Mitochondrial DNA haplotypes in a natural stand of the lichen *Peltigera membranacea*

Juho Erkki Oskari Rinne

Faculty of Life and Environmental Sciences
University of Iceland
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Thesis submitted as part of LÍF015M Rannsóknarverkefni í líffræði fyrir erlenda stúdenta, 20 ECTS

Advisor
Ólafur S. Andresson

Faculty of Life and Environmental Sciences
School of Engineering and Natural Sciences
University of Iceland
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Faculty of Life and Environmental Sciences
School of Engineering and Natural Sciences
University of Iceland
Askja, Sturlugötu 7
107, Reykjavík
Iceland

Telephone: 525 4000

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Abstract

Around 30 polymorphic sites in the mtDNA genome of the lichen *P. membranacea* were identified from 454- and Illumina sequencing data. To verify the frequencies of these polymorphisms in a natural population 46 thalli were collected. Also 8 additional thalli samples were collected from two sites to see if there is a difference in polymorphisms within a single lichen thallus. From the data collected a haplotype map of *P. membranacea* was compiled. Two newly designed primer sets were used to amplify the regions of polymorphisms and then sequenced using the Sanger sequencing method. Only two SNPs (positions 7721 and 54447) were found in the samples used in this study. No difference in polymorphisms was found within the same lichen thallus. This was expected because the mitochondrial mutation rate is not fast enough to cause readily detectable mutations. The frequencies of the two SNPs were substantially higher (3 to 4,5 times) in this study compared to the preliminary data. Almost 1500 bp were sequenced from each sample and only the two common SNPs (7721 and 54447) were found. The samples for the preliminary study were collected from a different site than the samples used here which most likely explains the difference in the frequencies and degree of SNPs. Only two different haplotypes were present in the population used for this study: the reference type and the variant type. No intermediate haplotypes were present. The distribution of the haplotypes within the sampling grid is compatable with two or three founders and vegetative expansion down the hillside.

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1 Introduction

1.1 Introduction to lichen

Lichens are often classified as plants next to mosses even though they consist of a fungal partner (a mycobiont) and a photosynthetic partner (a photobiont) that is usually either a green alga or a cyanobacterium. Lichens are a textbook example of symbiosis. The symbiotic relationship between the two organisms has been considered to be mutualistic which means that both individuals benefit. There are over 14 000 known lichen forming fungi species but only about 100 known photobionts. Most of these lichen forming fungi belong to the phylum *Ascomycota*. The symbiosis of the organisms is so successful that one can find lichens growing almost everywhere on land. Lichens are known to inhabit all sorts of substrates both inside and outside of surfaces for example on rocks and plants or even man made materials like concrete.

Sexual reproduction is common in lichens. When they reproduce sexually the spores of the mycobiont need to establish the symbiotic relationship with the photobiont a new. If the lichen reproduces asexually it spreads vegetative particles that consist of both fungal and photobiont partners.

What makes lichens really interesting as a research object is the strong symbiotic relationship of the different types of organisms and their wide diversity of unique secondary metabolites. For more basic information on lichens see Lutzoni and Miadlikowska 2009.

1.2 Intoduction to mitochondria

Many textbooks have been written on mitochondria so a lot of literature can be found on this matter. Information presented here is found in the textbook by Alberts et al. (2008). Mitochondria are eukaryotic cell organelles. They are bounded by two membranes, the outer and inner membrane. The inner membrane is usually highly folded to increase the surface area. These folds are called cristae. The space within the inner membrane is called the matrix. The inner membrane as well as the matrix is rich in proteins that perform most mitochondrial functions. Mitochondria have a variety of duties ranging from metabolism to cell apoptosis. Mitochondria are the power plants of the cell. In the matrix oxidation of fatty acids and pyruvate produce acetyl coenzyme A for further oxidation in the citric acid cycle. Captured electrons from these oxidation-reduction reactions are then used in the electron-transport chain in the inner mitochondrial membrane to pump protons from the matrix to the intermembrane space. Proton pumping produces an electrochemical gradient over the inner membrane. The ATP synthase complex uses this electrochemical gradient to synthesize ATP molecules.

Mitochondria have their own genetic material that is generally accepted to be a remnant of their bacterial heritage. The mitochondrial DNA (mtDNA) molecule is a circular double stranded DNA, the size varying between different organisms from 6 000 bp in a human malaria parasite to 300 000 bp in some plants (Alberts et all 2008). The number of DNA

molecules in each mitochondrion can vary from only one to several hundred depending on the organism again. The mutation rate in mitochondria is several times greater than in the nucleus mainly because of lack of proper DNA repair mechanisms and a high presence of radical oxygen species. These facts introduce a mitochondrial characteristic called heteroplasmy. It means that cells or tissues of an organism have both mutated and wild type DNA molecules. The mutations can be single nucleotide polymorphisms (SNPs): a situation when corresponding DNA molecules have different bases in the same location. If all the mtDNA molecules are of the same type the situation is known as homoplasmy. Mitochondrial DNA also has a special type of inheritance called uniparental inheritance: a new organism gets it mitochondria from only one parent e.g. in humans only from the mother. These properties of the mitochondrial genome have proven useful in phylogenetics and other genetic studies (Dakubo 2010). SNPs are a useful mtDNA characteristic for a phylogenetic study. The collection of SNPs on a single DNA molecule is called a haplotype and it can be used to determine its relationship to other individuals in the same species or between populations.

1.3 Haplotyping in *P. membranacea*

The lichen *Peltigera membranacea* is one of the most common lichens in Iceland. The photobiont in this lichen is a cyanobacterium from the genus *Nostoc*. Because this lichen is so common in Iceland it is a good model organism for genetic and population studies, especially mitochondrial genetics because the fungal partner is the only mitochondrial contributor. Before this study my colleagues found out from 454- and Illumina-sequencing data of mtDNA of *P. membrancea* (unpublished), that it was highly polymorphic. There were at least 30 polymorphic sites but the frequencies of most polymorphisms were low. The study presented here was done to shed some light on this finding. The goals of this study are i) to find out if there are differences in polymorphisms within a single lichen thallus ii) to verify the frequencies of different polymorphisms, iii) and finally construct a haplotype map of *P. membranacea* and iv) describe the distribution of haplotypes in a small plot.

This kind of intrathallus SNP mapping has not been done with lichen-associated fungi previously. In plants there is generally no difference in polymorphisms intraleaf so we can expect not to find any intrathallus polymorphisms here either. Mitochondrial haplotype mapping has been done previously with lichen associated fungi (Prinzen et al. 2003) and also with plant pathogen fungi (Martin 2008).

2 Materials and methods

Samples were collected from two different sites in Iceland inside the capital Raykjavík: from Keldur (N 64° 07,769'; W 21° 46,608') and Perlan (N 64° 07,055'; W 21° 55,192'). Altogether 54 thalli were collected. For the first part of the study, two thalli were collected from Perlan and six from Keldur. In Keldur the thalli were collected in pairs from three locations approximately five meters apart. For the second part of the study 46 thalli were collected from Keldur inside a 20 m^2 square. A schematic drawing of the Keldur sampling area is presented in Figure 1.

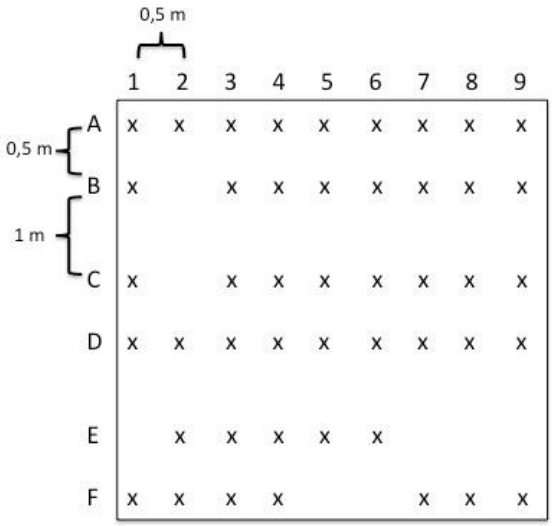

Figure 1. Sample collection area in Keldur. The sampling grid was formed by 9 columns, half a meter apart, and six rows in three pairs, the pairs one meter apart and rows within a pair half a meter apart. *P. membranacea* was not found at all gridmarks. X marks the samples collected.

After sample collection thalli were cleaned from dirt and other macroscopic organisms using a brush and water and then left to dry. 20 to 30 mg or approximately 1 cm^2 -of dried thallus was used for DNA extraction (see DNA extraction protocol in appendix A1). For the first part of the study two to four 1 cm^2 pieces were cut from a single thallus but for the second part only a single piece was cut per thallus. From each sample 4 µg of DNA was recovered on average.

To amplify the region of mtDNA with polymorphisms two sets of primers were designed. The primer sets with sequences are in table 1. The first SNP (7721) is in the end of a group IC intron and the second SNP (54447) is in an intergenic region. For the first part of the study only primer set Pmemmito1 (SNP 7721) was used. For the second part both primer sets were used. The PCR reactions for primer set Pmemmito1 are illustrated in appendix A2. For the primer set Pmemmito3 additional $MgCl_2$ had to be put into the reaction. 2,5 µl of double distilled water was replaced by a 25 mM $MgCl_2$ solution. The PCR cycle was the same with both primer sets.

PCR products were depleted of extra nucleotides and oligonucleotides by phosphatase and exonuclease enzymes. See exo sap protocol in appendix A3.

The treated PCR products were then used as templates for Sanger sequencing reactions. Sequencing reactions were done using the Big Dye® -ready reaction mix (Applied Biosciences). Sequencing reactions were done for both sense and antisense strands of the PCR product. The Sanger sequencing protocol is also found in the appendix (A4).

Finally sequencing reaction products were precipitated and the DNA pellet was dissolved in HiDi (see appendix A5 for precipitation protocol). Sequencing was done with an Applied Biosciences 3500XL Genetic Analyzer.

The sequences were read through manually using XL CLC Genomics Workbench software. Sequences were checked for double peaks indicating heteroplasmy of the mtDNA. SNPs were also check manually and compared to the mtDNA genome compiled from 454 and Illumina whole genome sequencing.

Table 1. Primer sets used. Name, sequence, length and position in reference genome are shown.

Primer name	Primer sequence	Size	Primer position in reference
Pmemmito1F	5' AGCCTTACATCAGCTTTCCTG	21	7606-7626
Pmemmito1R	5' AATCTTCACGATCCCCCAATG	21	8201-8221
Pmemmito3F	5' TTGCTTTTGTCTGGTTGCGATT	22	54367-54388
Pmemmito3R	5' ACATAGAAATCTTTCACCCACTC	23	54761-54783

3 Results and discussion

Intrathallus polymorphisms were not observed: all the samples from the same thallus either had or did not have the SNP. Samples were sequenced only for SNP 7721. Table 2 shows how many samples from each thallus were sequenced and the SNP found at position 7721.

Table 2. Multiple samples from one thallus sequenced with primer set Pmemmito1. Sample count from each thallus is shown as well as the base of the polymorphic site 7721.

Thallus nr.	sample count	SNP
67	3	T
68	4	T
70	4	C
71	2	C
72	3	T
73	3	C
74	3	C
75	3	C

As it turned out no intrathallus polymorphism was detected. The mtDNA polymorphisms that one might expect would most likely arise from spontaneous mutations. Polymorphisms are not frequently observed because the mutation rate in mitochondria ($3 \cdot 10^{-5}$/bp/20 y generation in humans (Schneider 1999)) is too low to cause significant amounts of polymorphisms inside a lichen thallus.

The total number of samples with reference and variant bases to each SNP are presented in table 3. Frequencies of polymorphisms are counted only for the total number of samples. Frequencies from the large scale whole genome data are also shown.

Table 3. Number and frequencies of each SNP. For each SNP both the number of samples with reference and variant bases are shown. The total frequency is presented as well. 20 m^2 refers to samples collected inside 20 m^2 area and Other thalli to the 8 thalli collected for the first part of the study. Whole genome data is the data from the 454- and Illumina sequencing.

SNP	7721		54447	
	reference	variant	reference	variant
Base	C	T	A	C
20 m^2	11	31	11	26
Other thalli	5	3	5	3
Total	16	34	16	29
Frequencies	32 %	68 %	35,6 %	64,4 %
Whole genome data	76,1 %	23,9 %	85,8 %	14,2 %

The frequencies of the variant SNPs found were substantially greater than in the large scale preliminary data. The frequency of the SNP 7721 variant was over 3 times greater and in the SNP 54447 the variant was 4,5 times more frequent. However no other polymorphisms were detected unlike in the whole genome sequencing data. Approximately 1 500 bp of sequence was checked for SNPs and only the two considered here were found. The samples for these two studies were collected from close but different sites, which most likely explains the difference shown here.

Only two of the four theoretical haplotypes appeared in the study. The samples were either had the same bases as the reference or they had variaant bases in both loci considered here. The numbers of each possible haplotype are shown in table 4.

Table 4. Haplotype distribution of *P. membranacea*. Number of different haplotypes are shown.

	Reference		Haplotype 1		Haplotype 2		Haplotype 3	
SNP	7721	54447	7721	54447	7721	54447	7721	54447
Base	C	A	C	C	T	A	T	C
Count	10		0		0		24	

Some mtDNA molecules have acquired spontaneous mutations over many generations and some have become fixed into the mtDNA molecules through the bottleneck of sexual reproduction. In sexual reproduction mitochondria with different mtDNA molecules can fuse allowing recombination between the different mtDNA molecules. This is one possible explanation for how the two SNPs have paired into the same mtDNA. Another possibility is that first one variant becomes frequent in a subpopulation and then another variant. It may be possible to distinguish between these possibilities by extensive sampling since one predicts the occurance of three haplotypes (serial mutations) and the other predicts four

haplotypes (recombination). The samples collected for the study are most likely descendants from three different founder lichens that represent these two predominant haplotypes. This is easy to observe from the projection of SNPs to the collection site at Keldur shown in figure 2.

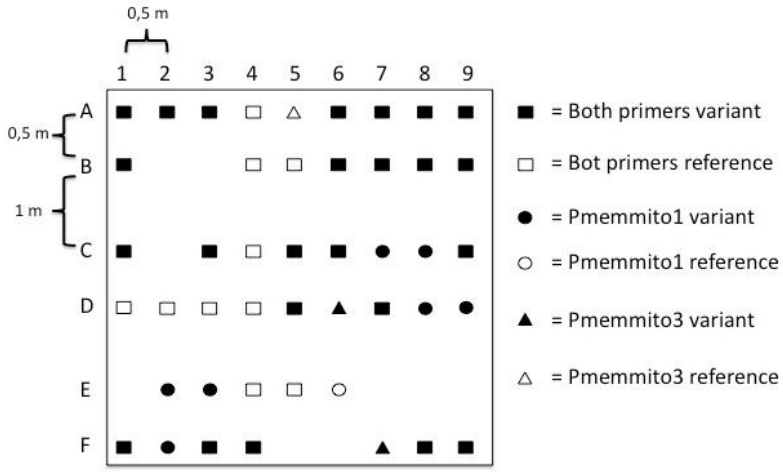

Figure 2. Geographical distribution of polymorphisms. There seem to be three points of origin from where the lichens start to grow: one variant type on each side of the reference type. The site is actually in a slope and it therefore seems therefore natural that the different types spread and cross as they grow.

Assuming that the lichens spread downhill, there are three different points of origin for subpopulations. The reference type subpopulation separates the two variant type subpopulations. They seem to also spread to different degrees and cross when growing down the hill.

4 Conclusions

It seems that the frequency and number of polymorphisms in *P. membranacea* is very site specific. Because this lichen is so common in Iceland it would be interesting to make a large-scale haplotype mapping with samples around the country. It would also give a more realistic estimate of differences in the frequencies of SNPs.

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Appendix A

A1. DNA extraction

1. Cut the cleaned and dried 1 cm^2 piece of thallus into small segments. Put the pieces into a 1,5 ml microcentrifuge tube.
2. Adjust heat block to 65°C.
3. Add 500 µl of room temperature lysis buffer and start homogenization with tube homogenator. After enough homogenization add another 500 µl of lysis buffer and mix.
4. Incubate at 65°C for 20 minutes. (Mix the contents of the tube during incubation.)
5. Centrifuge at 14 000 rpm for 4 minutes
6. Transfer the supernatant into a new tube and add $3/7^{th}$ volume of 10,5 M ammonium acetate.
7. Mix the contents and incubate on ice for 20 minutes.
8. Centrifuge at 14 000 rpm for 4 minutes.
9. Transfer the supernatant to a new tube, discard the pellet and add $9/14^{th}$ volume of isopropanol.
10. Mix the contents and incubate on ice for 20 minutes.
11. Centrifuge at 14 000 rpm for 4 minutes.
12. Discard the supernatant.
13. Add TE buffer and RNase A (10 mg/ml) to the pellet (1 ml TE + 4 µl of RNase A). Adjust the volume to 250 µl with TE buffer.
14. Incubate the tubes at 50°C for 10 minutes until the pellet is dissolved. (Gently tap the tube during incubation).
15. Spin the samples down.
16. Add an equal volume of Phenol:Chloroform:Isoamyl alcohol (25:24:1).
17. Vortex briefly.
18. Centrifuge at 14 000 rpm for 4 minutes.
19. Transfer the aqueous phase to a fresh tube without disturbing the interphase.
20. Add an equal volume of saturated chloroform.
21. Vortex briefly.
22. Centrifuge at 14 000 rpm for 4 minutes.
23. Transfer the aqueous phase to a fresh tube without disturbing the interphase.
24. Add $1/10^{th}$ volume of 3 M sodium acetate pH 7,0 (NaOAc) and twice the volume of 96% ethanol.
25. Incubate the tubes on ice for over an hour.
26. Centrifuge at 14 000 rpm for 4 minutes.
27. Discard the supernatant.
28. Add 500 µl of 70% ethanol.
29. Centrifuge at 14 000 rpm for 4 minutes.
30. Discard the supernatant.
31. Air dry the pellet.
32. Add 20 µl of TE buffer.
33. Incubate at 50°C for 2 minutes, tap gently during incubation to dissolve the pellet.
34. Check the condition of DNA by loading 0,2 µg of sample into a 0,7% agarose gel.

A2. PCR protocol

1. For one 25 µl reaction mix:
   13,8 µl of double distilled water (ddH_2O)

   2,5 µl of 10x ThermoPol Reaction Buffer (New England Biolabs)

   2,5 µl of 2 mM dNTPs (each)

   0,5 µl of forward and reverse primers (10 µM)

   0,2 µl of Taq-polymerase

   5 µl of template DNA

2. Mix the contents of the tubes and spin them down.
3. Put the samples into a PCR machine with the following program:
   1. 94°C for 2 minutes
   2. 94°C for 20 seconds
   3. 55°C for 20 seconds
   4. 72°C for 1 minute and 20 seconds
   5. Go back to step 2 33 times
   6. 72°C 10 minutes
   7. 4°C infinity
4. Check the size of the product on a 1,2% agarose gel.

A3. Exo sap -treatment of PCR products

1. For one 10 µl reaction mix
   3,7 µl of ddH_2O

   1 µl of Antarctic Phosphatase buffer (New England Biolabs)

   0,2 µl of Antarctic Phosphatase 5U/µl (New England Biolabs)

   0,1 µl of Exonuclease I 20U/µl (New England Biolabs)

   5 µl of PCR product

2. Gently pipet mix when adding template DNA.
3. Spin samples down.
4. Put the samples into a PCR machine with the following program:
   1. 38°C for 35 minutes
   2. 80°C for 20 minutes
   3. 12°C infinity

A4. Sequencing reactions

1. For one 15 µl reaction mix
   5,75 µl of ddH_2O

   2,75 µl of 5x Sequencing Buffer (Applied Biosciences)

   0,5 µl of Big Dye (Applied Biosciences)

   1,5 µl of 10µM forward OR reverse primer

   4,5 µl of Exo sap product.

2. Gently pipet mix when adding Exo sap product.
3. Spin samples down.
4. Put the samples into a PCR machine with the following program:
   1. 96°C for 10 seconds
   2. 96°C for 10 seconds
   3. 55°C for 5 seconds
   4. 60°C for 2 minutes
   5. Go back to step 2 25 times
   6. 60°C for 7 minutes
   7. 12°C infinity

A5. Ethanol precipitation

1. Perform all the steps on ice.
2. Precipitation mix for 30 samples:
   1350 µl of double distilled water

   150 µl of 3M NaOAc pH 7,0

   7,5 µl of Glycogen (Fermentas)

3. Pipet 50 µl of Mix and 10 µl of sample (sequencing reaction product) together and mix gently.
4. Add 125 µl of 96% ethanol (ice cold).
5. Centrifuge the samples at 10 500 rpm for 20 minutes at 4°C.
6. Discard the supernatant with the help of air suction.
7. Add 250 µl of 70% ethanol (ice cold).
8. Centrifuge the samples at 10 500 rpm for 5 minutes at 4°C. (Repeat this step one more time).
9. Discard the supernatant with the help of air suction.
10. Leave the sample in a dark place for one hour or until complete evaporation of ethanol.
11. After completely drying add 15 µl of HiDi at room temperature and vortex samples.
12. Then heat the samples at 95°C for 2 minutes.
13. After heating transfer the samples immediately to ice and keep there for 5 minutes.

14. Vortex and spin the samples. Now samples are ready for loading into the sequencing tray.