specimens of *P. furfuracea* were classified into different groups according to colour and size. Specimen colour was assessed by eye and the samples were then divided into two groups:

light or dark. Lichen specimens were measured across the thallus, as dry herbaria-material, to get a rough approximation of their size. Dry-weight was also recorded for each specimen and the lichens were accordingly divided into two groups: large or small.

Secondary substances

To detect secondary substances, high performance thin layer chromatography (HPTLC) was used. HPTLC has several advantages compared to standard TLC, it is for example a more sensitive method and the samples can be run in a shorter period of time (Arup *et al.* 1993). Samples were applied on silica gel plates, 10 x 10 cm, and chromatographic development was performed according to Arup *et al.* (1993), using solvent system A, B and C. References used were acetone extracts of *Pleurosticta acetabulum*, *Platismatia glauca*, *Hypogymnia tubulosa* and purified samples of atranorin- and olivetoric acid. The plates were studied under UV light at 366 nm, before and after heating, and the colour of each spot was recorded. This is important for the identification of each substance.

Haplotype network construction and statistical approach

Haplotype network were constructed from alignments using TCS version 1.21 (Clement *et al.* 2005). This software provides an unrooted network with 95 % plausible set for all linkages, utilizing the statistical parsimony procedure (Templeton *et al.* 1992). Gaps were treated as a fifth characteristics. Thirty-four of the 36 specimens were sequenced and included in the network. When performing statistical analyses, haplotypes were divided into two groups when tested as a response variable.

Generalized linear models with a binomial error distribution, using the computer software R version 2.0.1 (r-project.org 5), was analysed to find any significant correlations between characteristics. Each feature was divided into two groups when tested as a response variable, with other characteristics as explanatory variables.

Results

Genetic variation

The alignment length was 502 nucleotide sites for the ITS region. A total of seven different haplotypes (H1–H7) were found, with nine different polymorphic nucleotide sites (Table 2). The haplotype network is presented in Figure 2. The most frequent haplotype is presented in the middle of the network and other haplotypes are connected with branches. One branch represents one mutational step.

Table 2. Haplotype No. in first column, followed by polymorphic sites. No. of individuals for each haplotype and in which country specimens were collected. Total number of individuals = 34.

Haplotype	063	065	158	162	414	444	445	446	447	No. of individuals (% of total)	Country
H1	T	T	T	G	G	-	-	-	-	19 (56 %)	Sweden, Estonia, Finland
H2	C	.	.	.	C	-	-	-	-	1 (3 %)	Estonia
H3	.	C	.	.	.	-	-	-	-	6 (18 %)	Sweden, Estonia, Finland
H4	.	.	C	.	.	-	-	-	-	1 (3 %)	Sweden
H5	.	.	.	A	.	-	-	-	-	1 (3 %)	Sweden
H6	C	-	-	-	-	5 (15 %)	Sweden, Estonia, Finland
H7	T	A	T	C	1 (3 %)	Sweden

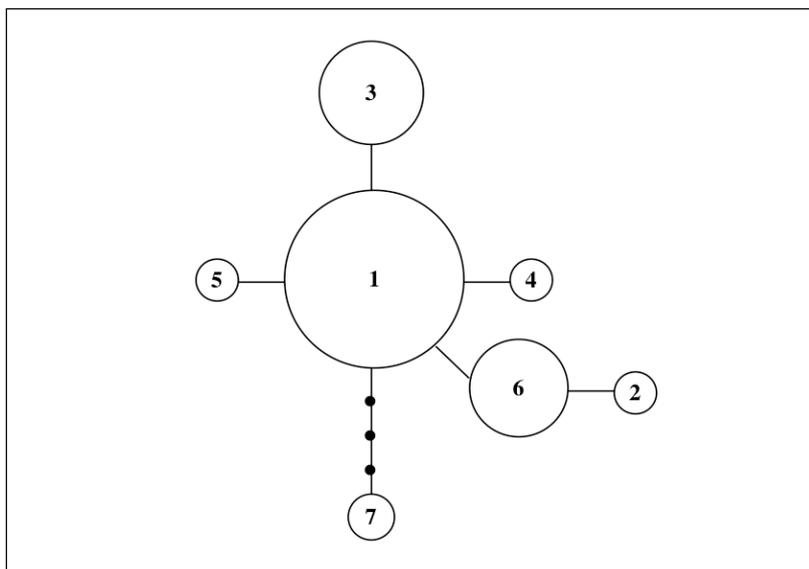


Figure 2. Haplotype network constructed in TCS 1.21, including 34 sequenced specimens. One line is equal to one mutational step. Size of circles is equivalent to number of specimens for each haplotype and figures in circles refer to haplotype number, H1-H7. Filled circles represent missing haplotypes.

Most abundant haplotype was haplotype 1, which was found in all three countries, in most populations and on most substrates. Three haplotypes (H4, H5 and H7) were found exclusively in Sweden and one haplotype (H2) was found exclusively in Estonia.

Morphology

Thallus size ranged from 18 mm to 97 mm, with a mean of 55 mm and dry-weight of the specimens ranged from 0.024 g to 1.338 g, with a mean of 0.311 g. The colour of the lichen specimens ranged from light grey-white to dark grey-green. The upper surface of all specimens was more or less isidiate. No classification could be made, using the abundance of isidia.

Secondary chemistry

Four different secondary substances were detected in *P. furfuracea* (Table 3).

Table 3. Description of chemical strains found after performing HPTLC. Chemicals used for each solvent system, according to Arup *et al.* (1993). Rf/Rf_{total} values refers to distance in mm reached by the substance compared to distance reached by the solvent front. Substances identified from table 1 in Arup *et al.* (1993).

Chemical strain	Solvent system	Rf/Rf _{total} (Class)	Colour in UV _{366nm} before heating	Colour in UV _{366nm} after heating	Substance
I	A	36/43 (8)	Light green with dark orange in the middle	Brownish yellow	Atranorin
		19/43 (4)	Violet	Pale pink	Physodic acid
		13/43 (3)	Violet	Pale violet	Oxyphysodic acid (?)
	B	28/43 (8)	Green	White brownish yellow	Atranorin
		23/43 (6)	Violet	White pale pink	Physodic acid
		17/23 (5)	Light violet	White	Oxyphysodic acid (?)
	C	30/43 (8)	Green	Brownish yellow	Atranorin
		10/43 (3)	Violet	Pale pink	Physodic acid
		4/43 (2)	Violet	White pale pink	Oxyphysodic acid (?)

III	A	36/43 (8)	Light green with dark orange in the middle	Brownish yellow	Atranorin
		17-22/43 (4-5)	Light blue-green	Pale pink with darker pale pink center	Olivetoric acid
	B	28/43 (8)	Green	White-brownish yellow	Atranorin
		24/43 (7)	Light blue-green	Pale pink with darker pale pink center	Olivetoric acid
	C	30/43 (8)	Green	Brownish yellow	Atranorin
		13/43 (4)	Light green-blue	Pale pink with darker pale pink center	Olivetoric acid

Twenty-nine of the specimens (81 %) were identified producing physodic and probably oxyphysodic acid (chemical strain I) and seven specimens (19 %) contained olivetoric acid (chemical strain III). Names of the chemical strains follows Halvorsen & Bendiksen (1982). Within both chemical strains, atranorin acid was also found. No specimen was identified producing both physodic- and olivetoric acids (chemical strain II).

Substrate ecology

Most specimens were collected from birch (*Betula spp.*) and spruce (*Picea abies*), 68 % all together (Figure 3). Other substrates on which *P. furfuracea* was collected from were scots pine (*Pinus sylvestris*), common juniper (*Juniperus communis*), common alder (*Alnus glutinosa*), oak (*Quercus robur*), willow (*Salix spp.*), aspen (*Populus tremula*) and rowan (*Sorbus aucuparia*).

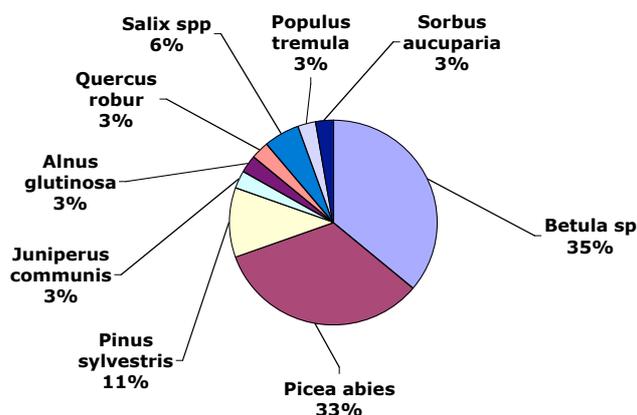


Figure 3. Figure presents proportion of different substrates (tree species) for total sampling size (n=36).

Combining characteristics

One significant correlation was found: between colour of the lichen and haplotype. Dark colouration had a significant positive effect on haplotypes, as did haplotypes H2-H7 have a significant positive effect on colouration of the lichen (Table 5).

Table 5. Response variables and significant explanatory variables used in statistical analysis. Response variables were divided into two groups within each character as showed in brackets.

Response variable	Significant explanatory variable (<i>p-value</i>)
Haplotype (H1 or H2-H7)	Colour, dark (0.0347)
Haplotype (H2+H6 or H1+H3-H5+H7)	No significance found
Weight (small or big)	No significance found
Length (small or big)	No significance found
Colour (light or dark)	Haplotype, H2-H7 (0.0347)
Substrate (poor bark or not poor bark)	No significance found
Chemical strain (I or III)	No significance found

Discussion

Haplotype distribution

Considerable variation in the ITS region of *P. furfuracea* was found in this study: seven different haplotypes were detected. Several other studies have revealed significant intraspecific variation in lichenized fungi, based on ITS sequences (Printzen & Ekman 2002; Lindblom & Ekman 2006). These studies show that ancestral haplotypes are shared between habitats (and populations), and that other and more rare haplotypes have not been removed by processes like e.g. genetic drift or have a more recent origin and thus, is not yet common. This could be the case with *P. furfuracea* since all three investigated countries shared several haplotypes. From the Finnish specimens, no unique haplotype could be found though, probably due to the low number of sampled specimens in Finland.

Morphology

The *P. furfuracea* specimens showed an amazing morphological plasticity, from very tiny light coloured specimens to larger individuals with broad lobes. Some specimens were very isidiate, whereas others had a smooth thallus when examining the lichen by eye. When studying the specimens under hand lens, all specimens in this study were producing isidia though. Therefore abundance of isidia was not included as a character.

A previous study of *P. furfuracea* in Finland (Rikkinen 1997) describes the colouration of lichen thalli as dark purplish black to pale grey, almost white. Pale grey/white specimens could be found also in this study, but the purplish black ones were not observed.

Correlation between haplotype and morphology

One significant correlation between the different characters could be found: between haplotype and colouration of the lichen specimen. The lichen thallus of haplotypes H2-H7 were more likely to be dark than light in colouration. This means that if one finds a light coloured specimen of *P. furfuracea* in the field, it is most likely the presumably ancestral haplotype 1.

Substrate ecology

The most common substrate for *P. furfuracea* in this study was birch followed by spruce and pine. These tree species are all classified as nutrient poor bark trees, and have a less diverse lichen flora (Moberg & Holmåsén 1990). What this could imply for conservation decision makers is that these kinds of habitats/substrates are often not regarded as “as valuable”, as other habitats and substrates with a more diverse lichen flora. Current study and unpublished results (Mattsson et al. *in prep*) suggest that these poor bark lichen societies can be regarded as highly genetically diverse. Therefore, studying common lichen species can have implications when deciding where conservation efforts should be made.

No specimens were collected from rock, another substrate of *P. furfuracea*. Collecting specimens from rock could affect the results, since rock is quite a different substrate from bark. Two recent studies of the lichen *Xanthoria parietina* in Scandinavia revealed significant differences between rock and bark populations, and provided evidence of restricted gene flow between populations in close proximity but on different substrates (Lindblom & Ekman 2006, 2007). One might suspect to find similar patterns for populations of *P. furfuracea* as well. This could mean that for lichens, the substrate on which they grow is often more important for

the individual lichen, than surrounding habitat (Hallingbäck 1995). Taken together, this supports earlier suggestions that it might be of importance to leave some large trees uncut when an area is being clear-cut (Vinter 2006).

Secondary substances

Of the thirty-six examined specimens of *P. furfuracea*, a major part, 81 %, were found to produce physodic acid, whereas 19 % contained olivetoric acid. These results are quite different from Halvorsen & Bendiksen's (1982) study from Norway. In that study, the distribution of chemical strains was almost equal between individuals producing physodic acid and individuals producing olivetoric acid. The Norwegian study also found a few specimens (1.2 %) producing both physodic and olivetoric acids, this study found none. A possible explanation to our different results, could be sampling bias. Sampling size in this study was relatively small compared to the 728 specimens in the Norwegian study.

Sampling bias

The sample size has most likely affected the results given in this study. A relatively small sample size can reveal patterns of variation or connections between characteristics within a species, or give ideas for further studies, even though it may be less statistically sound than a larger sample size. The sampling method in this study involved collecting a few samples in many locations, thus giving patterns of diversity from a wide geographical area. It would also be interesting to investigate chemical and genetic variation in populations of *P. furfuracea* on a smaller scale, collecting more specimens on fewer locations. Is it possible to find diversity in specimens collected from the same tree or even the same branch? It would also be interesting to investigate if a possible correlation between haplotype and colouration of the lichen can be found within populations on a smaller scale.

Conclusions

Intraspecific variation in the ITS region of *Pseudevernia furfuracea* was detected. One dominating haplotype was found in Sweden, Estonia and Finland, which suggest that ancestral haplotypes can be found in all three countries. Statistically significant correlation between haplotype and colouration of the lichens was found, but not between any other characters. Altogether, genetic variation in the ITS region could be used to support that *P. furfuracea* in the Baltic Sea area should be regarded as one single species.

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References

- Arup U, Ekman S, Lindblom L & Mattsson J-E (1993) High performance thin layer chromatography (HPTLC), an improved technique for screening lichen substances. *Lichenologist* 25: 61–71.
- Clement M, Posada D & Crandall KA (2000) TCS: a computer program to estimate gene genealogies. *Molecular Ecology* 9: 1657–1659.
- Culberson CF (1969) *Chemical and Botanical Guide to Lichen Products*. The University of North Carolina Press, USA.
- Culberson WL, Culberson CF & Johnson A (1977) *Pseudevernia furfuracea-olivetorina* relationships: chemistry and ecology. *Mycologia* 69: 604–614.
- Du Rietz GE (1945) Om fattigbark- och rikbarksamhällen. *Svensk Botanisk Tidskrift* 39: 147–150.
- Gardes M & Bruns TD (1993) ITS primers with enhanced specificity for basidiomycetes – application to the identification of mycorrhizae and rusts. *Molecular Ecology* 2: 113–118.
- Hallingbäck T (1995) *Ekologisk katalog över lavar*. ArtDatabanken, Uppsala.
- Halvorsen R & Bendiksen E (1982) The chemical variation of *Pseudevernia furfuracea* in Norway. *Nordic Journal of Botany* 2: 371–380.
- Huovinen K & Ahti T (1982) Biosequential patterns for the formation of depsides, depsidones and dibenzofurans in the genus *Cladonia* (lichen-forming ascomycetes). *Ann. Bot. Fennici* 19: 225–234.
- Lindblom L & Ekman S (2006) Genetic variation and population differentiation in the lichen-forming ascomycete *Xanthoria parietina* on the island Storfosna, central Norway. *Molecular Ecology* 15: 1545–1559.
- Lindblom L & Ekman S (2007) New evidence corroborates population differentiation in *Xanthoria parietina*. *Lichenologist*, in press.
- Lutzoni F, Kauff F, Cox CJ, McLaughlin D, Celio G, Dentinger B, Padamsee M, Hibbett D, James T Y, Baloch E, Grube M, Reeb V, Hofstetter V, Schoch C, Arnold AE, Miadlikowska J, Spatafora J, Johnson D, Hambleton S, Crockett M, Shoemaker R, Sung G-H, Lücking R, Lumbsch T, O'Donnell K, Binder M, Diederich P, Ertz D, Gueidan C, Hansen K, Harris RC, Hosaka K, Lim Y-W, Matheny B, Nishida H, Pfister D, Rogers J, Rossmann A, Schmitt I, Sipman H, Stone J, Sugiyama J, Yahr R and Vilgalys R (2004) Assembling the fungal tree of life: progress, classification, and evolution of subcellular traits. *American Journal of Botany* 91: 1446–1480.
- Mattsson J-E (1994) Lichen proteins, secondary products and morphology: a review of protein studies in lichens with special emphasis on taxonomy. *J. Hattori Bot. Lab* 76: 235–248.

Mattsson J-E, Hansson A-C & Lindblom L (2006) *Genetic diversity and substrate preference in Hypogymnia physodes in northern Europe*. Abstract of the 8th International Mycological Congress, Cairns, Australia.

Mattsson J-E, Hansson A-C & Lindblom L (In prep) Genotypic variation in relation to morphology, substrate preferences, and geographic distribution of *Hypogymnia physodes*.

Moberg R & Holmåsen I (1990) *Lavar, en fälthandbok*. 3rd ed. Stenströms Bokförlag AB/Interpublishing, Stockholm.

Printzen C & Ekman S (2002) Genetic variability and its geographical distribution in the widely disjunct *Cavernularia hultenii*. *Lichenologist* 34: 101–111.

Printzen C, Ekman S & Tønsberg T (2003) Phylogeography of *Cavenularia hultenii*: evidence of slow genetic drift in a widely disjunct lichen. *Molecular Ecology* 12: 1473–1486.

Purvis W (2000) *Lichens*. The Natural History Museum, London.

Rikkinen J (1997) Habitat shifts and morphological variation of *Pseudevernia furfuracea* along a topographical gradient. *Symb. Bot. Ups* 32: 223–245.

Templeton A R, Crandall A & Sing C F (1992) A Cladistic Analysis of Phenotypic Associations With Haplotypes Inferred From Restriction Endonuclease Mapping and DNA Sequence Data. III. Cladogram Estimation. *Genetics* 132: 619–633.

Vinter T (2006) *Habitat associations and fitness in Daphne mezereum*. Master Thesis in Biology, Södertörn University College.

Wedin M, Döring H & Gilenstam G (2004) Saprotrophy and lichenization as options for the same fungal species on different substrata: environmental plasticity and fungal lifestyles in the *Stictis conotrema* complex. *New Phytologist* 164: 459–465.

White TJ, Bruns T, Lee S & Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR protocols: a guide to methods and applications* (M A Innis, DH Gelfand, JJ Sninsky & TJ White, eds): 315–322. Academic press, San Diego, CA.

Online resources:

1. biology.duke.edu. Vilgalys lab, Duke University. “Conserved primer sequences for PCR amplification and sequencing from nuclear ribosomal RNA”. 22 December 2006. <<http://www.biology.duke.edu/fungi/mycolab/primers.htm>>.
2. mbio.ncsu.edu. “BioEdit, Biological sequence alignment editor for Win 95/98/NT/2K/XP”. 18 May 2007. <<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>>.
3. dnastar.com. “DNASTar Lasergene EditSeq, Utility for Editing and Importing Unusual File Type”. 17 May 2007. <<http://www.dnastar.com/products/editseq.php>>.

4. dnastar.com. “DNASTar Lasergene MegAlign, Multiple and Pairwise Sequence Alignment”. 17 May 2007. <<http://www.dnastar.com/products/megalign.php>>.
5. r-project.org. “The R Project for Statistical Computing”. 17 May 2007. <<http://www.r-project.org>>.