Occurrence and distribution of *Phytophthora spp.* affecting European beech *(Fagus sylvatica)* within Söderåsen National Park

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Abstract

There has been an increase of damage on beech in Europe since the 1990s, especially in central Europe and Germany. The first report on beech in Sweden was in 2010 in Malmö and since then there have been additional observations of symptomatic beech trees at multiple locations, among which Söderåsens national park is one such location. The aim of this thesis was to investigate which *Phytophthora* species are present at Söderåsen and to confirm previous observations of symptomatic trees within the park. Through tissue plating and environmental sequencing the presence of *Phytophthora* at Söderåsen was confirmed, specifically *Phytophthora plurivora* and *Phytophthora cambivora*, of which *P. plurivora* is a newly confirmed species. The main spreading pathway of *Phytophthora* in Europe is thought to be via nursery stocks. Between 2002 and 2006 many trees were planted at Söderåsen, grown at nursery stocks in Germany and Poland. This is one possible spreading pathway through which *Phytophthora* may have become established at Söderåsen. Finally, this thesis gives further suggestions for management strategies within the park, of which monitoring of new symptomatic trees is the most pressing one. The success of sampling *Phytophthora* is heavily dependent on the freshness of the sampled lesions. For further research it is of importance to sample symptomatic trees soon after discovery for best chance of isolation success. These results give further understanding of the *Phytophthora* situation at Söderåsen, however there is still a lot to understand about these pathogens and how they interact with their surroundings. Further monitoring and management strategies will be needed for the future ensuring of Söderåsen as a hotspot for nature conservation in Sweden and Scandinavia.

Keywords: Beech, *Fagus sylvatica*, Söderåsens nationalpark, *Phytophthora cambivora*, *Phytophthora plurivora*, soil baiting, tissue plating, environmental sequencing.

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1. Introduction

Global distribution of different *Phytophthora* species

Phytophthora is one of the most important genera in the world in regards to microorganisms causing damage to plants. These pathogens cause disease on both agricultural crops and woody tree species, especially in temperate and subtropical regions. The most famous *Phytophthora* species is *Phytophthora infestans*, also known as potato blight. *P. infestans* was the causal agent of the Irish famine in mid 19th century, which in turn caused the great emigration from Ireland to the USA. *Pytophthora infestans* specifically affects potato and other *Solanum* spp., and causes major crop loss for potato growers in both Europe and North America. Another major agricultural disease caused by *Phytophthora spp.* include *Phytophthora sojae*, which, as the name indicates, affects soybean (*Glycine max*), mainly in the USA. Also, *Phytophthora capsisi* is of great concern to farmers in the USA, where it destroys vegetable crops such as chilli peppers, from which its name was derived (Lamour, 2013).

Global outbreaks of different *Phytophthora* species have led to severe damage to forest ecosystems. In North America, *Phytophthora ramorum* has decimated large forest areas (Grunwald et al., 2012). *P. ramorum* is the first known *Phytophthora* species, among the forest infecting *Phytophthora* species, that successfully has been able to spread via aerial spores (Lamour, 2013). In Australia *P. cinnamomi* has devastated entire ecosystems associated with eucalyptus (*Eucalyptus marginata*), but also has a broad host range. *P. cinnamomi* is known as one of the world's worst invasive species; it can infect more than 900 woody perennial species (Lowe et al., 2000). In the tropics, *Phytophthora palmivora* is the most destructive pathogen on tropical tree species, it causes major damage to cocoa production mainly in South-East Asia (Lamour, 2013).

During the last 20 years we have seen an increase of *Phytophthora* introductions around Europe. The major introduction pathways being global transportation and trade of plants via infected nursery stocks. The introductions have led to decimated stands of oak (*Quercus spp.*), chestnut (*Castanea spp.*), alder (*Alnus spp.*) and European beech (*Fagus sylvatica*) (Jung et al., 2005).

Phylogeny and evolutionary origins of *Phytophthora* species

The genus *Phytophthora*, together with the genus *Pythium*, belong to the family *Phythiaceae*, and the class *Oomycetes*. *Oomycetes* share morphological similarities with fungi, but lack a common evolutionary origin (Cooke et al., 2000). The differences between oomycetes and fungi consist of fundamental physiological differences. The oomycete cell wall consists of cellulose, in contrast to the fungal chitin and their oomycete cells are diploid, while fungal cells usually are haploid (Lamour, 2013).

The evolutionary origins of most invasive *Phytophthora spp.* are uncertain. However, for the most destructive species of *Phytophthora*, the origin is somewhat known. The potato blight pathogen, *P. infestans*, is thought to have originated from Mexico (Grunwald and Flier, 2005). Among *Phytophthora* species that attack woody plant species, it has been indicated through isozyme analysis that *P. ramorum* have an origin of north Australia (Oudemans and Coffey, 1991). Vannini et al. (2009) showed that *Phytophthora citricola* (which are now considered a complex of many species, of which *Phytophthora plurivora* is one (Jung and Burgess, 2009)) has an origin of Nepal or geographically surrounding areas.

Biology of the *Phytophthora* genera

Phytophthora thrives in moist areas and has both sexual and asexual reproductive stages. They form oogonium during sexual reproduction and sporangia (containing motile zoospores) during asexual reproduction (Figure 1). The pathogen can lie dormant in the soil for long periods of time. When soil moisture increases, it forms sporangia and releases large quantities of zoospores (Hardham and Cahill, 2010), which in turn migrate through the water and infect surrounding trees. Different species of *Phytophthora* have different ways to infect a potential host. Spores infect via the roots, stem wounds and leaves (Osswald et al., 2014). However, the majority of *Phytophthora* species are soil-borne pathogens, and infect the roots, via motile zoospores that are attracted by root exudates from the growing root tips (Khew and Zentmyer, 1973, Portz et al., 2011). Grooves, located between epidermal cells of the host plant, make it easier for the pathogen to penetrate the epidermis to enable further infection. The pathogen usually colonizes the elongation zone, located just above the root tip, or wounds on the surface of the roots. The elongation zone appears to be the most vulnerable due to the stretching of the plant cell walls, which are thinner and easier to penetrate by the pathogen (Hardham, 2001).

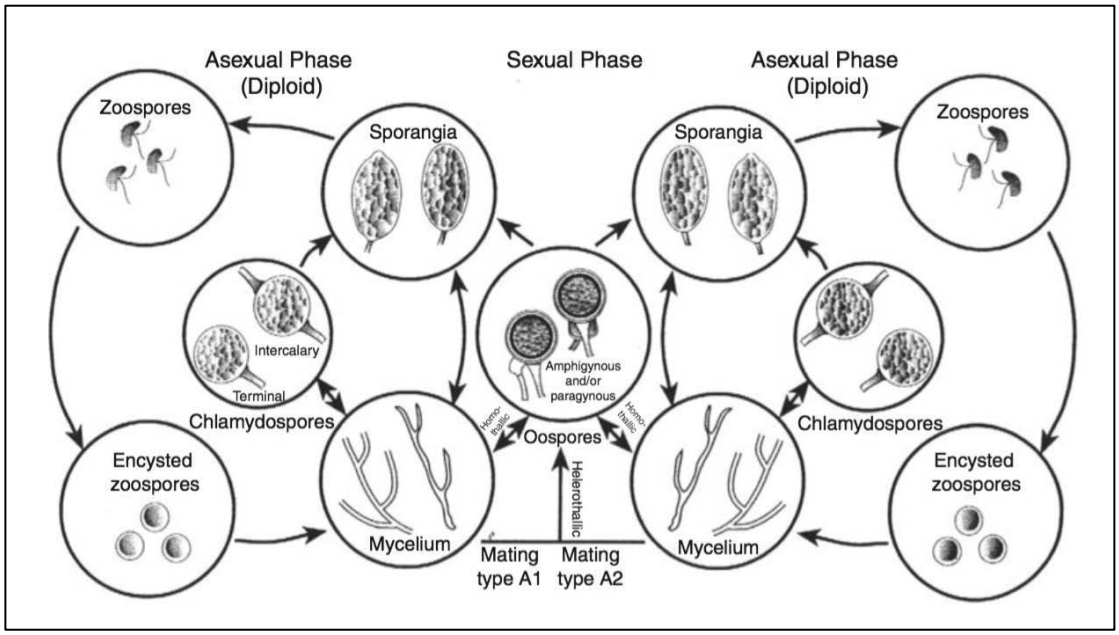

Figure 1. The life cycle of *Phytophthora spp*. showing both sexual (oospores) and asexual (zoospores and chlamydospore) reproduction stages (Lamour, 2013).

When zoospores attach to the root, they secrete adhesive substances, encysts on the surface of the root and then forms a germ tube. Germ tube formation results in a penetration hypha that directly penetrate epidermal cells and make it possible for the pathogen to advance into the apoplast (Hardham, 2001). Afterwards, it will infect the root by penetrating cell walls and producing haustoria. A haustorium is a structure that sits within the plant cell wall, but outside the plant cell plasma membrane, this is where the pathogen will have most of its nutrient uptake from and molecular interactions with the host plant (Hardham and Cahill, 2010).

Initially the pathogen does not kill the host, but will live as a biotroph within the plant, requiring living tissue for growth. At a later stage of infection, *Phytophthora* will

change its life stage from biotrophy to necrotrophy, killing the infected host cells as it advances through the plant tissue (Lee and Rose, 2010). Having both life stages is known as Hemibiotrophy. As a hemibiotroph, *Phytophthora* is a potent primary infector, and usually the first pathogen to infect the root or stem of a host plant. As the pathogen advances into the root it will rapidly grow through the cortex, the pericycle and the phloem. When reaching the phloem, it rapidly spreads along the roots, up to the collar and the stem (Osswald et al., 2014). After initial infection other secondary agents usually follow, such as necrotrophic, saprotrophic fungi and wood degrading insects (Jung et al., 2005).

Infection biology of *Phytophthora*

One reason for the success of many introduced *Phytophthora spp*. is the initial geographical isolation, restricted to interactions with indigenous species. Increased globalization, transportation and trading of plants from nursery stocks is thought to be the primary reason for the major outbreaks of damage of *Phytophthora* that we see today (Lamour, 2013, Jung et al., 2016). Evolutionary adaptations to pathogens occur over long periods of time (generations). Plants growing together with pathogenic *Phytophthora spp.* over tens of thousands of years may have developed resistance towards the locally occurring pathogens. When *Phytophthora spp*. have been introduced in different parts of the world, other plant species, previously unaffected by *Phytophthora*, do not have the same means of resistance. As with most introduced pests and pathogens, where the host plant lacks a history of co-evolution, the damage is devastating, in many cases leading to major economic and ecological losses (Jung, 2009).

Plant resistance biology

The plant immune system consists of mainly two different branches, the first branch comprises of transmembrane recognition receptors for pattern recognition of microbial- or pathogen-associated molecular patterns (MAMPs or PAMPs respectively) (Jones and Dangl, 2006). These transmembrane proteins induce a PAMP triggered immunity (PTI), which initiates antimicrobial reactions within the plant cell. Defences to pathogen infections include for example, deposition of callose (a polysaccharide, which do not form the same linear structure as cellulose) and activation of the hypersensitive reaction (HR) (Osswald et al., 2014). A distinct symptom of *Phytophthora* infection is the dark bleeding sap that exudes from bleeding cankers on the stem. This symptom is thought to be due to the production of polyphenols, which is triggered by the pathogen, and diffuse out into the exuding sap (Brown and Brasier, 2007).

One way for pathogens of overcome PTI is by deploying effectors into the apoplast or into the host cells via haustoria (Huckelhoven, 2007). Effectors are defined by Harham and Cahill (2010) as *"molecules that manipulate host cell structure and function, and thereby facilitating infection and/or triggering defence responses*" (Hardham and Cahill, 2010). Effectors are separated into two kinds, those who are deployed into the apoplast, and those that are deployed, via a haustorium into the cell cytoplasm. The effectors that are found inside the cytoplasm of host cells all have a conserved N-terminal motif necessary for translocation over the plasma membrane of the plant, from the haustorium into into the plant cell (Whisson et al., 2007). Effectors can be cell-wall degrading enzymes, enzyme inhibitors, toxins or elicitins (small conserved polypeptides that induce the HR) (Fleischmann et al., 2005). These effectors can also act as inhibitors for the downstream induction of PTI (Hardham and Cahill, 2010).

The second branch of the plant immune system, the pathogen effector recognition gives an effector-triggered immunity (ETI). ETI is mainly initiated by nucleotide binding and leucine rich repeats (NB-LRR) protein products, which recognise pathogen effectors and inhibit the negative effect of the effectors. The NB-LRR proteins are named for their nucleotide binding (NB) and leucine rich repeat (LRR) domains (Jones and Dangl, 2006). This way, the plant can deploy defence strategies against the evolving pathogens by neutralizing deployed pathogen effectors. As a response, the pathogen may produce new effectors that override the induced resistance of the plant. There are some pathogens that produce effector molecules that inhibit PTI, which in turn is called effector-triggered susceptibility (ETS) since the plant will show a higher susceptibility towards the pathogen. These evolutionary mechanisms go back and forth, they are slow and evolve over long periods of time. Evolution acts towards more resistant plant species while the pathogens try to override the defence mechanisms of the plant. One general way of decreasing the negative effects of these pathogen outbreaks could be to find resistant hosts that will be better adapted to these invasive pathogens and to understand the complex interactions involved in ETI and ETS therein.

Decline of important broadleaved tree species in Europe

Different invasive species of *Phytophthora* have, over the last two decades, shown an increased prevalence throughout Europe, resulting in large-scale decline and mortality of many broadleaved tree species. There are mainly four tree species that have seen a large-scale decline due to *Phytophthora* in Europe: oak (*Quercus spp.*), chestnut (*Castanea spp.*), alder (*Alnus spp.*) and European beech (*Fagus sylvatica*) (Lamour, 2013). It is thought that the increased prevalence and spread of *Phytophthora* is due to climatic extremes becoming more pronounced, such as heavy rainfalls, alternated with periods of dryer climate and an general increased temperature. Providing a more favourable habitat for *Phytophthora* as well as increasing abiotic stress on the Beech trees, lowering their immune system, making them more susceptible towards pathogenic infection. The increased frequency of these climactic extremes are thought to be a result of global warming (Jung, 2009).

The decrease of vitality of oak stands was first encountered in southern Europe, mainly in Portugal and Spain, but has over the last decades become more evident throughout Europe. In Europe, there are different species responsible for the decline of oak, among these are *Phytophthora quercina*, *P. cinnamomi, P. cambivora* and *P. plurivora* the most noted. The decrease in vitality of chestnut trees due to *Phytophthora* was first encountered in Portugal and Spain. The disease has generally been known as the chestnut ink disease, named after the symptomatic lesions created on the stem of the chestnut trees. Today this disease has spread throughout larger areas of Europe, and is primarily caused by species of *Phytophthora*, namely *P. cinnamomi* and *P. cambivora*. Alder tends to grow in wetter areas, due to higher water tolerance. Since it is able to grow in waterlogged areas it also becomes quite vulnerable towards *Phytophthora* infections traveling through water systems. The host-specific pathogen responsible for alder decline is *Phytophthora alni* and its subspecies. *P. alni* has been located at numerous locations throughout Europe. Beech decline was first observed in the UK in the 1930's (Day, 1939). Today *Phytophthora* infecting beech is a common problem throughout Europe. The biggest outbreaks have been observed in Germany where pure beech forests are common. The major pathogens recognised as the causing agents for this spread are *P. cambivora*, *P. cactorum* and *P. plurivora* (Lamour, 2013).

Phytophthora affecting broadleaves in Southern Sweden

Symptoms of *Phytophthora* infections have been noticed on beech trees in southern Sweden, particularly throughout Skåne. The first reports were from trees in Pildamsparken in Malmö where initial surveys showed that a significant number of the trees were infected with *Phytophthora* and also exhibited symptoms of decay (Jung, 2011). There have also been reports of *Phytophthora* occurrence at Söderåsen National Park. A first survey of Söderåsen was conducted in spring/ early summer of 2012 (Agostinelli and Witzell, 2012). There have also been various reports from Kullaberg nature reserve, Torup castle outside of Malmö (unpublished) and at Slottsskogen in Gothenburg.

Söderåsen National Park

During Bronze age (3300-1200 BC) and earlier, the area that today constitutes Söderåsen National Park, was used for farming. There are still signs of manmade cairns (cairns were usually made when stones, removed to create farmland, were piled) within the park. Today, Söderåsen is the largest protected beech forest in Scandinavia. It was established 2001 after approximately 35 years of gradual land acquisition to the state. It all started in the end of the 19th century when Theodor Jönsson, who at that time owned the land, protected the rocky slopes of the valley from exploitation. The area had been used for commercial forestry for a couple of hundred years. As early as the beginning of the 20th century the area around Skäralid was frequently visited for recreational purposes by people from all over Skåne. The first formal land acquisitions were made in 1980, and further acquisitions were made throughout the 90's and the last piece of land was acquired the year 2000. In 1989 Naturvårdsverket suggested that the area that today consists of Söderåsen would become a national park. The park are especially important for nature conservation, since biodiversity conservation is a primary goal for the future of the national park, all logging at Söderåsen ceased in 1989 (Länsstyrelsen, 2016).

The park was consecrated in 2001 and spans over 1625 hectares of mainly forest. The composition within the park is mainly beech forest, but also contains alder and birch forest, oak forest and other mixed forests trees such as (*Acer platanoides*), elm (*Ulmus glabra*), linden (*Tilia cordata*), alder (*Alnus glutinosa*) and more (Naturvårdsverket, 2001). Aside from the beech forests, there are also areas left from the former logging period, which consists mainly of spruce (*Picea abies*). Between 2002 and 2006 the LIFE-Nature project were implemented at Söderåsen. The LIFE-Nature project is one of the largest nature conservation projects that have been implemented in Sweden. The project aimed at replacing the spruce stands within Söderåsen by planting beech and by natural regeneration (Naturvårdsverket, 2006). The trees that were planted with seeds collected within Söderåsen and sent to German and Polish plant nurseries. In the ravine area there is a diverse assortment of woody tree species. The conservational values, consists mainly of rare bryophyte, plant and insect species, but also to some extent birdlife. The areas of high conservational value are constrained mainly to the ravine, however, the plateaus flanking the ravine are thought to have the potential to establish a more diverse flora and fauna at a timescale of approximately 200 years. Many of the important and rare species found in Söderåsen are closely connected to old trees and availability of dead wood (Naturvårdsverket, 2001).

Since *Phytophthora spp.* have proven to be hazardous to beech forests in central Europe (Jung et al., 2005), it is of importance to take precautions to prevent new introductions and lessen the spread from infected trees to adjacent non-infected areas. It is already known that *Phytophthora spp*. are damaging trees within Söderåsen, however, it is important know which species of *Phytophthora* are present and causing damage to trees to further understand what could be done to limit the negative effects of *Phytophthora*. The species richness of the pathogens, together with the geographical distribution of these species could give an indication to which kind of disease mitigation strategies would be suitable to make, to limit the negative effects of these pathogens.

Study aims

The objectives for this thesis will be to shed light on which species of *Phytophthora* are present at Söderåsen, using direct tissue plating, soil baiting and environmental sequencing of both soil and tissue samples (Catala et al., 2015, Perez-Sierra et al., 2010, Vannini et al., 2013). By taking samples throughout the park, the goal has been to map the distribution, species composition and to further discuss possible management strategies for containing future spread.

2. Methods

Field work

Selection of trees at Söderåsen

Trees were selected by using coordinates from an earlier inventory, conducted by Marta Agostinelli during spring/summer 2012 (unpublished, Figure 2), together with guidance by park staff on the locations of non-vital beech trees, and by traversing along walking paths. GPS points were used from previous inventory, covering most of the official walking paths. All points that were previously investigated in 2012 were revisited in this study.

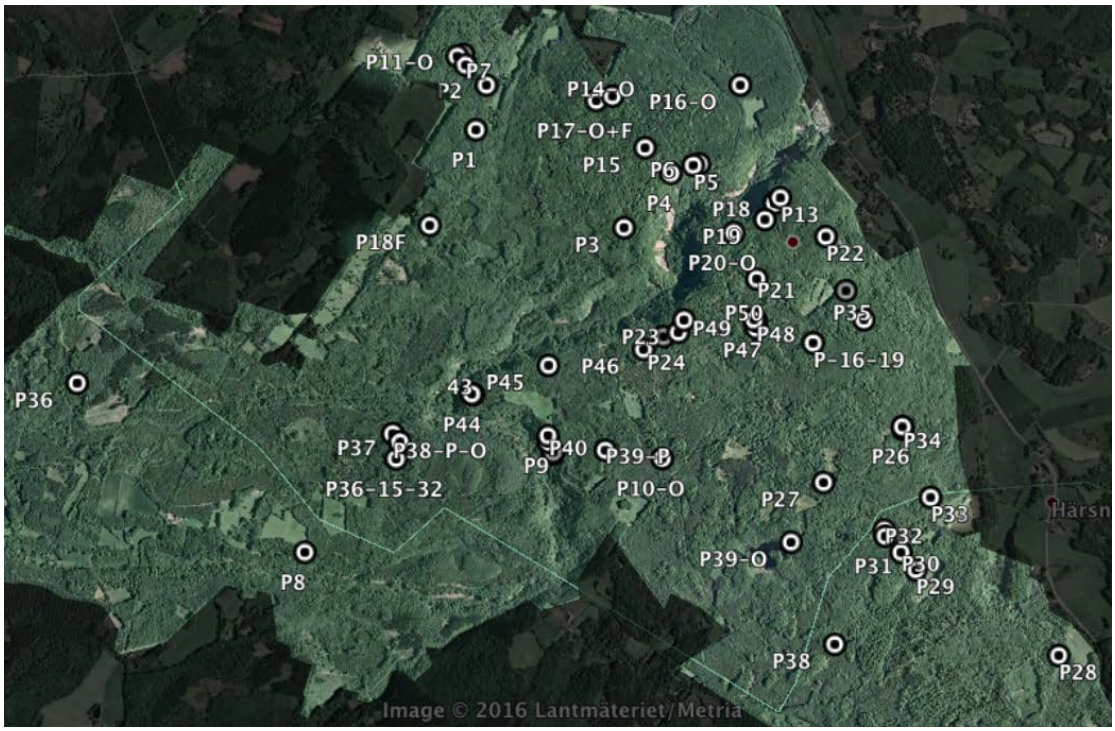

Figure 2. Map of Söderåsen national park, showing positions of trees inventoried in 2012. Map made in Google Earth, with information from Swedish Lantmäteriet.

Geographical distribution of collected samples

In total, 22 symptomatic trees were sampled covering a wide geographical distribution throughout the park (Figure 3). A majority of the sampling was focused on the areas around Skäralid, which is the most frequently visited part of the park. Some parts of the park, which in the previous inventory showed a high density of symptomatic trees, had been felled as a result of forest management, du to safety reasons. Those areas were around Serpentinvägen extending up to Liagården, including areas around Liagården extending up to Stora vägen (Figure 4, b). The removal of trees in the centre of the park becomes evident when comparing the map in figure 2 with figure 3, and explains the lack of sampled trees in the centre. Most of the sampled trees were old, with large trunks, having a mean dbh of 64 cm (Appendix I).

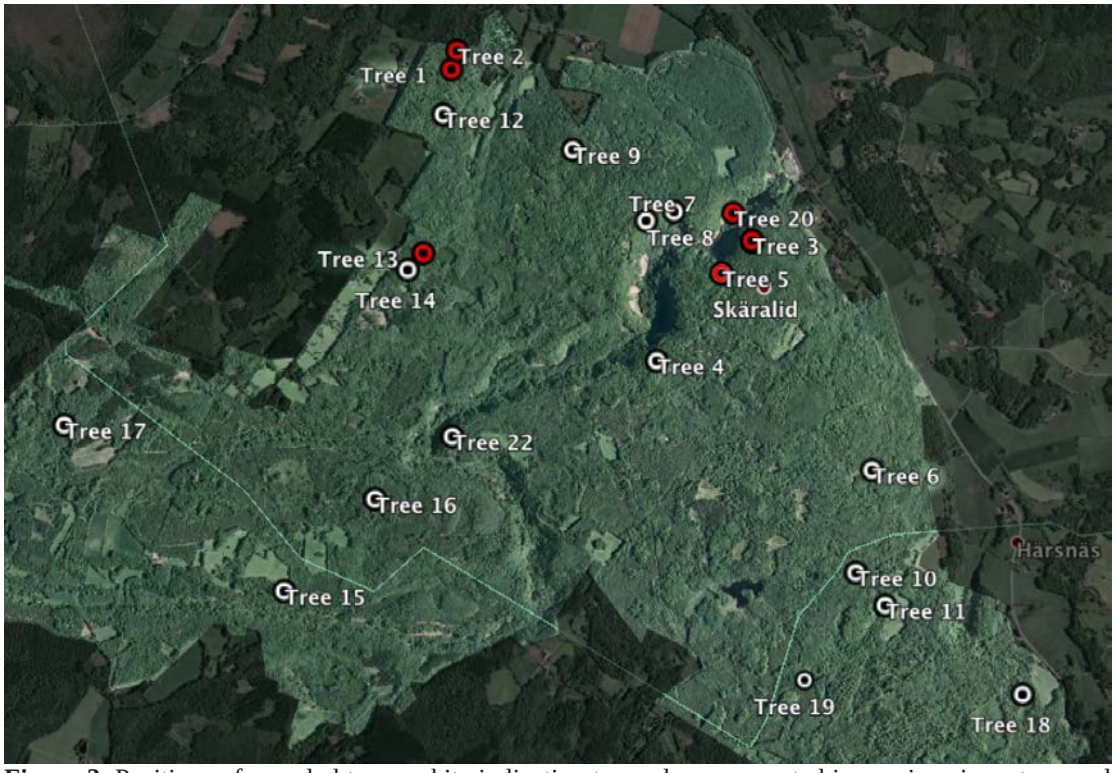

Figure 3. Positions of sampled trees, white indicating trees also represented in previous inventory, red indicating trees not previously noted. Map made in Google Earth, with information from Swedish Lantmäteriet.

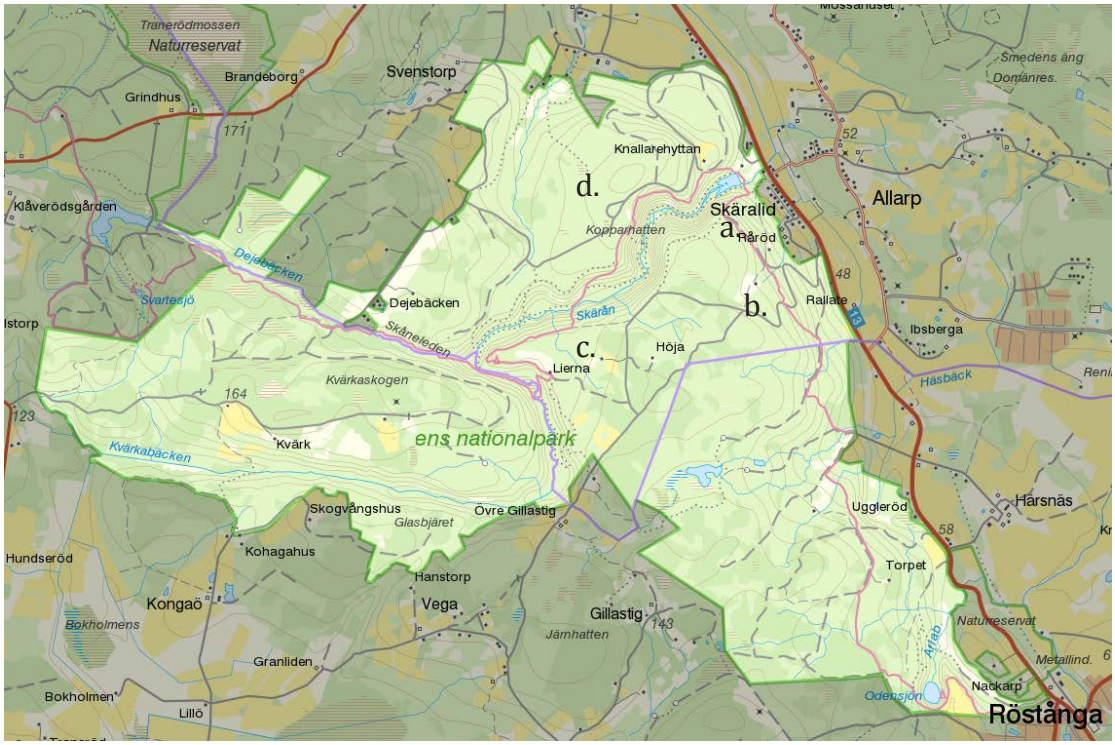

Figure 4. Map of Söderåsens nationalpark. Notable places on the map is: a. Skäralid, location of the park rangers. b. The serpentine road, which leads up towards Lierna. c. Lierna, which is a lodge open for free accommodation. d. Kopparhatten. Picture taken from the Swedish Lantmäteriet.

Collecting soil and tissue samples

The symptoms of infected trees usually show as a red lip shaped lesion on the trunk, exuding a coloured watery substance. Also, as a result of infection, crown thinning and branch tip necrosis is a typical symptom of infection (Figure 5), together, they give a clear indication of *Phytophthora* presence.

Soil was collected around symptomatic trees, at a depth of up to 20 cm, using a 2-cm diameter soil core. The total volume of soil collected at each tree was approximately 0,25 L. soil per sampled tree. Two subsamples were taken at four different points around the tree, located 50 cm from the root collar. The samples were pooled for each tree and thoroughly mixed in a zip lock plastic bag.

Using a sterilized chisel, tissue samples were collected at the margin of bleeding cankers on the trunk of the symptomatic tree (Figure 6). The area of interest was the border between necrotic and healthy tissue. Tissue samples were placed in a plastic bottle filled with sterile water and labelled appropriately. The water was changed at least four times within a 24 h period to minimize inhibitory effect of phenolic compounds towards microbial growth during culturing. Samples from each lesion were placed in aluminium foil and stored in a – 20 °C freezer for later DNA extraction.

Figure 5. Left: Picture showing typical symptoms of tongue shaped lesions of *Phytophthora* on Beech. Right: Beech tree showing signs of foliar thinning, (Adriaan van Tour, 2015).

Figure 6. Figure showing a lesion with removed phloem tissue revealing a rose-coloured area underneath, (Adriaan van Tour, 2015)

Lab work

Soil baiting and tissue samples

The common way of isolating *Phytophthora* from soil or waterways is by baiting. *Phytophthora* has motile zoospores that activate, as the soil gets moist and wet. This is used in laboratories for isolation; by flooding collected soil samples the zoospores will be move freely in the water and infect susceptible leaf tissue floating on top of the water. This way, pathogens with freely swimming zoospores, such as *Phytophthora* is separated from other pathogenic fungi found in the soils.

Baiting of soil samples

For soil baiting, a 1,2 L plastic container (Curver, Poland) was filled with soil to approximately 2 cm, after which approximately double volume (Figure 7) of distilled water was added to the container. Some soil was saved for DNA extraction in a zip-lock bag.

Figure 7. Left: a plastic container filled with approximately 2 cm of soil. Left: a plastic container tiller with the double amount of water, (Adriaan van Tour, 2015).

Rhododendron (Cunninghams white) and *Camelia japonica* leaves were surface sterilized in 1 % sodium hypochlorite (Colgate-palmolive, France) for 30 seconds, washed three times in sterile water, and blotted dry on filter paper, before being placed floating on top the distilled water. The leaves were left for 4-7 days or until they showed visible lesions. The leaf lesions were plated by punching circular holes in the leaves or by cutting out small pieces with a scalpel and directly placing the pieces onto selective V8-PARPH media (Jeffers and Martin, 1986), which is a media based on V8 vegetable juice amended with chemicals, making it *Phytophthora* and *Pythium* specific (Appendix 2).

Tissue samples
After tissue samples were kept in distilled water, they were blotted on filter paper prior to dissection and plating on media. A clean face of the tissue surface was cut with a sterile scalpel. Small pieces of approximately 3x3x5 mm were then dissected from the cut surface and plated directly on selective V8-PARPH agar.

Subculturing of colonies
Colonies that emerged within 3-7 days from plating of baited leaves or phloem tissue were transferred onto Potato Dextrose Agar (PDA). Pure isolates were achieved by replating cultures on PDA several times by hyphal tipping.

Freeze drying of pure isolates
After achieving pure cultures, three agar plugs were transferred from cultured plate to 50 mL falcon tubes containing 30 mL of liquid malt extract media (Oxoid, UK). The cultures were stored in darkness at room temperature for two weeks. Afterwards, cultures were filtered through a büchner funnel covered by a filter paper with connected suction created by water flow, resulting in the waste fluid ending up in an Erlenmeyer flask. The mycelium was rinsed thrice with 300 mL distilled sterile water. The mycelium were transferred into 1,5 mL micro centrifuge tubes covered by perforated parafilm. The isolates were then freeze dried in a Coolsafe freezedryer (Scanvac, Denmark) for 48 h at a temperature of -110 C° and a pressure of 7 Pa. The freeze-dried mycelia were stored at - 20 °C until further processing. The samples were grinded to a fine powder. Two small glass beads were inserted into the sample tubes and run on a Fast Prep-24 machine (MP-biochemicals, USA) at 5000 rpm for 30 s.

DNA-extraction
DNA was extracted from the pure isolates by using the E.Z.N.A. SP Plant DNA Kit (Omega Bio-Tek, USA). The protocol followed manufacturer's instructions with some exceptions: using 200 µL of SP1 buffer instead of 400 µL. The final volume was set to 50 µL of elution buffer. Eluted samples containing extracted DNA were measured using Nano drop NP-1000 spectrophotometer (Saveen Werner, Sweden) and subsequently diluted to a final concentration of 2 ng/µL using the same elution buffer from the corresponding kits.

PCR amplification
DNA was amplified using polymerase chain reaction (PCR). For the PCR protocol, the total reaction volume was 50 µL. The reagent concentrations were 1x Deam Taq PCR buffer, 1,5 µM $MgCl_2$. 0,2 µM dNTP solution, 0,4 µM ITS4 primer, 0,4 µM ITS6 primer, 0,02 Units Taq DNA polymerase, 0,06 ng/µL template DNA and added up with sterile dH_2O. The positive control was 2 ng/µL of extracted DNA from *Phytophthora*

cambivora reference isolate. The protocol for PCR amplification was set to an initial step of denaturation for 3 min at 94 °C, followed by 35 cycles of denaturation for 1 min at 94 °C, annealing for 1 min at 55 °C, extension for 1 min at 72 °C and a final extension step of 10 min at 72 °C. The PCR product was mixed with loading dye on a piece of parafilm, and run on 1,5 % agarose gel (Saveen Werner, Sweden), together with a GeneRuler 100 bp DNA ladder (Thermo fisher, USA).

Gel electrophoresis

The gel for the gel electrophoresis was made by making 1 X Tris-acetate-EDTA (TAE) buffer from a 50 X stock solution and adding 1,5 % agarose (Saveen Werner, Sweden) together with 1 µL Gel Red (Biotium, USA) per 10 mL of 1 X TAE buffer. The Gel was heated in a microwave (Husqvarna, micronett, Sweden) until the boiling point. The gel was left to cool to 50 °C before pouring and any remaining gel was stored in a 60 °C incubator for later use. The gel was run in electrophoresis chambers of various sizes (Fischer Scientific, USA). The voltage and run-time was depending on the size of the gel, approximately 160 V for 40 minutes for a large gel or 100 V for 20 minutes for a smaller gel. The DNA bands were visualized using a UV machine (UVP, BioDoc-It Imaging system, UK) with a mounted camera (Computar, Japan).

PCR cleaning and sequencing

The PCR products were cleaned using a kit called USB ExoSAP-IT PCR Product Cleanup (Affymetrix USB, USA). The procedure was performed according to the manufacturer's instructions by adding 2 µL of ExoSAP product to every 5 µL of PCR product volume. The cleaned product was then measured using a Qubit Fluorometer (Life Technologies, USA) and a Qubit dsDNA high sensitivity (HS) Assay Kit (Life Technologies, USA). The final product was divided into two replicates for each isolate sample. The final PCR product was adjusted to 20 ng, together with forward and reverse primer to a final concentration of 4 mM, dilutions were made with sterile water. The samples were sent to Uppsala Genome Center – SciLife Labs in Uppsala enclosed in dry ice for Sanger sequencing.

DNA extraction and sequencing of environmental (tissue and soil) samples

Preparation of environmental samples

Two replicates were made for each soil and tissue sample that was collected from each tree in the field. For each replicate, 1 mL of tissue or soil was added to a 2 mL screw cap tube (Sarstedt, Germany), together with three small glass beads for the soil samples and two small metal nuts for the tissue samples. The soil and tissue samples were freeze dried for the purpose of excluding water for DNA extraction process and making the samples easy to pulverize with a Fast prep machine. The samples were put into a well-ventilated carton box covered with perforated Parafilm. Thereafter, the samples were freeze dried in a Coolsafe freezedryer (Scanvac, Denmark) for 48 h at a temperature of -110 C° and a pressure of 7 Pa. The freeze died samples were stored at - 20 °C until processed in a Fast Prep-24 (MP-biochemicals, USA) at 5000 rpm for 30 s, creating a fine powder.

DNA-extraction

The DNA extractions were performed by using two different kits, one kit was specific for soil samples and another for plant and/or fungal DNA extraction. For the soil samples a kit called "DNA, RNA, and protein purification" (Macherey-Nagel, Germany) was used according to manufacturer's instructions. For the tissue samples

the kit called E.Z.N.A. SP Plant DNA Kit (Omega Bio-Tek, USA) was used according to the kit's protocol. The final volume was set to 50 µL of elution buffer. Eluted samples containing extracted DNA were measured using a Nano drop NP-1000 spectrophotometer (Saveen Werner, Sweden) and subsequently diluted to a final concentration of 2 ng/µL using elution buffer from the corresponding kits.

PCR amplification

DNA from soil and tissue samples were amplified using PCR with barcoded primers. Three technical replicates were produced for each sample. For the PCR protocol the total volume was 50 µL. The reagent concentrations were 1x Deam Taq PCR buffer, 1,5 µM $MgCl_2$. 0,2 µM dNTP solution, 0,3 µM ITS4 barcoded primer, 0,4 µM ITS6 primer, 00,2 Units Taq DNA polymerase, 0,06 ng/µL template DNA and added up with sterile dH_2O. The positive control were 2 ng/µL of extracted DNA from *P. cambivora*. The protocol for PCR amplification were set to an initial step of denaturation for 3 min at 94 °C, followed by 35 cycles of denaturation for 1 min at 94 °C, annealing for 1 min at 55 °C, extension for 1 min at 72 °C and a final extension step of 10 min at 72 °C. The first technical replicate for each sample were mixed with loading dye on a piece of parafilm, run on a 1,5 % agarose (Saveen Werner, Sweden) gel.

PCR cleaning

Each PCR product that produced a band on the agarose gel was cleaned using the USB ExoSAP-IT PCR Product Cleanup kit (Affymetrix USB, USA). The procedure was performed according to the protocol of the kit, adding 2 µL of ExoSAP to every 5 µL of PCR product volume. The concentrations of the cleaned product was then measured using a Qubit Fluorometer (Life Technologies, USA) with the Qubit dsDNA HS Assay Kit (Life Technologies, USA).

Pooling of samples, final cleaning and sequencing

The PCR products were pooled together in an equi-molar mix by adding a sample volume that gave a final amount of 75 ng of DNA for each sample. The pooled samples had a final amount of 300 ng of DNA and a total volume of 1,2 mL. The pooled sample was subject to a final cleaning using the E.Z.N.A. Cycle Pure Kit (Omega Bio-Tek, USA). Three subsamples were prepared, each by cleaning 100 µL of pooled sample according the manufacturer's instructions. The final volume was fixed to 50 µL of elution buffer for each subsample. The final pooled sample was sent to Eurofins Genomics in Germany, for 454 sequencing.

Data analysis

Identification of sequences for isolates.

Sequences for individual samples were aligned and manually edited using the Lasergene software package SeqMan (version 5.07, DNAStar, Madison, WI, USA). The Basic Local Alignment Search Tool (BLAST) was used to find regions of local similarity between sequenced samples and that in reference databases at the National Center for Biotechnology Information (NCBI) website.

Identification of sequences for tissue and soil samples

Data derived from 454-amplicon sequencing was processed using the QIMME bioinformatics pipeline SCATA. Quality filtering removed sequences considered too short (<200 base pairs), with low mean read quality of <20, and those missing either primers. Sequences that passed the quality control thresholds were clustered into

operational taxonomic units (OTUs), which were considered to be taxonomically distinct at 2% dissimilarity. OTUs were identified by comparing them with reference sequence databases: Phytophthora-ID (Grunwald et al., 2011), UNITE (Abarenkov et al., 2010), and by alignment with BLASTN tool at Genbank (NCBI website).

3. Results

Inventory observations
Most of the sampled trees were those previously noted in the 2012 inventory. Six of 22 trees were not previously noted (1, 2, 3, 5, 13 and 20) (Figure 3). Most trees had bleeding lesions, though some appeared more fresh than others, of which tree number 2, 7 and 13 had the freshest lesions. Six trees (no. 10, 11, 12, 14, 17 and 21) out of the 22 sampled trees were cleaved or split in half by wind at the time of sampling, and one tree (no. 11) was removed by park rangers shortly after the time of sampling due to safety reasons.

Plated soil and tissue samples
Seventeen isolates grew from direct tissue plating on selective media, from trees 2, 8 and 13. Of the 17 isolates, two different morphotypes could be distinguished (see figure 8). All of the isolated cultures from tissue samples produced bands of the gel following PCR amplification with primers ITS 4 and ITS6, as seen in figure 9.

Two isolates (from tree 4 and 5) were obtained from soil baiting. The colonies that grew were very thin and transparent, and did not produce enough biomass for DNA extraction.

The result from the Sanger sequencing confirmed *Phytophthora spp*. in all isolates. Table 1 shows the isolates present at trees 2 and 8, which were identified as *P. cambivora*, and isolates from tree 13 were identified as *P. plurivora*.

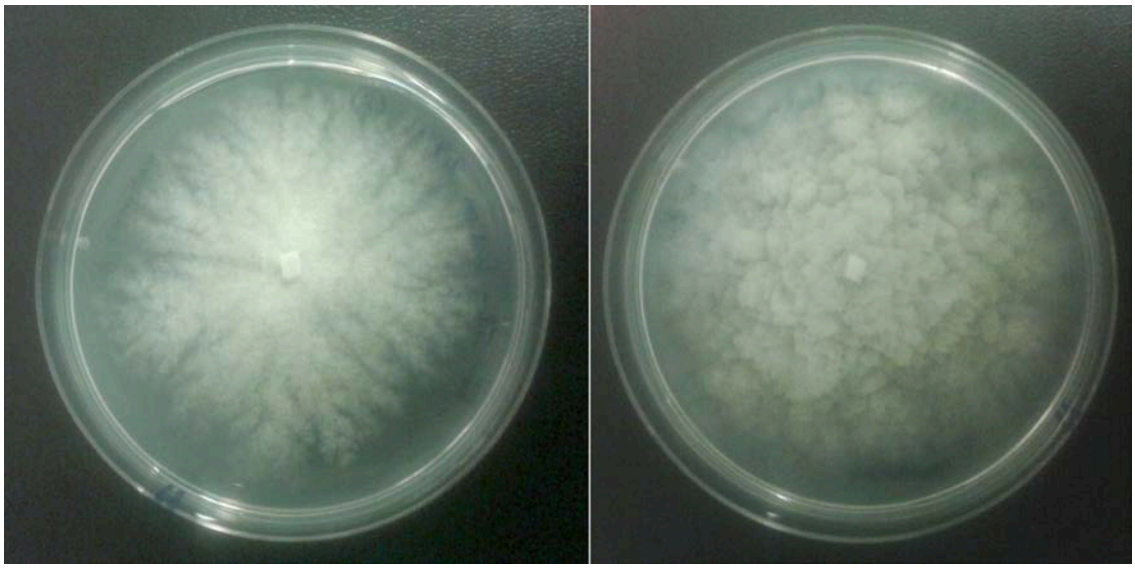

Figure 8. Representative morphologies of isolated colonies plated on PDA. Left: *P. cambivora.* Right: *P. plurivora*, (Adriaan van Tour, 2015).

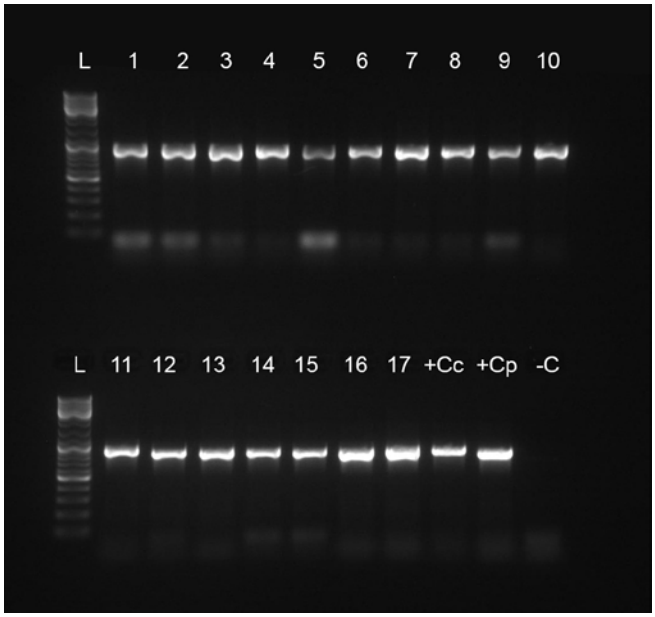

Figure 9. Gel showing PCR products from DNA extraction of isolates. L indicates the 100 bp ladder, +Cc the positive control of *P. cambivora*, +Cp the positive control of *P. plurivora*, -C negative control, numbers 1-7 indicate amplified DNA from tree 2, numbers 8-13 indicate amplified DNA from tree 8, numbers 14-17 indicate amplified DNA from tree 13.

Table 1. Species identification of isolates, based on sequenced DNA; values from BLAST.

Isolate no.	*Tree No.*	*Species name*	*Sequence length*[a]	*Closest GenBank accession No.*[b]	*Max identity [%]*[c]	*Type of sample*
1	*2*	*P. cambivora*	*459*	*KU053267.1*	*100*	*Tissue*
2	*2*	*P. cambivora*	*475*	*KU053271.1*	*99*	*Tissue*
3	*2*	*P. cambivora*	*444*	*KU053271.1*	*100*	*Tissue*
4	*2*	*P. cambivora*	*444*	*KU053271.1*	*100*	*Tissue*
5	*2*	*P. cambivora*	*444*	*KU053271.1*	*100*	*Tissue*
6	*2*	*P. cambivora*	*444*	*KU053271.1*	*100*	*Tissue*
7	*2*	*P. cambivora*	*444*	*KU053271.1*	*100*	*Tissue*
8	*8*	*P. cambivora*	*440*	*KU053270.1*	*100*	*Tissue*
9	*8*	*P. cambivora*	*444*	*KU053270.1*	*99*	*Tissue*
10	*8*	*P. cambivora*	*444*	*KU053270.1*	*100*	*Tissue*
11	*8*	*P. cambivora*	*431*	*KU053270.1*	*100*	*Tissue*
12	*8*	*P. cambivora*	*444*	*KU053270.1*	*100*	*Tissue*
13	*8*	*P. cambivora*	*444*	*KU053270.1*	*99*	*Tissue*
14	*13*	*P. plurivora*	*747*	*KU310937.1*	*100*	*Tissue*
15	*13*	*P. plurivora*	*685*	*KU682581.1*	*99*	*Tissue*
16	*13*	*P. plurivora*	*756*	*KU682581.1*	*100*	*Tissue*
17	*13*	*P. plurivora*	*749*	*KU682581.1*	*100*	*Tissue*

[a] Length of the sequence after the edges was trimmed.

[b] GenBank is database containing all publicly available DNA sequences; it can be accessed via www.ncbi.nlm.nih.gov.

[c] Max identity defines the percentage of identical nucleotides between length of the query and the matching sequence.

Environmental sequencing of tissue samples

Forty-four tissue samples and 44 soil samples were prepped for next generation sequencing. However, not all collected samples produced PCR products, as shown in figure 10. Of the 88 samples, 78 produced enough amplified DNA with concentrations high enough to be sent for sequencing. Of these samples, 66 were successfully sequenced. *Phytophthora* was detected in environmental samples of 3 tissue and 2 soil samples at trees 2, 11, 13, 14 and 19.

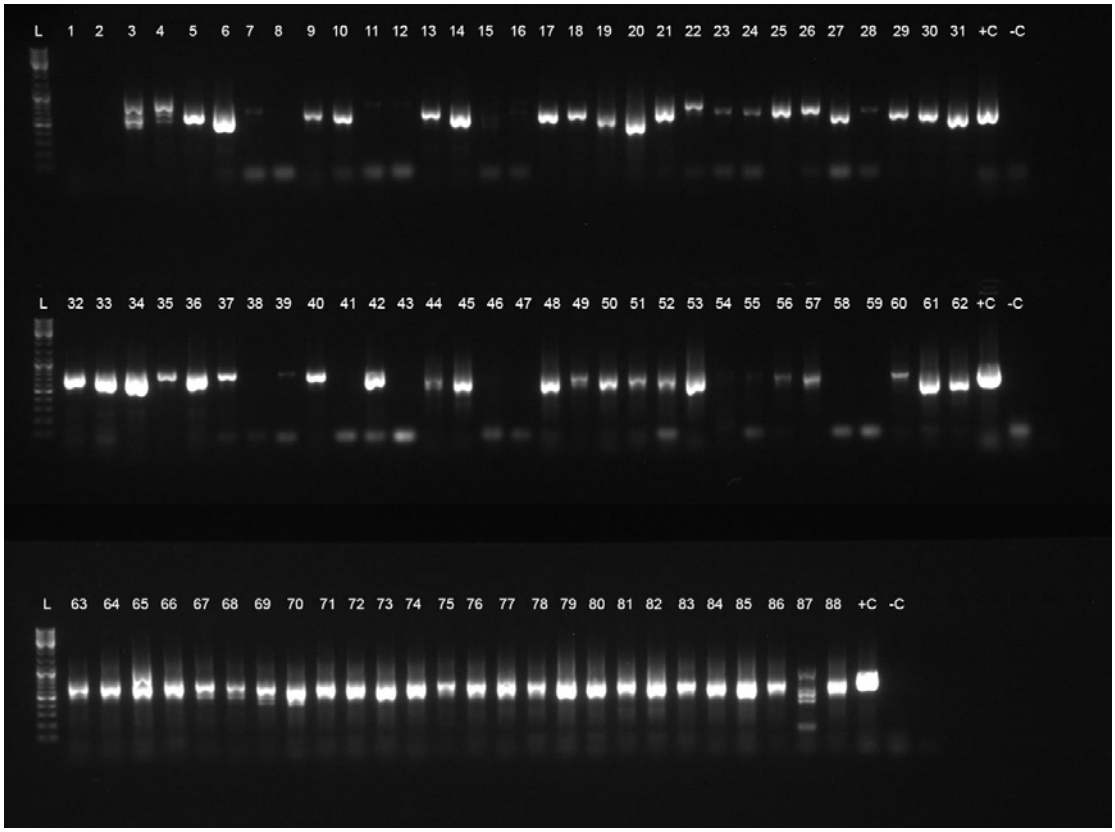

Figure 10. Gel shows PCR products of extracted DNA from tissue and soil samples for environmental sequencing. L indicates the 100 bp ladder, +C the positive control of *P. cambivora*, -C the negative control, numbers 1-44 indicates lesion samples from tree 1-22 paired with replicates, numbers 45-88 indicates soil samples from tree 1-22 paired with replicates.

Sequenced for trees 2 and 11 were identified as *P. cambivora*. Tree 13 was identified as *P. plurivora,* which correlate to obtained isolates for that tree (Table 2). Trees 14 and 19 also were identified as being infected by *Phytophthora sp.,* but sequence quality was too low to delineate at the species level.

Geographical distribution of Phytophthora species

Symptomatic trees were found throughout Söderåsen national park (Figure 3). Out of 22 sampled trees 6 trees were confirmed as being infected by *Phytophthora*. As seen in the distribution map of the indicated *Phytophthora* species (Figure 11), the most widespread species that was detected is *P. cambivora*, located at opposite ends of the park. *P. plurivora* was found in the northern part of the park. *Phytophthora sp.*, which was of too low sequencing quality for definite species identification, were found in both the southern and northern parts of the park. *P. plurivora*, *P. cambivora* and *Phytophthora sp*. were all found at Kopparhatten (Figure 4, d), where 4 out of 6 confirmed trees were situated.

Table 2. Species classification of operational taxonomic units OTU:s occurring at tree no.; values from BLAST check.

Tree No.	*OTU No.*	*Species name*	*Sequence length*[d]	*Relative total abundance [%]* [e]	*Phytophthora database No.*[f]	*Closest GenBank accession No.*[g]	*Max identity [%]*[h]	*Type of sample*
2	*OTU572*	*P. cambivora*	*408*	*2*	*PD_00691_ITS*	*AF087479.1*	*99*	*Tissue*
11	*OTU572*	*P. cambivora*	*408*	*2*	*PD_00691_ITS*	*AF087479.1*	*99*	*Tissue*
14	*OTU602*	*Phytophthora sp.*	*545*	*0*	*PD_00095_ITS*	*Yet to be submitted*	*90*	*Soil*
19	*OTU602*	*Phytophthora sp.*	*545*	*0*	*PD_00095_ITS*	*Yet to be submitted*	*90*	*Soil*
13	*OTU621*	*P. plurivora*	*439*	*1*	*PD_00446_ITS*	*KC602469.1*	*98*	*Tissue*
13	*OTU921*	*P. plurivora*	*533*	*1*	*PD_01136_ITS*	*KC602469.1*	*99*	*Tissue*

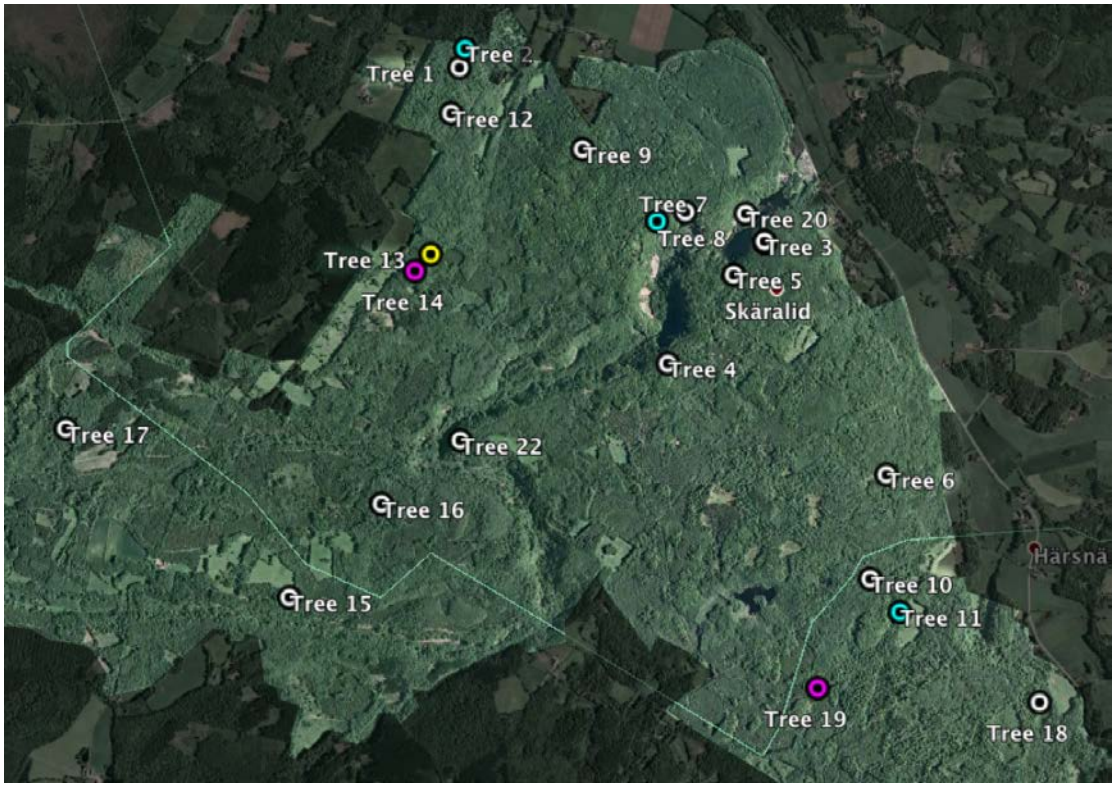

Figure 11. Sampled trees indicated in white with confirmed cases of *Phytophthora* indicated in yellow, teal and magenta. Yellow: *P. plurivora and P. cambivora*, Teal: *P. cambivora* and Magenta *Phytophthora sp.* Map made in Google Earth, with information from Swedish Lantmäteriet.

d Length of the sequence after the edges was trimmed.

e Relative total abundance indicates the percentage of DNA of interest in comparison the total amount of DNA found in the specific sample.

f *Phytophthora* database No. contains collected sequences of *Phytophthora spp.* www.phytophthoradb.org of which not all are added to GenBank.

g GenBank is database containing all publicly available DNA sequences; it can be accessed via www.ncbi.nlm.nih.gov.

h Max identity defines the percentage of identical nucleotides between length of the query and the matching sequence.

4. Discussion

In this study, I examined the occurrence and species composition of *Phytophthora* at Söderåsen national park. The discussion aims at providing some explanation to the results, some possible management strategies for containing and minimizing the spread to and within Söderåsen, as well as, the possible implication for Söderåsen in regards to the aims and goals of nature conservation in its management plan (Naturvårdsverket, 2001).

Results of isolates and environmental sequencing

Results showed two species of *Phytophthora* predominant in Söderåsen, *P. cambivora* and *P. plurivora.* These two species have proven to be especially hazardous to beech. The tree growth, nutrient uptake and pigment content of the leaves is significantly decreased by these two species of *Phytophthora*. *P. cambivora and P. plurivora* can cause up to 50 % loss of root biomass, as well as significant loss of above ground biomass. Trees afflicted by *P. cambivora* and *P. plurivora* also show a significant reduction in N and P uptake infection, as well as a reduction in plastid pigments, which in turn will decrease the efficiency of carbon fixation, also leading to decreased biomass (Fleischmann et al., 2004).

Out of 22 sampled trees, five showed positive results. The low number of successful isolates could be explained partly by the sampling relying on the previous inventory. Two out of three trees that showed positive results were sampled from trees that were not previously noted in 2012. Most of the trees included in this report are trees present in previous inventory (Figure 3). Many of the trees already had advanced symptoms when revisited, which has also predisposed these trees to infection by other agents thereby reducing the likelihood of isolating only *Phytophthora* (Drenth and Sendall, 2001, Jung, 2009).

It would be expected that the results of the environmental sequencing would show positive results even for samples that otherwise would not produce any living *Phytophthora* from tissue plating. However, the results of this report, do not show any significant difference between the two methods. Both tree no. 2 and 13 show positive results on both Sanger sequencing and environmental sequencing. Tree 8 only showed positive results for tissue plating. Tree no. 11, 14 and 19 only showed positive results from the environmental sequencing. Environmental sequencing is able to detect lower concentrations of *Phytophthora* than direct plating from active tissue (O'Brien et al., 2009). This is because there still can be intact DNA from *Phytophthora* present even after the pathogen loses viability. Since *Phytophthora* is a primary pathogen, other organisms will follow, degrading any remaining *Phytophthora*. The sampled trees were of different age, some lesions were more fresh than others. Taking the freshness of the lesions in consideration, it could be one explanation for the low detection of *Phytophthora* in samples taken from symptomatic trees. For future tissue sampling, it is important that the lesions on the trees are fresh, preferably performed at the same time or within a narrow timeframe of inventory. Despite the small number of trees positively identified as *Phytophthora* it can be inferred that most of the trees showing symptoms actually are infected by *Phytophthora* at Söderåsen, and the symptoms are till a strong indicative of the pathogen (Jung et al., 2005).

The lack of positive results from soil baiting could be a case of bad timing, since the process demands plating the leaf lesions before other microbial species inhibit the growth of *Phytophthora*. The process of soil baiting has a tendency to give false negatives due to its time-consuming nature and optimal timing is needed for successful isolation due to competing microorganisms (O'Brien et al., 2009).

Geographical distribution of Phytophthora within Söderåsen.
Previous studies show a correlation between public access areas and streams and *Phytophthora* incidence (Peterson et al., 2014). Söderåsen has many hiking trails through the woods and a stream running through a ravine, as well as wetlands in the centre of the park. Most of the symptomatic trees were located in close proximity to, or directly adjacent to, roads. This observation suggests, humans as a probable pathway for spread of the disease within Söderåsen. Vehicles are also generally seen as one of the common pathways for *Phytophthora* within a forest. The first inventory indicated many symptomatic trees around the serpentine road (Figure 4, b), where a lot of traffic occurs. As for the distribution of *Phytophthora* within the park, the highest frequency of symptomatic trees was at Kopparhatten (Figure 4, c), while symptomatic trees were far more dispersed at other parts of the parks (Figure 4). One explanation for this spatial pattern of infected trees, with a fairly wide distribution of *Phytophthora* within the park except for the central parts of the park, could be due to the logging of trees in the central parts around serpentine road (Figure 4, b), mainly due to safety reasons. In the western part of the park, only three trees were sampled of which, all has old lesions. The western part of the park has far less visitors that other parts of the park, such as Kopparhatten and Lierna. These circumstances could help to explain the pattern seen in Figure 11.

Introduction of *Phytophthora* to Söderåsen could be a result of multiple pathways. The most common pathway of introduction for *Phytophthora spp*. via infected nursery stocks (Jung et al., 2016, Schoebel et al., 2014). While the trees standing in and around the ravine are mostly naturally germinated, there are other areas within Söderåsen that have been planted from nursery stocks. However, other possible infection pathways would be hikers, bringing spores on the soil on their boots and on vehicles tires (Jung et al., 2016, Ribeiro, 2014). The stream that runs through Söderåsen has its water sources at two points, one at Traneröds mosse northwest of the park, and from Bokholmens mosse and Svatensjö southwest of the park (Naturvårdsverket, 2001). Areas around these water sources consist mostly of planted spruce forests. *Phytophthora* is known to efficiently spread via waterways (Peterson et al., 2014). If the forests around these water sources would be planted with plants from infected nursery stocks, this could also be a possible pathway of introduction to Söderåsen. Plants that would normally not show any symptoms of *Phytophthora* could carry the pathogen if the pathogen is present at the nurseries stocks. Studies have shown that *Phytophthora spp*. are ubiquitously present at most nurseries around Europe and even coniferous nursery stocks have shown positive signs of *P. plurivora* among other *Phytophthora spp*. (Jung et al., 2016). This could be subject for future investigation at Söderåsen, testing the occurrence of *Phytophthora* in the streams Dejebäcken and Kvärkbäcken, which conflux into Skärån that runs through the ravine.

Management of diseased areas
Since *Phytophthora* is an aggressive invasive pathogen, it is of interest to know which species are present in the park and in which areas they are present. *Phytophthora* has been known at Söderåsen since at least 2011. There has been a restrictive approach

towards management and pathogen control for *Phytophthora* at Söderåsen, mainly due to a lack of information about the spread and possible long-term implications of the pathogen to the ecosystem. However, with the occurrence known it would be of interest to implement management strategies to limit the negative effect of *Phytophthora* to the natural biodiversity. In the following section, I present some possible management strategies, some easier implemented than due to costs, resources, etc. An important aspect to be taken into account when discussing management strategies at Söderåsen is its size. Management strategies that would otherwise be easier in a smaller, urban park for example monitoring a smaller area or application of phosphite to a limited number of trees, can become financially questionable for a larger area such as Söderåsen.

Continuous monitoring of Phytophthora at Söderåsen

Phytophthora is a pathogen that spreads via soil and waterways. To deal with this pathogen in an effective way it is important to know where *Phytophthora* is present. There was an initial survey done 2012, and this report adds five new trees to the old survey, however a lot of the trees from the old survey have since died, been removed or no longer show any visible symptoms. Further mapping of known trees and inventory would be of interest to further investigate the spatial dynamics of the disease. For this reason, it is important to make continuous surveys, tentatively on a yearly basis (Jung et al., 2016, Ribeiro, 2014). Another possible resource that could be inquired is to use citizen monitoring, where tourists and the public report on suspected *Phytophthora* findings within the forest. It has been shown that citizen monitoring also creates more awareness among the public for issues regarding disease spread and invasive species (Fernandez-Gimenez et al., 2008).

Removal of diseased material to minimize spread

Phytophthora is very difficult to eradicate once established. Minimizing the spread of *Phytophthora* could be achieved by establishing general procedures when handling infected material. Ribeiro (2014) suggests several management procedures that could minimize the spread within a contained management area: among them, removing trees around confirmed diseased trees to create a “buffer zone”, avoid dragging infected material or material that has grown in close proximity of infected trees. Other procedural strategies is to regularly wash tools and avoid contaminating non-infected areas by washing boots and vehicle tires (Hansen, 2015).

Nursery stocks

It has been shown that the primary spread of *P. plurivora*, as well as other *Phytophthora spp*. in Europe and the US is through trade of infected nursery stock material (Jung et al., 2016, Schoebel et al., 2014). One way of minimizing the spread via nursery stocks is to regenerate new forest from seeds and ensuring nursery stocks have been tested and are certified to be free from *Phytophthora* (Jung et al., 2016, Ribeiro, 2014).

Quarantine zones

One way of containing *Phytophthora* is by implementing quarantine zones, mainly to isolate disease free areas and newly introduced stocks of beech. This has been implemented in various places, for example both in Oregon (Grunwald et al., 2012), and in Australia (Jung et al., 2011), where the devastation has been extensive due to *P. ramorum* and *P. cinnamomi* respectively. Quarantine zones as a management solution is mainly used at commercial sites in Oregon and Australia, the solution would be difficult to implement due to the importance of the park as a tourist attraction. A formal quarantine of newly established areas of beech could be difficult to implement, however, a continuous monitoring of newly planted areas should be of interest to detect indication of *Phytophthora* in introduced plants from nursery stocks (Ribeiro, 2014).

Selection of planting stock

When planting new trees within Söderåsen it is of interest to plant beech trees with broad genetic diversity to ensure better resistance towards *Phytophthora*. According to Jules et al. (2014), increased host heterogeneity slows the rate in which a pathogen advances through a forest; this heterogeneity could also be through planting different tree species (Jules et al., 2014). For Söderåsen, birch (*Betula spp.*), alder (*Alnus spp.*), aspen (*Populus tremula*), linden (*Tilia cordata*) and oak (*Quercus spp.*) are options to consider (Naturvårdsverket, 2001). In time, with additional research, it might be possible to find beech trees, which are more resilient towards infection by *Phytophthora*.

Phosphite treatment

It has been shown that inorganic phosphite has an inhibitory effect on *P. plurivora* attacking seedlings of beech. Inorganic phosphite has two means of protecting the trees against pathogens, partly by inducing host defence enzymes, and partly through direct inhibition of growth of *Phytophthora*. The induced host defence occurs mainly at low phosphite concentrations, while pathogen control occurs at high phosphite concentrations (Dalio et al., 2014).

Phosphite can be applied in various ways. Soil drenching and foliar application has shown to be less effective than more local treatments. In urban areas, injections of phosphite has shown to be effective at inhibiting growth of *P. ramorum* growth on coast live oaks (*Quercus agrifolia),* giving the trees a longer lifespan. Superficial aerial applications have been shown to give very short-term effects while injection of phosphite is thought to have effect for months. The injections as performed by Garbelotto et al. (2007) was applied by several injections around the trunk of 10 mL each, at an interval distance of 15 cm, with a depth of injection entering the outer xylem of the trees. The injections should be done during dry and warm days to increase the translocation of the phosphite.

From a park management perspective, making large-scale applications of phosphite could turn out to be both expensive and impractical. However, making more specific applications, treating specific trees might be a more cost efficient approach. This would require frequent inventory of symptomatic trees within Söderåsen, preferably performed during late spring early summer. Also, the treatments would have to be closely monitored to investigate the responsiveness of the trees, evaluating to ensure successful treatment. The aim of a phosphite treatment would be to slow down the spread of *Phytophthora* and to give individual trees a better opportunity to

compartmentalize the infection subsequently giving the trees a longer lifespan (Jung, 2009), and hopefully creating habitats for endangered species to create a more sustainable nature conservation.

Impact of *Phytophthora* at Söderåsen

Söderåsen is a key location for Swedish nature conservation, it is an ecological hotspot in Scandinavian standards, with many red listed species of mosses and lichens (Naturvårdsverket, 2001). The area is also suggested to be one of Sweden's most species rich areas in regards to soil- and wood-living fungi with a rough estimate of 400 observed species. About 60 red listed insect species are currently found at Söderåsen, of which most are tied to old trees and dead wood (Naturvårdsverket, 2001). The long-term impact of a widespread *Phytophthora* infestation throughout the park is difficult to assess. It could have implications to the age composition of the beech forest, possibly leading to a situation where most beech trees not being able to reach their full potential lifespan. The age of the trees could in turn affect many of the red listed species that are dependent of old beech trees for their survival.

Biodiversity aspect

The primary focus of the management of Söderåsen is biodiversity and creating a sustainable environment for flora and fauna within the park. There is a lot of ecological value connected to old trees, usually trees at an age older than 200 years. There is concern whether *Phytophthora* infections have negative effect in regard to these older trees with a high ecological value (Jules et al., 2014).

One approach that has been discussed by the park is implementing veteranization. The concept of veteranization is based upon creating habitats within a tree that has not yet achieved the natural age for these ecological values. The approach is therefore to inflict artificial damage to the trees to, for instance, create cavities within the tree for wood boring insects to exploit. The last 300 years most of Söderåsen has been subject to forestry, except for the areas in and around the ravine. Large parts of the forest therefore consist of even aged stands. In this aspect, *Phytophthora* could in the long run lead to stands that are more variable in age, and potentially creating new niches for new fauna. An important aspect of how a forest reacts towards an invasive pathogen, is the rate in which the destruction may occur (Hansen, 1999). Within Söderåsen, the infected trees are quite dispersed. There are not a lot of infected trees standing in close proximity to each other, a pattern that is more commonly seen at urban park areas such as Pildamsparken in Malmö or Slottskogen in Gothenburg. *Phytophthora* could lead to a natural form of veteranization, creating habitat for insects to colonize and in the long run also for birds and smaller mammals.

Conclusions

This thesis gives some insight in the nature of *Phytophthora*, the situation worldwide and locally, a confirmation of which species are present at Söderåsen, a broad picture of its distribution and some suggestions of possible management strategies to minimize the negative effects of *Phytophthora* at Söderåsen.

Some things are known about the *Phytophthora* infection at Söderåsen.

- *Phytophthora* is an invasive species, that cause mortality to woody plant species.
- The species *P. plurivora* and *P. cambivora* has been confirmed present at Söderåsen, with a wide distribution throughout the park.
- *P. plurivora* and *P. cambivora* has proven especially hazardous to beech forests in other parts of Europe.
- It is virtually impossible to remove *Phytophthora* from soils when established.

These points of knowledge are important when discussing future management to actively minimize the negative effects of *Phytophthora* at Söderåsen. The main goal of Söderåsen National Park is nature conservation, recreating natural environments within the park, creating and upholding valuable habitats for endangered species (Naturvårdsverket, 2001).

There is two main strategies of management at Söderåsen in the light of the above stated facts: either to let nature take its course, or to actively try to limit the negative effects of the pathogen. The previous approach has been not to actively interfere with the situation due to lack of knowledge of species composition and spread. Knowing that *Phytophthora* has a destructive effect towards beech (Jung et al., 2005), but without knowledge of how it will affect the habitats for endangered species within Söderåsen, it would be advisable to take active measures to limit additional spread of the pathogen within Söderåsen.

In this thesis some suggestions, to possible management strategies to mitigate the threat of *Phytophthora* at Söderåsen, are given. Of these, the most pressing would be monitoring the trees already known to be infected, and finding new infected trees. Preferably an inventory of infected trees is performed once a year in late spring or early summer. Other measures that would be easy to implement would be routines for cleaning tools when handling infected plant material, for instance by washing gear, boots and tires between used in different areas within Söderåsen. Also avoid dragging infected material in the forest, since this could help spread *Phytophthora* uninfected areas. It could be of interest to do specific efforts to treat individual trees with phosphite injections. This would be a more costly approach, and would also demand continuous monitoring on the development of the treated trees. If such an approach would be implemented, it would be of essence to further investigate the development of the individual trees to properly evaluate the successfulness of the treatment. In the areas, which are still spruce forests, and subject to nature restoration, it is of special importance to take into consideration *Phytophthora*. When establishing new plantations, only trees from certified disease free nursery stocks should be used, and special efforts should be taken to establish a genetically heterogeneous tree population. Furthermore, it would be of interest to investigate the long-term effects of *Phytophthora* at Söderåsen, especially the interactions between the pathogen, its environment and how it will affect the red listed species that are associated to beech. It would also be of further interest to assess how Söderåsen could work together with the public and tourists to find infected trees, and to create awareness about *Phytophthora* and the risks of spread between different localities.

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Appendix I

Recipes

PAR(PH)-V8 media Selective medium for *Phytophthora* species

Table I. PAR(PH)-V8, ingredients and amounts

Basal medium ingredients	***Amount***
Vegetable juice	*50 ml*
Deionized water	*950 ml*
Agar	*15 g*
Amendments	
Rifampicin	*10 mg*
Dimethyl sulfoxide (DMSO)	*1 ml*
Sodium ampicilin	*250 mg*
Pimaricin	*5 mg*
Pentachloronitrobenzene (PCNB)	*100 mg*
Hymexazol	*50 mg*
Benomyl	*10 mg*

Procedure

1. Mix vegetable juice, deionized water and agar
2. Autoclave at 121°C for 15 minutes
3. Cool down the media to 50 °C in a water bath
4. Dissolve rifampicin in a test tube with DMSO
5. Dissolve the remaining amendments in 10 ml sterile water each and vortex until dissolved
6. Add the amendments into the 50°C medium and rinse the tubes once with sterile water
7. Mix the media slowly with a magnetic stirrer
8. Pour the media into Petri dishes
9. Let the plates cool to room temperature
10. Store the petri dishes in a refrigerator, upside down in plastic bags away from sunlight.

Potato Dextrose Agar (PDA)

Table II. PDA, ingredients and amounts

Ingredients	***Amount***
Potato dextrose agar	*39 g*
Distilled water	*1000 mL*

Procedure

1. Mix potato dextose agar with distilled water and mix thoroughly
2. Autoclave at 121°C for 15 minutes
3. Mix the agar before pouring into Petri dishes
4. Let the plates cool to room temperature
5. Store the petri dishes in a refrigerator, upside down in plastic bags away from sunlight.

Malt extract broth

Table III. Malt extract broth

Ingredients	***Amount***
Malt extract	*20 g*
Deionized water	*1000 mL*

Procedure

1. Mix the ingredients and stir thorough
2. Autoclave at 121°C for 15 minutes
3. Pour 35 ml into falcon tubes

Appendix II

Tree coordinate data

Table IV. Coordinates of collected samples and diameter at breast height (dbh) of each tree.

Tree no.	*Dbh (cm)*	*N°*	*E°*	*2012 inventory*
1	*53,5*	*56°02.659'*	*013°13.339'*	---
2	*45,5*	*56°02.717'*	*013°13.368'*	---
3	*50,5*	*56°02.147'*	*013°14.949'*	---
4	*70,4*	*56°01.786'*	*013°14.436'*	*P23*
5	*54,1*	*56°02.047'*	*013°14.783'*	---
6	*96,0*	*56°01.459'*	*013°15.591'*	*P34*
7	*46,2*	*56°02.232'*	*013°14.531'*	*P6*
8	*57,6*	*56°02.204'*	*013°14.380'*	*P4*
9	*60,0*	*56°02.416'*	*013°13.988'*	*P17-O+F*
10	*63,0*	*56°01.151'*	*013°15.509'*	*P31/P32-P*
11	*72,0*	*56°01.053'*	*013°15.666'*	*P29*
12	*76,4*	*56°02.522'*	*013°13.296'*	*P7*
13	*64,0*	*56°02.107'*	*013°13.190'*	---
14	*57,4*	*56°02.057'*	*013°13.107'*	*P18-F*
15	*81,0*	*56°01.099'*	*013°12.448'*	*P8*
16	*39,0*	*56°01.375'*	*013°12.936'*	*P36-15-32*
17	*68,5*	*56°01.594'*	*013°11.281'*	*P36*
18	*103,8*	*56°00.783'*	*013°16.413'*	*P28-26h-9*
19	*79,0*	*56°00.832'*	*013°15.224'*	*P38*
20	*48,5*	*56°02.232'*	*013°14.856'*	---
21	*73,0*	*56°02.141'*	*013°14.950'*	*P13-O*
22	*54,0*	*56°01.560'*	*013°13.344'*	*P44-O*
Mean	*64,2*			

Table V. Coordinates from previous inventory, performed by Marta Agostinelli June 2012.

Name	*N°*	*E°*
P-Plot 15-13	*56°01.518'*	*13°15.173'*
P-plot16-19	*56°01.426'*	*13°15.067'*
P01	*56°02.200'*	*13°13.212'*
P02	*56°02.279'*	*13°13.244'*
P04	*56°02.123'*	*13°14.223'*
P05	*56°02.137'*	*13°14.293'*
P06	*56°02.140'*	*13°14.312'*
P07	*56°02.313'*	*13°13.179'*
P08	*56°01.060'*	*13°12.277'*
P09	*56°01.263'*	*13°13.438'*
P10-P	*56°01.224'*	*13°14.196'*
P11-O	*56°02.329'*	*13°13.153'*
P12-O+F	*56°02.332'*	*13°13.173'*
P13-O	*56°02.081'*	*13°14.568'*
P14-O	*56°02.259'*	*13°14.040'*
P15	*56°02.167'*	*13°14.142'*
P16-O	*56°02.281'*	*13°14.445'*
P17-O+F	*56°02.251'*	*13°13.590'*
P18	*56°02.071'*	*13°14.549'*
P18-F	*56°02.032'*	*13°13.068'*
P19	*56°02.042'*	*13°14.518'*
P20-O	*56°02.019'*	*13°14.421'*
P21-Tree24	*56°01.538'*	*13°14.491'*
P22	*56°02.013'*	*13°15.112'*
P23	*56°01.466'*	*13°14.264'*
P24	*56°01.445'*	*13°14.246'*
P25	*56°01.437'*	*13°14.200'*
P26	*56°01.279'*	*13°15.352'*

Name	*N°*	*E°*
P27	*56°01.182'*	*13°15.101'*
P28-26h-9	*56°00.473'*	*13°16.252'*
P29	*56°01.027'*	*13°15.396'*
P3	*56°02.028'*	*13°14.077'*
P30	*56°01.057'*	*13°15.349'*
P31	*56°01.086'*	*13°15.300'*
P32-P	*56°02.097'*	*13°14.297'*
P33	*56°01.154'*	*13°15.444'*
P34	*56°01.274'*	*13°15.353'*
P35	*56°01.467'*	*13°15.230'*
P36	*56°01.356'*	*13°11.169'*
P36-15-32nd	*56°01.224'*	*13°12.565'*
P37	*56°01.254'*	*13°12.578'*
P38	*56°00.498'*	*13°15.136'*
P38-P-O	*56°01.268'*	*13°12.554'*
P39-O	*56°01.077'*	*13°14.600'*
P39-P	*56°01.238'*	*13°14.018'*
P40-O,felt	*56°01.252'*	*13°13.440'*
P41-N.3-46th	*56°01.234'*	*13°13.455'*
P42-N9-46.	*56°01.231'*	*13°13.453'*
P43-O	*56°01.336'*	*13°13.197'*
P44-O	*56°01.335'*	*13°13.205'*
P45-O	*56°01.387'*	*13°13.440'*
P46	*56°01.415'*	*13°14.138'*
P47	*56°01.466'*	*13°14.483'*
P48-O	*56°01.452'*	*13°14.490'*
P49-O	*56°01.461'*	*13°14.486'*
P50-O	*56°01.466'*	*13°14.488'*

Appendix III

Detailed results of environmental sequencing

Table VI. *Relative occurrence of Phytophthora spp. of soil samples, shown as percentage of sample.*

Sample name	*P. cambivora*	*P. citricola*	*P. plurivora*	*Phytophthora sp.*
1AS	*0.0 %*	*0.0 %*	*0.0 %*	*0.0 %*
1BS	*0.0 %*	*0.0 %*	*0.0 %*	*0.0 %*
3AS	*0.0 %*	*0.0 %*	*0.0 %*	*0.0 %*
3BS	*0.0 %*	*0.0 %*	*0.0 %*	*0.0 %*
4AS	*0.0 %*	*0.0 %*	*0.0 %*	*0.0 %*
5AS	*0.0 %*	*0.0 %*	*0.0 %*	*0.0 %*
5BS	*0.0 %*	*0.0 %*	*0.0 %*	*0.0 %*
7AS	*0.0 %*	*0.0 %*	*0.0 %*	*0.0 %*
7BS	*0.0 %*	*0.0 %*	*0.0 %*	*0.0 %*
9AS	*0.0 %*	*0.0 %*	*0.0 %*	*0.0 %*
9BS	*0.0 %*	*0.0 %*	*0.0 %*	*0.0 %*
10AS	*0.0 %*	*0.0 %*	*0.0 %*	*0.0 %*
10BS	*0.0 %*	*0.0 %*	*0.0 %*	*0.0 %*
11AS	*0.0 %*	*0.0 %*	*0.0 %*	*0.0 %*
11BS	*0.0 %*	*0.0 %*	*0.0 %*	*0.0 %*
12AS	*0.0 %*	*0.0 %*	*0.0 %*	*0.0 %*
12BS	*0.0 %*	*0.0 %*	*0.0 %*	*0.0 %*
13AS	*0.0 %*	*0.0 %*	*0.0 %*	*0.0 %*
13BS	*0.0 %*	*0.0 %*	*0.0 %*	*0.0 %*
14AS	*0.0 %*	*0.0 %*	*0.0 %*	*0.9 %*
14BS	*0.0 %*	*0.0 %*	*0.0 %*	*0.0 %*
15AS	*0.0 %*	*0.0 %*	*0.0 %*	*0.0 %*
15BS	*0.0 %*	*0.0 %*	*0.0 %*	*0.0 %*
16AS	*0.0 %*	*0.0 %*	*0.0 %*	*0.0 %*
16BS	*0.0 %*	*0.0 %*	*0.0 %*	*0.0 %*
17AS	*0.0 %*	*0.0 %*	*0.0 %*	*0.0 %*
17BS	*0.0 %*	*0.0 %*	*0.0 %*	*0.0 %*
18AS	*0.0 %*	*0.0 %*	*0.0 %*	*0.0 %*
18BS	*0.0 %*	*0.0 %*	*0.0 %*	*0.0 %*
19AS	*0.0 %*	*0.0 %*	*0.0 %*	*1.2 %*
19BS	*0.0 %*	*0.0 %*	*0.0 %*	*0.0 %*
20AS	*0.0 %*	*0.0 %*	*0.0 %*	*0.0 %*
20BS	*0.0 %*	*0.0 %*	*0.0 %*	*0.0 %*
21AS	*0.0 %*	*0.0 %*	*0.0 %*	*0.0 %*
21BS	*0.0 %*	*0.0 %*	*0.0 %*	*0.0 %*
22AS	*0.0 %*	*0.0 %*	*0.0 %*	*0.0 %*
22BS	*0.0 %*	*0.0 %*	*0.0 %*	*0.0 %*

Table VII. Relative occurrence of *Phytophthora spp.* of tissue samples, shown as percentage of sample.

Sample name	*P. cambivora*	*P. citricola*	*P. plurivora*	*Phytophthora sp.*
2AL	*23.8 %*	*0.0 %*	*0.0 %*	*0.0 %*
2BL	*60.6 %*	*0.0 %*	*0.0 %*	*0.0 %*
3AL	*0.0 %*	*0.0 %*	*0.0 %*	*0.0 %*
3BL	*0.0 %*	*0.0 %*	*0.0 %*	*0.0 %*
4AL	*0.0 %*	*0.0 %*	*0.0 %*	*0.0 %*
5AL	*0.0 %*	*0.0 %*	*0.0 %*	*0.0 %*
5BL	*0.0 %*	*0.0 %*	*0.0 %*	*0.0 %*
7AL	*0.0 %*	*0.0 %*	*0.0 %*	*0.0 %*
7BL	*0.0 %*	*0.0 %*	*0.0 %*	*0.0 %*
9AL	*0.0 %*	*0.0 %*	*0.0 %*	*0.0 %*
9BL	*0.0 %*	*0.0 %*	*0.0 %*	*0.0 %*
10AL	*0.0 %*	*0.0 %*	*0.0 %*	*0.0 %*
10BL	*0.0 %*	*0.0 %*	*0.0 %*	*0.0 %*
11AL	*47.7 %*	*0.0 %*	*0.0 %*	*0.0 %*
13BL	*0.0 %*	*60.5%*	*7.9 %*	*0.0 %*
14AL	*0.0 %*	*0.0 %*	*0.0 %*	*0.0 %*
14BL	*0.0 %*	*0.0 %*	*0.0 %*	*0.0 %*
15AL	*0.0 %*	*0.0 %*	*0.0 %*	*0.0 %*
15BL	*0.0 %*	*0.0 %*	*0.0 %*	*0.0 %*
16AL	*0.0 %*	*0.0 %*	*0.0 %*	*0.0 %*
16BL	*0.0 %*	*0.0 %*	*0.0 %*	*0.0 %*
17AL	*0.0 %*	*0.0 %*	*0.0 %*	*0.0 %*
17BL	*0.0 %*	*0.0 %*	*0.0 %*	*0.0 %*
18AL	*0.0 %*	*0.0 %*	*0.0 %*	*0.0 %*
18BL	*0.0 %*	*0.0 %*	*0.0 %*	*0.0 %*
19AL	*0.0 %*	*0.0 %*	*0.0 %*	*0.0 %*
19BL	*0.0 %*	*0.0 %*	*0.0 %*	*0.0 %*
21AL	*0.0 %*	*0.0 %*	*0.0 %*	*0.0 %*
22AL	*0.0 %*	*0.0 %*	*0.0 %*	*0.0 %*

Appendix IV

Schematic overview of sampling and plating of soil and tissue samples

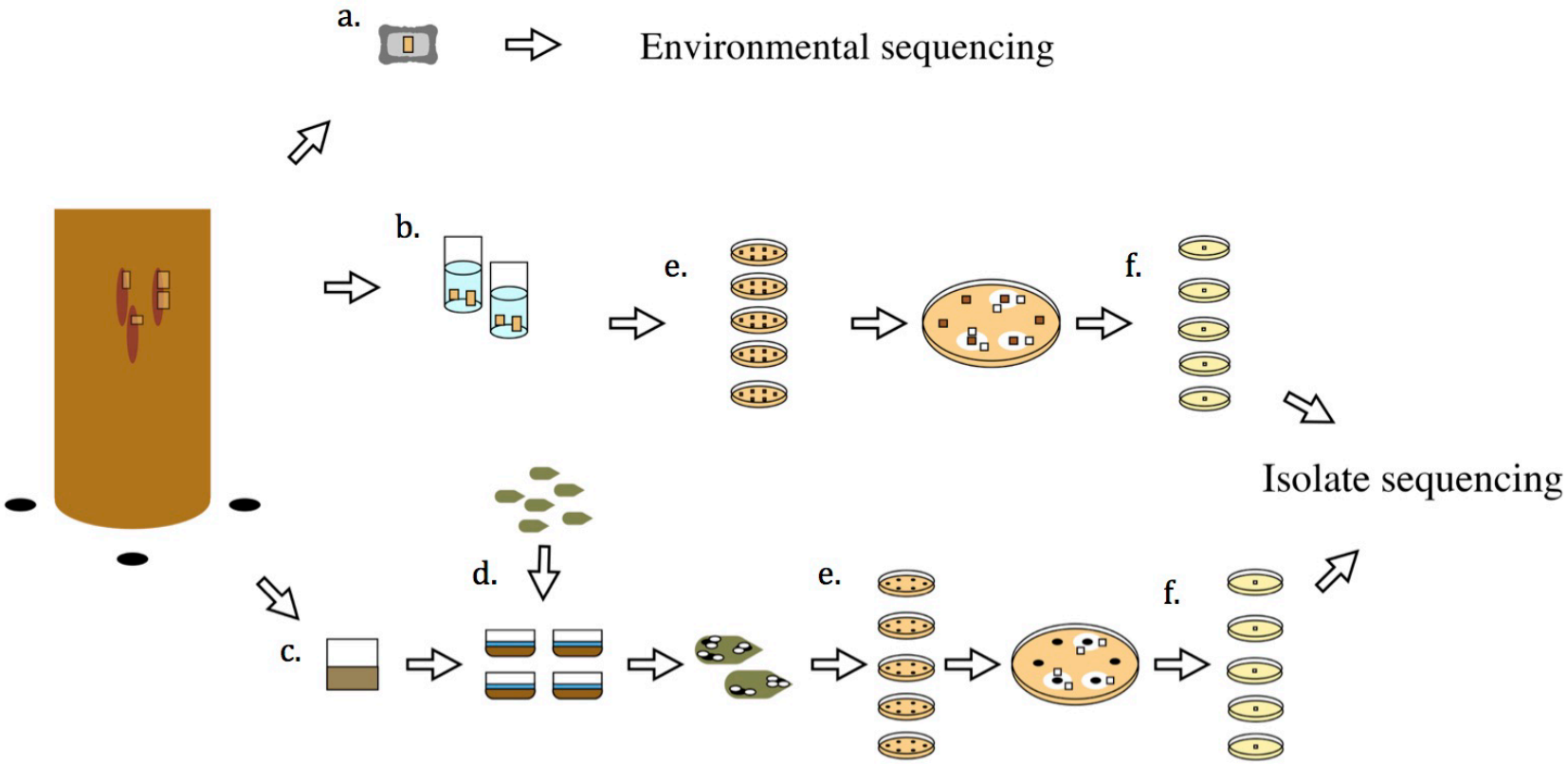

Figure I. Schematic overview of sampling and plating of soil and tissue samples. Tissue samples were taken for environmental sequencing (a) and direct plating (b) and soil samples (c) were taken for soil baiting (d) and subsequent leaf tissue plating (e). The positive results were replated (f) to get pure cultures for DNA extraction and sequencing.