Invasive *Phytophthora* species affecting broadleaved tree species in urban and landscape settings in Southern Sweden

Mimmi Blomquist

Supervisor: Michelle Cleary SLU, Southern Swedish Forest Research Centre

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Examiner: Magnus Löf SLU, Southern Swedish Forest Research Centre

Picture front cover: Michelle Cleary

Abstract

Phytophthora translates to 'plant destroyer' and are fungal-like, hemibiotrophic plant pathogens. *Phytophthora* was discovered in the mid 19th century, as the causal agent of the widespread collapse of potato crops throughout Europe. Today, more than 150 *Phytophthora* species have been identified and they continue to cause massive problems, both in agriculture and forest ecosystems. There are many *Phytophthoras* species responsible for dramatic forest diebacks. In Europe, *Phytophthoras* are casual agents of oak decline, ink disease of chestnut, beech decline and dieback of alders. The aim of this study was to examine which forest *Phytophthora* species are present in southern Sweden. Symptomatic trees were identified in five urban forest settings in southern Skåne. Stem tissue and soil surrounding symptomatic trees were collected. *Phytophthora* isolates were cultured on artificial media and their DNA sequenced by sanger-sequencing. The obtained sequences were compared with the known reference sequences using the basic local alignment search tool (BLAST) at NCBI. Five *Phytophthora* species were identified: *P. cactorum, P. cambivora, P. gonapodyides, P. plurivora* and *P. syringae*. The result includes the first findings of *P. gonapodyides* causing stem lesions on trees in Sweden. Confirmation of pathogenicity of *P. gonapodyides* in an inoculation trial using obtained *P. gonapodyides* isolates confirmed that they were able to infect healthy beech seedlings, resulting in stem lesions. Many common tree species in southern Sweden are susceptible to the *Phytophthora* species found in this study. Trade with nursery seedlings is an important pathway of dispersal and establishment of *Phytophthoras* in natural environments. There is no efficient treatment and it is practically impossible to eradicate *Phytophthora* once it has become established in a site. If the identified *Phytophthora* species become widely established in Sweden, they may cause severe damages to forest ecosystems as well as for the Swedish forestry sector.

Key words:
Fagus sylvatica, *Phytophthora*, forest, pathogen, invasive, *P. gonapodyides*, Pildammsparken, Sweden.

Sammanfattning

Phytophthora betyder "växtförgörare" på grekiska och är svampliknande, hemibiotrofa växtpatogener. Släktet upptäcktes under mitten av 1800-talet, då *Phytophthora infestans* orsakade en utbredd kollaps av potatisskörden runt om i Europa. Idag har fler än 150 arter av *Phytophthora* identifierats och de fortsätter att orsaka stora problem, både inom jordbruket och i skogsekosystem. Det finns många arter av *Phytophthora* som bidrar till hög mortalitet i skogar. I Europa är *Phytophthora* inblandad i kastanjeblödarsjukan och orsakar dessutom betydande mortalitet och vitalitetsnedsättning på framförallt ek, bok och al. Syftet med denna studie var att undersöka vilka trädlevande arter av *Phytophthora* som finns i södra Skåne. Stamvävnad från symptomatiska träd och närliggande jord insamlades och *Phytophthora* isolerades och odlades på artificiell media och dess DNA sekvenserades genom Sanger-sekvensering. De erhållna sekvenserna jämfördes med referens-sekvenser med hjälp av *basic local alignment search tool* (BLAST) vid NCBI. Fem arter av *Phytophthora* identifierades; *P. cactorum, P. cambivora, P. plurivora, P. syringae* och för första gången påvisades *P. gonapodyides* orsaka blödande stamsår på träd i Sverige. Inokulationstester bevisade att de erhållna isolaten av *P. gonapodyides* kunde återinfektera friska bokplantor, vilket resulterade i stamsår. Många vanliga trädarter i södra Sverige är mottagliga för de identifierade arterna av *Phytophthora* och smittan sprids ofta med växter som drivits upp i plantskolor runtom i Europa som planteras ut i trädgårdar och i naturen. Det finns i nuläget ingen effektiv behandling och det är i praktiken omöjligt att utplåna *Phytophthora* efter etablering i ett område. Om de identifierade arterna etablerar sig runtom i Sverige kan detta därför resultera i omfattande skogsskador.

Nyckelord:
Bok, bokskog, algsvamp, skog, patogen, invasiv, skadegörare, Sverige.

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And finally, I would like to dedicate this thesis to my father, I miss you and wish you were still here.

Mimmi Blomquist
April 2016, Alnarp

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Introduction

Phytophthoras, the plant destroyers

In 1845-1846 the accidental import of a *Phytophthora* species to Europe resulted in an epidemic that destroyed potato crops, causing mass starvation throughout the continent (Reader, 2009). It affected Ireland particularly hard since many Irish farmers couldn't afford other crops, and in only a couple of years the famine led to starvation and emigration of two of the eight million Irish inhabitants (Ribeiro & Lamour, 2013). At this time it was not clear what caused the potato crop to collapse throughout more than two million square kilometers in Europe (Reader, 2009). Almost three decades later, in 1876, Anton de Bary formally named the pathogen *Phytophthora infestans*, meaning plant destroyer, derived from the Greek word for plant φυτόν (phytón) and destruction φθορά (phthorá) (Ribeiro & Lamour, 2013). It is often claimed that de Bary initiated the science of plant pathology, when he described the life cycle of *P. infestans* and showed correlations between the life cycle and the progress of the disease (Matta, 2010).

Despite more than 130 years of intensive research, *P. infestans* continues to cause severe damages (Kamoun *et al.*, 2015). Breeding of potato for resistance has not been very successful (Haverkort *et al.*, 2008), and this has made potato one of the most chemically dependent crops (Reader, 2009). Due to reduced yields and control measures the annual economic losses on potato in Europe amounts to € 1 billion (Haverkort *et al.*, 2008). Following the formal recognition of the genus, many new species have been found. Today roughly 150 species have been described (Jung *et al.*, 2015), with the majority of those found after the year 2000 (Scott *et al.*, 2013).

Biology and life cycle of *Phytophthora*

The phylum Oomycota, belonging to the kingdom *Stramenopila* includes many plant pathogens, such as *Phytophthora*, downy mildews and white blister rusts (Oßwald *et al.*, 2014; Thines, 2014). Oomycetes are present in practically all ecosystems, but likely they evolved in marine environments and are in fact related to seaweeds and diatoms (Thines, 2014; Beakes *et al.*, 2012). Oomycetes and true fungi are similar in many ways, both groups are eukaryotic microorganisms that grow hyphae and produce decomposing enzymes, enabling osmotrophic digestion of nutrients (Richards *et al.*, 2006). However, the two groups are very distantly related, and the similarities are examples of convergent evolution (Richards *et al.*, 2006). One major difference is the cell wall construction, oomycete cell walls mainly contain cellulose and glucose polymers, whereas most fungal cell walls mainly consists of chitin (Judelson & Blanco, 2005). In contrast to fungi, oomycetes also form biflagellated zoospores, asexual spores that are motile in moist environments (Walker & van West, 2007).

Many *Phytophthora* species are important plant pathogens, able to infect many different hosts, while others are host specific (Osswald 2014; Kroon 2012). Most *Phytophthoras* are hemibiotropic,

initially parasitic to living cells and subsequently necrotrophic, living of dead tissue (Oßwald *et al.*, 2014). Since the saprophytic phase is limited, a key feature of *Phytophthora* is the ability to reach new hosts (Judelson & Blanco, 2005). This is achieved by biflagellate zoospores (Fig. 1), which are able to swim in water, or moist soils, towards healthy hosts (Judelson & Blanco, 2005; Hansen, 2015).
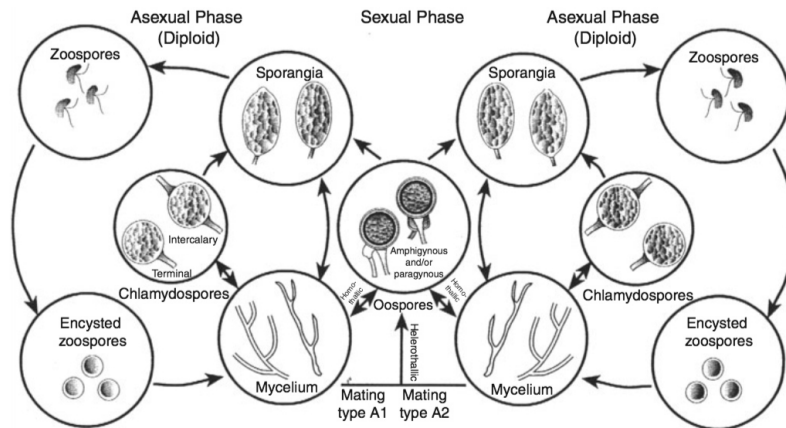

Figure 1. Characteristic life cycle for *Phytophthora* species (source: Ribeiro & Lamour, 2013)

In fact, fine root infection by zoospores are the most common way for *Phytophthora* to invade new hosts (Oßwald *et al.*, 2014). Zoospores are asexual spores, with two morphologically different flagella, which enables movement for hours or days (Judelson & Blanco, 2005). During nutrient uptake, roots secrete a number of compounds which attracts zoospores (Oßwald *et al.*, 2014; Judelson & Blanco, 2005). At the root surface the zoospores discharge the flagella and form cysts that attach to the root (Judelson & Blanco, 2005; Oßwald *et al.*, 2014). Subsequently a hypha is formed, which penetrates the plant surface and continues to grow both along and inside of the host cells (Oßwald *et al.*, 2014). Hyphae and cysts secrete a number of biologically active effector molecules, which suppress the plants defense responses and facilitate colonization (Oßwald *et al.*, 2014; Beakes *et al.*, 2012). Infection success depends on the *Phytophthora* species ability to neutralize the defense system of the particular plant species (Oßwald *et al.*, 2014).

In susceptible hosts, infection severely damages the tissue, especially phloem, e.g. by causing a significant decrease in cell turgor. The destruction of root cells drastically decreases the plants ability to absorb and transport water, which weakens, or kills the host (Oßwald *et al.*, 2014). In infected tissue, resting spores (sexually formed oospores, or asexual chlamydospores) may form (Oßwald *et al.*, 2014; Erwin & Ribeiro, 1996). These thick-walled spores cannot actively move, but unlike zoospores, they are able to survive unfavorable conditions, such as drought, for months or even years (Judelson & Blanco, 2005; Jung, 2011). During dry conditions, the resting spores eventually end up in the soil due to decomposition of infected tissue (Zobel *et al.*, 1985). However, during moist conditions both kinds of resting spores form sporangia, structures able to cause infection either by producing a hyphae that penetrates the host directly, or by producing and releasing new zoospores (Judelson & Blanco, 2005). Sporulation may occur many times each season, causing multi-cyclic infections (Oßwald *et al.*, 2014).

Forest *Phytophthoras*

Common symptoms of *Phytophthora* infection on trees

Most *Phytophthora* species are soil borne and attack hosts through fine root infections, but some species are able to aerially infect stem, needles or leaves of particular hosts, directly through lenticels or wounds (Oßwald *et al.*, 2014). Depending on the host-pathogen interaction and the condition of the host, primary infection of the roots may be limited to the root system, or spread up the trunk (Oßwald *et al.*, 2014). If it is limited to the roots, it may take several years before the root rot becomes evident through secondary symptoms such as leaf chlorosis, thinning of foliage and crown dieback. However, if the infection spreads up the stem, or if the trunk is infected aerially, the infection may lead to secretion of dark sap from the damaged bark tissue, a symptom often referred to as bleeding stem canker. Destruction of the root system limits the water uptake, which eventually reduces the photosynthetic activity, weakens the host, and may lead to death of the tree (Oßwald *et al.*, 2014).

Phytophthoras invasive to forest ecosystems worldwide

Various natural forest ecosystems host a number of *Phytophthora* species (Hansen, 2015). Usually indigenous *Phytophthora* species have co-evolved with the forest, and therefore do not threaten the ecosystem (Liebhold *et al.*, 2012). However, moving pathogens or hosts to new environments, introducing non-resistant hosts, or changes in the environment itself may alter the forest pathogen dynamics, causing previously harmless pathogens to become opportunistic and cause disease (Hansen, 2015). Some of the most devastating *Phytophthora* species, responsible for great destruction to forest ecosystems worldwide are *Phytophthora cinnamomi* and *Phytophthora ramorum*.

Phytophthora cinnamomi

Phytophthora cinnamomi is listed as one of the world's worst invaders (ISSG, 2007). For most plant species signs of infection include wilting and retention of dried foliage, which often leads to death of the plant (ISSG, 2007). This pathogen is likely of South Asian origin (Hansen, 2015), but has been spread globally for centuries through trade of ornamental plants (Zentmyer, 1988). It is limited by temperatures around 0°C and lower, but has a wide range of host species in warmer climates (Cahill *et al.*, 2008; Benson, 1982). Over 2500 host plant species are susceptible to *P. cinnamomi* in Australia alone (Hee *et al.*, 2013). It has caused great biodiversity destruction in several vegetation types, including more than 280 000 hectares of Jarrah (*Eucalyptus marginata*) forests in Australia, where it not only kills the trees, but also cause severe losses of the understory species (Boyd *et al.*, 2013; Weste, 2003). It has also caused widespread damages in the south eastern USA, where the most susceptible species, *Castanea dentata*, is no longer present (Hansen, 2015). Like most *Phytophthora* species, *P. cinnamomi* has the ability to survive for many years, even under adverse conditions by forming specialized resting spore structures in the soil. In addition, *P. cinnamomi* can survive on roots of less susceptible hosts.

Phytophthora ramorum

Phytophthora ramorum appeared in the 1990s in Germany, causing twig blight in ornamental plants (Werres *et al.*, 2001). A few years later it appeared in Northern California, killing large numbers of coast live oak (*Quercus agrifolia*) and tanoak (*Notholithocarpus densiflorus*) (Grünwald *et al.*, 2012). The oak dieback was named 'sudden oak death' (Svihra, 1999), but it was not until the year 2000 that *P. ramorum* was discovered to be the causal agent (Werres *et al.*, 2001). Today more than 100 susceptible plant species in 35 genera have been identified (USDA, 2013). The symptoms vary for different host species (Grünwald *et al.*, 2012). European oaks and beech develop bleeding stem cankers and the infection often causes mortality, while other species such as *Rhododendron* spp. develop necrotic foliage and shoot dieback, which normally is not lethal (Grünwald *et al.*, 2012). Aerial infections of certain hosts occur, and infection of less susceptible species enables continuous production of airborne spores, which is important for the spread of the pathogen in forest settings (Oßwald *et al.*, 2014; Balci *et al.*, 2013; Grünwald *et al.*, 2012). It is likely that this pathogen originates from the Himalayan region (Hansen, 2015), and less susceptible plant species used in plant trade have been identified as pathways of long distance dispersal (Grünwald *et al.*, 2012). Since 2009, *P. ramorum* has caused extensive dieback on a landscape scale of Japanese larch (*Larix kaempferi*) in the UK and in Ireland (Brasier & Webber, 2010).

Destructive forest *Phytophthoras* in Europe

Today, several *Phytophthora* species are involved in European forest declines (considerable tree mortality at genus or species level) (Haavik *et al.*, 2015; Jung *et al.*, 2013). A recent study highlights commercial nurseries as important pathways of dispersal for invasive *Phytophthora* species throughout Europe (Jung *et al.*, 2015). This study, which included over 700 forest- and ornamental nurseries in 23 European countries, found that 92% of the nurseries had *Phytophthora* infested stock, and in total 49 different *Phytophthora* taxa were found. Furthermore, regeneration with nursery grown seedlings proved to be an important pathway of dispersal and establishment of *Phytophthoras* in forest settings. Some of the most important forest diebacks caused by *Phytophthora* in Europe include oak decline, ink disease of chestnut, beech decline and dieback of alders.

Oak decline

Oak decline has occurred on a global scale repeatedly during the two last centuries (Haavik *et al.*, 2015). The European oak decline is complex and poorly understood, and includes the interaction of abiotic and biotic agents, resulting in loss of resilience, degradation of root systems, wilting of foliage and eventual tree mortality (Haavik *et al.*, 2015). Together with other damaging factors, multiple *Phytophthora* species are involved in the fine root degradation of European oaks, especially *P. quercina* (an oak specific species), *P. cinnamomi*, *P. cambivora* and *P. plurivora* (Jung *et al.*, 2013). The airborne *P. ramorum*, causal agent of sudden oak death in North America, is however not involved in the European oak decline (Jung, 2011). Symptoms of oak decline include tarry spots on the bark, epicormic branches, crown dieback and wilting of foliage (Jung *et al.*, 2013). Decades can pass before the fine root losses become evident through chronic loss of foliage. At this

stage the trees are more susceptible to abiotic stresses, such as drought, as well as to other biotic damaging agents (Jung *et al.*, 2013).

Ink disease of chestnut

The disease was noticed in Portugal in 1838 (Crandall, 1950). It was named ink disease because infected roots stain the surrounding soil black (Vettraino *et al.*, 2005). It is one of the most damaging diseases to the genus *Castanea* and is present throughout the natural range of sweet chestnut (*Castanea sativa*) in Europe (Jung *et al.*, 2013). Ink disease causes root and collar rot, necrosis of the inner bark, crown dieback and chlorotic foliage (Jung *et al.*, 2013). Both *P. cambivora* and *P. cinnamomi* are associated with ink disease on sweet chestnut, and infection usually leads to death of the tree within one to three years (Vannini & Vettraino, 2001). However, a suite of other *Phytophthora* species have also been associated to European sweet chestnut stands, mainly confined to fine root damages, including *P. cactorum, P. cryptogea, P. gonapodyides, P. megasperma, P. nicotinae, P. plurivora, P. pseudosyringae* and *P. syringae* (Jung *et al.*, 2013).

Beech decline

Beech have been increasingly declining in Europe and in the USA since the 1990s (Jung *et al.*, 2013). The decline can be characterized by symptoms of bleeding stem cankers, crown dieback, chlorotic foliage, collar rot and dieback of the roots (Jung *et al.*, 2013). Diseased stands often have a patchy distribution of symptomatic trees. Affected trees eventually die as a consequence of extensive root damages, which weaken the trees and predispose them to damage or death brought about by drought, windstorms, and/or attacks of secondary pathogens and pests (Jung *et al.*, 2013). In a large-scale investigation across Europe, 14 *Phytophthora* species were isolated and associated to the beech decline syndrome. The most common and aggressive species were *P. cambivora* and *P. plurivora* (Jung *et al.*, 2013). *Phytophthoras* have also been suggested to be associated with beech bark disease (Jung, 2009), acting as primary agents enabling subsequent colonization of bark beetles (such as *Cryptococcus fagisuga*) and secondary fungi (parasitic *Neonectria* spp. and other, wood decay fungi).

Dieback of Alders

In the beginning of the 1990s, alders in the UK started dying and showed classic symptoms of *Phytophthora* infection such as tarry spots on the bark, crown dieback, small and chlorotic leaves, epicormic branches, extensive seed production, necrosis of the inner bark and root rot (Jung *et al.*, 2013). The highest mortality rates were found in seasonally or permanently waterlogged sites, where mortality rates exceeded 50% (Jung *et al.*, 2013). The previously unknown causal agent was discovered to be *P. alni*, and subsequently divided into a hybrid complex of three subspecies; *P. alni* subsp. *alni*, *P. alni* subsp. *uniformis* and *P. alni* subsp. *multiformis* (Brasier et al., 2004)*.* The genetic relations among the subspecies are complex and the evolution of the subspecies is poorly known (Érsek & Lamour, 2013). However, hybridization probably happened several times in European nurseries and subsequently infested seedlings were planted in the UK (Adams *et al.*, 2008; Brasier *et al.*, 2004). By now *P. alni* causes dieback of *Alnus* spp. throughout the European continent (Érsek

& Lamour, 2013). It has also been found in Alaska, though its introduction from infested nursery stock there is unlikely. Rather it may have been introduced from tourists via contaminated shoes, or perhaps it is native to the area (Adams *et al.*, 2008).

Forest *Phytophthoras* in Sweden

Seven *Phytophthora* species have been found on six broadleaved tree species in Sweden. In 1996 invasive *P. alni* was first found on alders (*Alnus incana* and *Alnus glutinosa*) in riparian areas in southern Sweden (Redondo *et al.*, 2015a; Olsson, 1999). Today, all three subspecies have been found on alders in Sweden (Redondo *et al.*, 2015a). *Phytophthora alni* subsp. *uniformis* is found throughout southern Sweden along river systems, whereas the generally more aggressive *P. alni* subsp. *alni* is absent in areas with harsher winter climate, apparently limited by the duration of frost as well as the winter temperature (Redondo *et al.*, 2015a). Furthermore, *P. plurivora* has also been isolated from alders growing along three Swedish rivers (Redondo *et al.*, 2015a). In 2003, declining oaks (*Quercus robur* and *Quercus petraea*) were found to host *P. quercina*, *P. cactorum* and *P. cambivora* (Jönsson *et al.*, 2003). According to Jung *et al.* (2013) and Jung and Witzell (unpublished) *P. cactorum, P. cambivora, P. plurivora* and *P. syringae* were found in Swedish beech stands. Recently, *P. pseudosyringae* was isolated from aerial cankers on horse chestnut (Redondo *et al.*, 2015b). Furthermore, *P. ramorum* has been reported a few times on imported *Rhododendron* (Samuelsson *et al.*, 2012), but yet there have been no reports of *P. ramorum* infecting trees in Sweden.

Aim of the study

For management purposes, it is important to know which invasive *Phytophthora* species are causing disease on trees. However, a large knowledge gap exists on forest *Phytophthora* species and their distribution in Sweden. *Phytophthora* is often imported as passive companions to seedlings (Jung *et al.*, 2015), which makes urban forest settings particularly interesting for initial studies, since in many cases both tree seedlings and other ornamental seedlings are brought in and planted in these areas.

A previous study found *P. cactorum* and *P. plurivora* to be the casual agents of bleeding stem cankers on beech in Pildammsparken, Malmö (Jung, 2011). Later, all trees in the park were visually examined, with the aim to identify infected trees (Witzell & Agostinelli, 2012). However, a thorough investigation of the involved *Phytophthora* species was not conducted. The aim of this study is to address this knowledge gap by examining the presence of *Phytophthora* damages on beech in Pildammsparken and in other areas in the natural and urban landscape throughout Skåne where typical *Phytophthora* symptoms, such as bleeding stem cankers, have been reported, and confirm the species involved.

Method

The sampling areas

Between 8 Sept. and 20 Oct. 2015 trees with bleeding stem cankers were surveyed and sampled at various locations in Skåne including Pildammsparken in Malmö, Bokskogen in Torup, and the churchyards of Öja, Stora Köpinge and Tranås (Fig. 2).
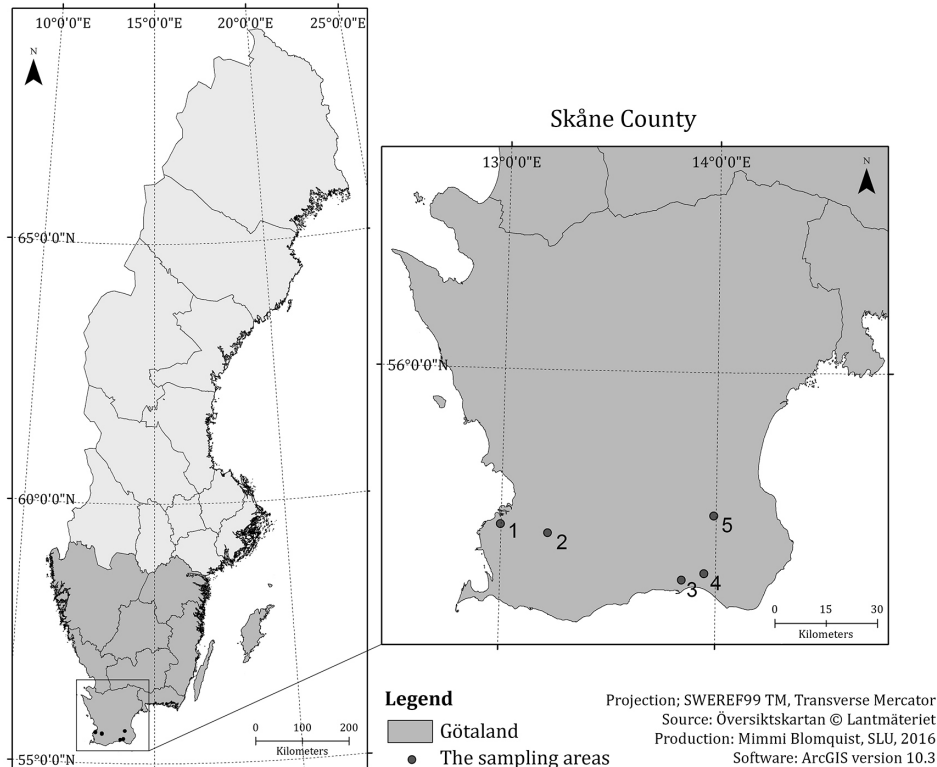
Figure 2. The sampling areas in Skåne. 1. Pildammsparken; 2. Bokskogen; 3. Öja; 4. Stora Köpinge; 5. Tranås.

Pildammsparken

Pildammsparken is a 45-hectare park situated in central Malmö, surrounding a pond that was established as water reservoir at the end of the 16th century (Witzell & Agostinelli, 2012). The area has been used for recreational purposes long before the park was established in 1914 (Gatukontoret, 2016a), and it continues to be a popular recreation area, today including two outdoor fitness facilities and numerous playgrounds. Roughly 100-year old beech trees (*Fagus sylvatica*) dominate the vegetation, but flowers and flowering shrubs are also important components of the park. In 2010, the park manager contacted researchers at the Swedish University of Agricultural Sciences, regarding reduced vitality of the beech trees (Witzell & Agostinelli, 2012). Following an initial visual inventory of trees with bleeding stem cankers (Witzell & Agostinelli, 2012), the managers were advised to remove trees with the lowest vitality, due to

their safety risks to the public. Later, the presence of *P. cambivora* and *P. plurivora* was determined from a small sample of symptomatic trees (Jung, 2011). The site was included in this study because of the documented, but not fully investigated, presence of *Phytophthora* species in the area.

Bokskogen

Bokskogen surrounds the renaissance castle in Torup, roughly 15 km east of Malmö city. The name Bokskogen translates to "the beech forest" and consists of 340-hectares, of mostly old growth beech. Bokskogen and Torup castle are owned by Malmö city and frequently used for recreational purposes (Gatukontoret, 2016b). The castle and its gardens are open to the public and the forest area provides an extensive trail system, a forest playground, a fitness center, a café and several museums. Bokskogen was included in this study since there had been previous reports about bleeding stem cankers on beech in the area. Due to time restrictions, the inventory mainly focused on the forest north of the castle.

The churchyards of Öja, Stora Köpinge and Tranås

All three churchyards were examined for trees with typical *Phytophthora* symptoms, based on previous reports of trees with bleeding stem cankers.

Field sampling

Phytophthora test kits

ELISA (enzyme-linked immunosorbant assay) *Phytophthora* test kits can be used as a quick method to identify *Phytophthora* in the field. These tests contain monoclonal antibodies, which bind to a generic *Phytophthora* antigen and can identify *Phytophthora* (O'Brien *et al.*, 2009). ELISA tests can however not identify *Phytophthora* to species level (O'Brien *et al.*, 2009), and the tests may also react to some *Pythium* spp. (closely related oomycetes) (Martin, 2009; MacDonald *et al.*, 1990). The "Pocket Diagnostic *Phytophthora* test kit" (Foresite diagnostics Ltd, York, UK) was used intermittently to confirm the presence of *Phytophthora* spp. on symptomatic trees prior to sampling (Fig. 3). The test included a buffer solution, in which small pieces of newly infected, i.e. necrotic, phloem tissue were added. The buffer and tissue were homogenized through shaking for half a minute, after which the solution settled for another 30 seconds. Subsequently 2-3 drops were pipetted into a well on the test strip. In case of positive result two lines appeared on the strip within 5-10 minutes. Although ELISA tests can react to *Pythium* spp. (MacDonald *et al.*, 1990), these species could be ruled out since they do not cause stem cankers to trees (Kageyama, 2014; Lane *et al.*, 2007).
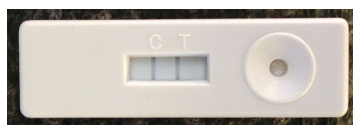

Figure 3. *Phytophthora* field test. The two lines indicate positive *Phytophthora* infection.

The sampling procedure

At each location, the positions of symptomatic trees were recorded by GPS (Oregon 650t, Garmin LTD, Canton of Schaffhausen, Switzerland) and the diameter at breast height was recorded. To improve the probability of successful identification of *Phytophthora*, both stem tissue from symptomatic trees and adjacent soil were used to isolate *Phytophthora* and obtain DNA for species determination. Figure 4 illustrates the steps involved in field and laboratory work (culturing and molecular techniques) to determine species identification.

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                    ┌──────────────────────────────────────────┐
                    │            Field sampling                │
                    │   Collection of soil and stem tissue     │
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                         │                          │
                ┌────────────────┐         ┌────────────────┐
                │      Soil      │         │     Tissue     │
                └────────────────┘         └────────────────┘
                         │                          │
                ┌────────────────┐         ┌────────────────┐
                │    Baiting     │         │ Soaking of     │
                │ Flooding soil  │         │ tissue         │
                │ and floating   │         │                │
                │ leaves         │         │                │
                └────────────────┘         └────────────────┘
                         │                          │
                ┌────────────────┐         ┌────────────────┐
                │ Plating of     │         │ Plating of     │
                │ leaves on      │         │ tissue on      │
                │ Phytophthora-  │         │ Phytophthora-  │
                │ selective agar │         │ selective agar │
                └────────────────┘         └────────────────┘
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Subculturing hyphae to potato dextrose agar

Culturing hyphae in malt extract broth

Freeze drying and homogenization

DNA extraction

DNA quantification - Nanodrop

PCR

Gel electrophoresis

Purification and Cleaning of PCR products

DNA quantification - Qubit Flurometer

Samples sent to SciLifeLab for Sanger sequencing

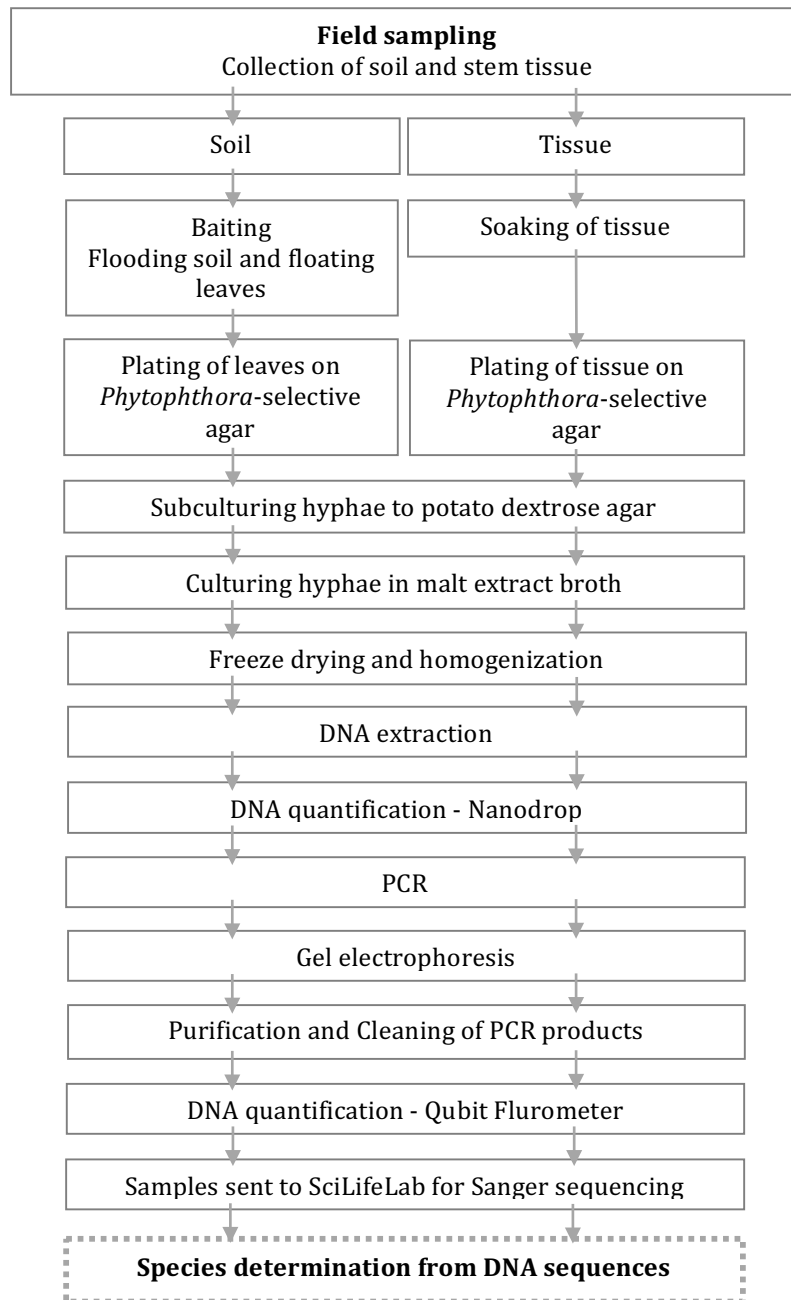**Species determination from DNA sequences**

Figure 4. Flowchart of practical work.

Phytophthora are primary invaders and therefore only attack living or recently wounded tissue (Drenth & Sendall, 2001). On symptomatic trees, the tissue samples were collected by excising the edge of the canker wounds, covering the actively growing lesion in the transition zone between healthy (whitish) and diseased (reddish) tissue under the bark (Fig. 5). Subsequently the tissue samples were transported in bottles containing deionized water to the lab. In addition, approximately 0.5 liter of soil was collected at four cardinal locations using a soil core surrounding the trees at a distance of between 0.5 and 1 m from the root collar, and at a depth between 5-15 cm since *Phytophthora* are disadvantaged in the dry soil close to the surface (Drenth & Sendall, 2001). The soil was transported to the lab in sealed plastic bags.

Figure 5. The transition zone between healthy and diseased tissue (left), tissue samples in deionized water (middle) and collection of soil (right).

Laboratory work

Isolation of *Phytophthora* from plant tissue

In the lab, the deionized water was replaced 3-4 times per day during 1-3 days, until the water became transparent, to remove excess polyphenols that might otherwise interfere with outgrowth of *Phytophthora* on artificial media (Drenth & Sendall, 2001). The bark tissue samples were surface sterilized in 70% ethanol for a few seconds, to reduce bacterial contamination in the later steps (Drenth & Sendall, 2001). The samples, covering the transition zone between healthy and necrotic parts, were cut into roughly 2x2x1 mm pieces and under sterile conditions, plated on 9 cm Petri dishes containing *Phytophthora*-selective PAR (PH)-V8 agar (Appendix I) (Fig. 6). The selective media contains antifungal and antibacterial chemicals, with the aim to allow *Phytophthora* hyphae grow without competition from fungi and bacteria. Since one of the chemicals (pimaricin) is light sensitive (Erwin & Ribeiro, 1996), the agar were subsequently kept in the dark. The plates were kept at room temperature (20 to 22 °C) for several days and checked for mycelia outgrowth.
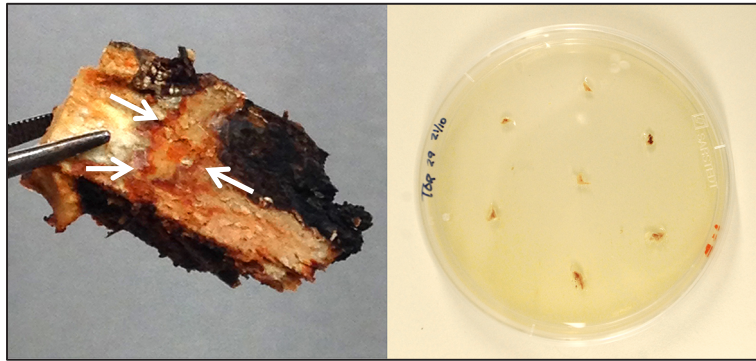
Figure 6. Stem tissue covering the transition zone (arrow) (left),
plated on *Phytophthora*-selective agar (right).

Isolation of *Phytophthora* by baiting of soils

In the lab, soil was placed in plastic boxes and flooded with deionized water, at a water:soil ratio of 4:1, as described by Drenth and Sendall (2001). The following day, any excess floating organic matter was removed. Healthy leaves of *Camellia japonica* and *Rhododendron caucasicum x ponticum* 'Cunningham´s White' were placed on the water surface (Fig. 7). Several members of the Ericales, such as *Rhododendron* and *Camellia* are susceptible to a wide range of *Phytophthora* spp., and are commonly used as baits for zoospores to infect the leaves close to the water surface, which eventually result in necrotic lesions (Erwin & Ribeiro, 1996). The boxes were covered with lids but not sealed, and kept at room temperature. After approximately 7-14 days the leaves with lesions were collected and rinsed in deionized water. The transition zone on the leaves were cut in 3x3 mm pieces and plated onto 9 cm petri dishes with *Phytophthora* selective PAR (PH)-V8 agar (Appendix I).

Figure 7. The baiting procedure. Soil samples were flooded with water and *Rhododendron* leaves were floated on the water surface (left). As zoospores infected the leaves, lesions occurred (middle) and subsequently pieces of the infected leaves were plated on selective agar (right).

Purification of the isolates

Hyphae of *Phytophthora* are semi-transparent and grows out in *Phytophthora* selective agar in only a few days (Erwin & Ribeiro, 1996). To get pure isolates, with no bacterial or fungal contamination, hyphae that grew out from the plated tissue of host trees and *Rhododendron* leaves after 2-5 days were transferred to 9 cm petri dishes with potato dextrose agar (PDA) (Appendix I), and allowed to grow for several days. Plugs of PDA containing pure cultures of hyphae were then transferred to 50 ml falcon tubes, filled with 35 ml malt extract broth (Appendix I) (Fig. 8). After inoculation in room temperature during two weeks in the dark residues of PDA were removed and the mycelia were filtrated (Munktell filter AB, Falun, Sweden) three times in 50 ml deionized sterile water.
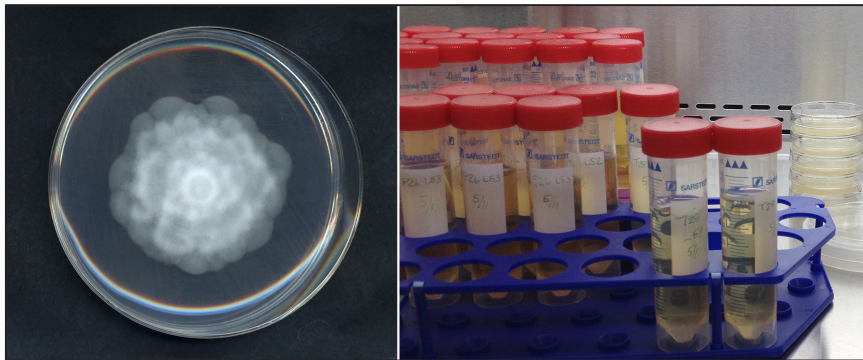

Figure 8. *Phytophthora* hyphae cultured on PDA (left), subsequently subcultured to liquid media (malt extract broth) (right).

Freeze-drying and DNA extraction

To prepare isolates for DNA extraction, mycelia were placed in 2 ml screw cap extraction tubes, together with three glass beads to improve the pulverization in later stages. The samples were freeze dried and collected after 2-3 days, and from this step onwards, the samples were kept on ice. In order to get access to the cell content (and therefore DNA) the cell walls need to be broken (Clemmensen & Ihrmark, 2014). This was done by grinding the samples in a "Fast Prep-24" bead beater machine (MP biomedicals, Santa Ana, USA) three times for 30 s at 5 m/s. To get rid of contaminants such as proteins, cellular debris or polysaccharides the DNA was extracted according to the manufacturer´s instruction using the kit "E.Z.N.A. SP Plant DNA Kit" (Omega Bio-Tek, Inc., Norcross, USA). The nucleic acid concentration of the extracted DNA was measured using NanoDrop® ND-1000 (Wilmington, USA).

DNA amplification via PCR

Polymerase Chain Reaction (PCR) is used to amplify selected parts of the genome (Clemmensen *et al.*, 2011). The template DNA is mixed with Taq DNA polymerase (a heat stable polymerase), $MgCl_2$ (Mg^{+2} is an important component for the polymerase), dNTP mix (nucleotides, the building stones of the new DNA strains), PCR-buffer, sterile water and primers (Klug, 2014). Primers are short

complementary sequences to the starting and endpoint of the target sequence (Klug, 2014). The samples are loaded in an automated thermal cycler that changes the temperature according to the three steps; denaturation, annealing and extension. Denaturation at 92-95°C opens the two DNA strains and all enzymatic activity is paused (Klug, 2014). Annealing between 45 and 65°C binds primers to the template DNA and the polymerase attaches and starts to synthesize new DNA, complementary to the template (Clemmensen *et al.*, 2011). The extension step which occurs between 65 and 75°C works in a way such that the higher temperature detaches primers that have a bad fit and weak bonds from the template, while primers with attached bases stay together (Klug, 2014; Clemmensen *et al.*, 2011). The polymerase continues to synthesize DNA.

The internal transcribed spacer (ITS) region of the rDNA is commonly used for species identification of fungi and oomycetes (Sapkota & Nicolaisen, 2015; Clemmensen *et al.*, 2011). Here primers that flank the ITS-4 (forward) and ITS-6 (reverse) regions were used (Cooke *et al.*, 2000). The DNA template was diluted to 2 ng/µl with the buffers included in the DNA extraction kit. Subsequently 1 µl of the template DNA (2ng) were added to 49 µl of a master mix containing 27.8 µl dH$_2$O; 5 µl 10x PCR buffer; 3 µl Mgcl$_2$ (1.5 mM); 5 µl dNTP mix (0.2 mM); 4 µl ITS-4 (0.4 µM); 4 µl ITS-6 (0.4 µM); and 0.2 µl Taq DNA-polymerase, and pipetted into a PCR plate. For positive and negative control the master mix was prepared, but in the template DNA 1 µl of a known sample of *P. plurivora* and dH$_2$O was used, respectively. Amplifications of each sample were performed under the following conditions: an initial step of denaturation for 3 min at 94 °C, followed by up to 35 cycles of denaturation for 1 min at 94 °C, annealing for 1 min at 55 °C, extension for 1 min at 72 °C finalized by an extension step of 10 min at 72 °C.

Gel electrophoresis

To make sure that PCR products formed, the samples can be visualized on an agarose gel and using a UV illuminator (Klug, 2014). The samples are pipetted into wells of a pre-prepared gel and placed in a buffer that conducts electricity. The DNA segments are negatively charged and will move through the porous gel from the negatively charged starting point towards the positively charged endpoint (Klug, 2014). The shorter segments move faster than larger segments and thus the DNA segments are sorted according to size. Here DNA quantification was checked using electrophoresis in 1.5% agarose gel containing GelRed and visualized by UV fluorescence (Fig. 9). The gel was prepared with 1 X Tris-acetate-EDTA (TAE) buffer and 1.5% agarose, together with 1 µl of GelRed per 10 ml of 1 X TAE buffer.
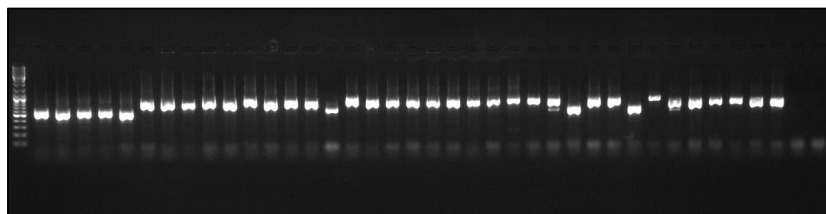

Figure 9. Gel electrophoresis showing PCR products under UV fluorescent light.

Sequencing

Sequencing reveals the order of the bases: adenine (A), guanine (G), cytosine (C) and thymine (T) in the DNA (Klug, 2014). Prior to sequencing the amplified PCR products were purified and cleaned using "HT ExoSAP-IT High-Throughput PCR Product Cleanup" (Affymetrix, Santa Clara, USA) and the DNA concentrations were measured with Qubit fluorometer, which is a measurement based on the fluorophore bindings of the double stranded DNA (Clemmensen *et al.*, 2011). The Qubit measurements were prepared with "Qubit dsDNA HS Assay Kit" (Life technologies, Carlsbad, USA). The PCR products were sequenced by automated Sanger sequencing at the National Genomics Infrastructure (NGI) at Science for Life Laboratory (Scilifelab, Solna, Sweden).

Data analysis

Identification of sequences

Visualization of the sequences were carried out using the software Chromas (version 2.4.4, Technelysium, South Brisbane, Australia) available on the website http://technelysium.com.au/?page_id=13. Since the individual samples were sequenced with one forward and one reverse primer, the files could be aligned and edited with Lasergene software package SeqMan (version 5.07, DNAStar, Madison, WI, USA). The consensus sequences were examined to uncover regions of local correspondence between samples and the reference databases at the National Center for Biotechnology Information (NCBI) website (http://blast.ncbi.nlm.nih.gov/Blast.cgi) by comparison of DNA sequences belonging to that of highly similar species. Isolates were determined to a species level based on greater than 98.5% sequence similarity and to a genus level based on greater than 96% sequence similarity.

Pathogenicity test

Phytophthora gonapodyides is a newly identified species to cause disease on trees in Sweden. To confirm the pathogenicity of this species, two isolates of *P. gonapodyides* was used in an inoculation test of healthy beech seedlings. Mycelial plugs of 3-week old cultures of each isolate grown on PDA were used as inoculum source. A small 5-mm flap of bark was excised from the stem surface and a similarly-sized diameter plug of either isolate was aseptically placed in the cambium zone, a droplet of Milliq H_2O was added and the wound was sealed with Parafilm®. Ten seedlings were inoculated for each isolate with two stem-wound inoculations per stem, separated by 20 cm. Controls consisted of stem-wound inoculation with a sterile plug of PDA, and no wounding at all. Lesion length on the bark surface and along the cambium was measured after 7 weeks. Paired t-tests for estimations of lesion development were performed in Minitab statistical software (version 16, Minitab, Ltd, Conventry, UK).

Result

In total 33 symptomatic trees with stem cankers (See figure 10 for symptoms) from the five sites; Pildammsparken (n=14), Bokskogen (n=14), Tranås (n=3), Stora Köpinge (n=1) and Öja (n=1) were sampled. The total number of tissue and soil samples amounted to 63. No soil samples were collected in Tranås due to how the churchyard was landscaped. Beech was the most frequently sampled tree species (n=28), followed by horse chestnut (*Aesculus hippocastanum*) (n= 4) and then lime (*Tilia cordata*) (n=1). The diameter at breast height of the sampled trees ranged from 21 cm to 155 cm.

Figure 10. Symptoms of *Phytophthora* infections such as bleeding stem cankers and crown dieback.

Phytophthora isolates were obtained from Pildammsparken, Bokskogen and Stora Köpinge, while no *Phytophthora* was obtained from Öja and Tranås. The culturing of isolates was successful for 13 of 63 samples (21%). Five *Phytophthora* species were isolated and identified; *P. cactorum, P. cambivora, P. gonapodyides, P. plurivora* and *P. syringae* (Fig. 11). Baiting of soil was more successful, yielding eight *Phytophthora* isolates compared to six from plating of tissue (Table 1).
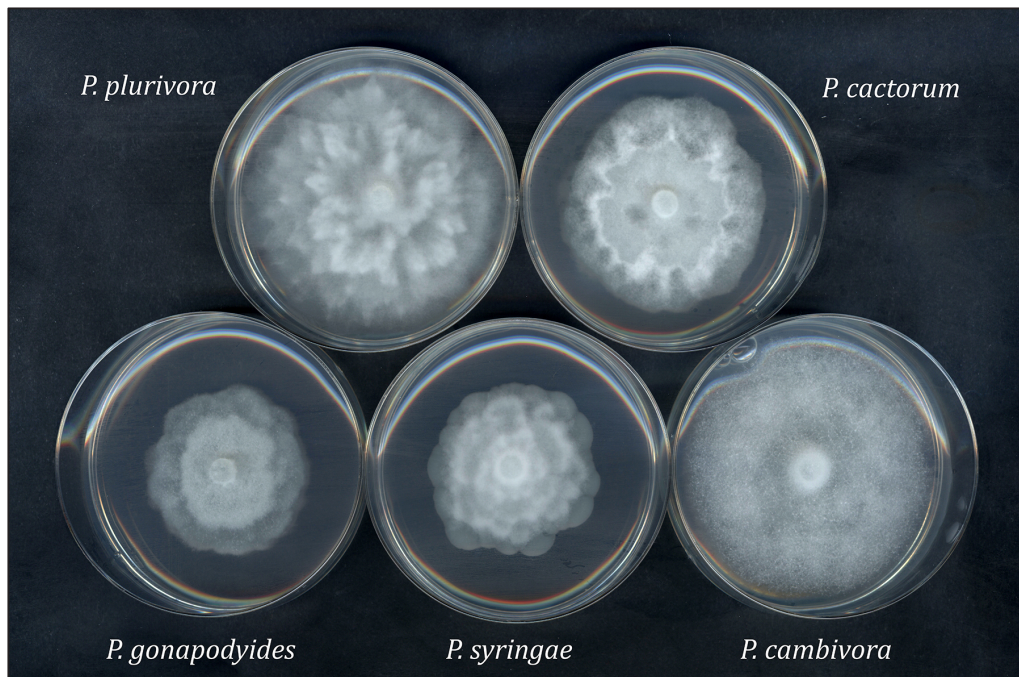
Figure 11. The isolated *Phytophthora* species obtained in this study.

Table 1. Location, tree number and isolation method used to detect *Phytophthora* spp. on beech ([b]) and horse chestnut ([h]).

Location	Tree	DBH (cm)	Soil sample	Stem lesion sample
Pildammsparken	1[b]	76		*P. gonapodyides*
Pildammsparken	6[b]	155	*P. plurivora*	
Pildammsparken	8[b]	68	*P. plurivora*	*P. plurivora*, *P. gonapodyides*
Pildammsparken	10[b]	82	*P. plurivora*	*P. plurivora*
Pildammsparken	12[b]	73	*P. cactorum*	*P. plurivora*
Pildammsparken	13[h]	61	*P. syringae*	
Pildammsparken	14[b]	152		*P. gonapodyides*
Bokskogen	21[b]	45	*P. plurivora*	
Bokskogen	26[b]	111	*P. cambivora*	
Stora Köpinge	29[b]	128	*P. cactorum*	

A few other pathogenic oomycetes closely related to *Phytophthora* (Thines, 2014), was isolated from soil samples (*Phytopythium citrinum*, *Pythium intermedium*, *Pythium perplexum* and one *Pythium* species which could only be determined to genus level). Even though selective media was used to exclude fungi during the culturing, some fungi did grow. These included *Neonectria coccinea* (generally associated with beech bark disease (Jung, 2009)), *Mortiella* spp. (common soil fungi) (Tsao & Guy, 1977) and *Clonostachys rosea* (mycoparasitic fungi) (Karlsson *et al.*, 2015).

Pildammsparken

In total 14 trees were sampled in Pildammsparken (Appendix II), including 13 beech trees and one horse chestnut (tree number 13). Cultivation of *Phytophthora* isolates was successful in 50% of the sampled trees in the park. *P. gonapodyides*, *P. plurivora* and *P. cactorum* were obtained from beech stem tissue and/or from soil adjacent to beech trees. *P. syringae* was isolated from soil surrounding a horse chestnut tree (tree number 13) (Table 1).

Isolations of other oomycetes included *Phytopythium citrinum, Pythium perplexum, Pythium intermedium,* and *Pythium* sp., all of which were obtained from soil samples. Isolated fungal samples included *Clonostachys rosea* and one *Mortiella* sp., derived from soil samples and *N. coccinea* obtained from tissue samples.

Bokskogen

In total 14 beech trees were sampled in Bokskogen (Appendix II). Out of these, only two trees (14%) were successful in isolating *Phytophthora* (Table 1). The soil surrounding both infected trees contained *P. plurivora* and *P. cambivora* respectively. *N. coccinea* was isolated from three of 14 stem lesions.

The churchyards of Stora Köpinge, Öja and Tranås

In total five symptomatic trees were sampled in the churchyards, including beech (n=1) in Stora Köpinge, lime (n=1) in Öja and horse chestnut (n=3) in Tranås. No soil samples were collected in Tranås since the churchyard was landscaped in a way that prevented proper collection of soil samples adjacent to the trees. Culturing of isolates was only successful in Stora Köpinge, where *P. cactorum* was obtained from the soil adjacent to the symptomatic beech tree.

Lesion development on beech caused by *P. gonapodyides*

P. gonapodyides isolate 1 and 2 were obtained from tree number 14 and 1, respectively. All beech seedlings inoculated with the two isolates developed stem cankers, in contrast to the controls, which did not develop stem wounds beyond the actual excision wound (Fig. 12). Cumulative lesion length on the bark surface after 7 weeks ranged between 17 and 29 mm for isolate 1 and between 18 and 32 mm for isolate 2. Necrosis along the cambium ranged between 18 and 35 mm, and between 19 and 35 mm for isolates 1 and 2, respectively. The cumulative lesion length (for both inoculation lesions), on bark and under bark, for each seedling is shown in Table 2. Paired t-tests showed that the lesions caused by the two isolates were significantly longer ($P<0.001$) for lesions on bark and under the bark, compared to the excision wounds of 5 mm on the controls, which were inoculated with a sterile plug of PDA. Untreated seedlings showed no symptoms. The mean cumulative lesion length was slightly higher with isolate 2 than isolate 1. However, paired t-tests revealed that there was no significant difference between the stem lesion length caused by isolate 1 and isolate 2 ($P = 0.232$ on the bark, and $P = 0.128$ along the cambium).

Figure 12. Lesions on bark and under the bark of beech seedlings inoculated for 7 weeks with a sterile plug of PDA (left) and with a mycelial PDA plug with *P. gonapodyides* (right).

Table 2. Cumulative lesion length (measured on and under the bark) resulting from inoculations of the two *P. gonapodyides* isolates at two locations on the stem

	Isolate 1		Isolate 2	
Replicate	On bark surface (mm)	Under the bark (mm)	On bark surface (mm)	Under the bark (mm)
1	23	27	29	35
2	29	35	25	30
3	20	24	32	33
4	29	31	23	26
5	22	23	18	19
6	23	23	25	29
7	27	29	25	27
8	17	18	23	28
9	24	25	31	33
10	19	19	27	29
Mean	**23**	**25**	**26**	**29**

Discussion

Sampling in five sites in southern Skåne revealed the presence of five *Phytophthora* species; *P. cactorum, P. cambivora, P. gonapodyides, P. plurivora* and *P. syringae.*

Phytophthora cactorum was found in connection to beech on the churchyard of Stora Köpinge and in Pildammsparken. *P. cactorum* occurs on a global scale and has a host range of more than 200 plant species (Erwin & Ribeiro, 1996), and is frequent in European nurseries (Jung *et al.*, 2015). Some of the known host trees species present in Sweden include; *F. sylvatica*, *Pinus sylvestris, Betula pendula, A. glutinosa*, *Sorbus aucuparia*, *Larix decidua*, *Fraxinus excelsior* (Orlikowski *et al.*, 2011), *Q.* robur, *Q. petraea* (Jung *et al.*, 1996), *Acer* spp., (Jung *et al.*, 2015), *Tilia* spp. (Jung *et al.*, 2009), *A. hippocastanum* (Oßwald *et al.*, 2014) *Populus* spp. (Keča *et al.*, 2015), *Salix* spp., *Malus* spp. and *Pyrus* spp. (Erwin & Ribeiro, 1996).

Phytophthora cambivora was found in connection to beech trees in Bokskogen. The species probably originates from Australia (Oudemans & Coffey, 1991), but is today present on all continents (CABI, 2014). *P. cambivora*, together with *P. plurivora,* constitutes the two most aggressive and common casual agents of beech decline throughout Europe (Jung *et al.*, 2013). Furthermore, *P. cambivora* plays an important role in European oak decline and ink disease of chestnut (Jung *et al.*, 2013). Other hosts include *A. hippocastanum* (Brasier & Jung, 2006), *Tilia* spp. and *Acer* spp. (Jung *et al.*, 2009). *P. cambivora* has also been found on in nurseries associated with *Picea abies* and *Alnus* spp. (Jung *et al.*, 2015).

Phytophthora gonapodyides obtained from stem lesions on beech in Pildammsparken are the first findings of the species causing disease on trees in Sweden. However, *P. gonapodyides* was discovered in neighboring Denmark in 1909 (Erwin & Ribeiro, 1996). Host tree species present in Sweden include; *A. glutinosa, Q. petraea* (Lakatos & Szabó, 2009), *Q. robur* (Jung *et al.*, 1996), *P. abies*, *F. sylvatica* (Jung *et al.*, 2015), *Tilia* spp., *Acer* spp., *Betula* spp. (Jung *et al.*, 2009) and *Salix* spp. (Brasier *et al.*, 2003). *P. gonapodyides* is often associated with aquatic environments such as rivers, riparian areas and wetlands (Brasier *et al.*, 2003), and has traditionally been regarded as a weak parasite with saprophytic abilities (Erwin & Ribeiro, 1996). It is able to colonize both fresh and withered leaf litter in water (Hansen & Delatour, 1999), and might be an important decomposer of plant litter (Brasier *et al.*, 2003). However, the damage caused by *P. gonapodyides* might be underestimated, as it also can inhibit germination of seeds (Martín-García *et al.*, 2015). In fact, *P. gonapodyides* may play an important role in the Iberian oak decline, by preventing the germination of *Quercus ilex* seeds (Martín-García *et al.*, 2015), the dominant tree species in the dehesas agroforestry systems in Spain and Portugal (Moreno & Pulido, 2009). The failure of natural regeneration, together with root and acorn damages caused by other pathogens, such as *P. cinnamomi*, and a problematic grazing regime, now threatens 3.1 million hectares of dehesas forests in the Iberian Peninsula (Martín-García *et al.*, 2015).

Phytophthora plurivora was obtained from soil and stem lesions of beech in Pildammsparken and from soil samples in Bokskogen. *P. plurivora* is highly aggressive to European oaks and beech (Jung *et al.*, 2013) and has a wide range of known host species present in Sweden including *Acer platanoides, A. hippocastanum, A. glutinosa, A. incana*, *B. pendula, Carpinus betulus, F. sylvatica,*

P. abies, P. sylvestris, *Q. petraea, Q. robur, T. cordata* (Jung & Burgess, 2009) and *Populus* spp. (Keča *et al.*, 2015). Molecular studies suggests that *P. plurivora* is native to Europe and that nurseries have had a key role in the spread throughout the European continent, and also in the introduction of the pathogen to the USA, where it has become established in forest settings (Schoebel *et al.*, 2014).

Phytophthora syringae   was found in soil in proximity to a horse chestnut tree in Pildammsparken. The literature regarding *P. syringae* is scarce, but its known host range include over 30 species (Erwin & Ribeiro, 1996). It has been found in connection to *F. sylvatica*, *Quercus* spp., *Alnus* spp., (Jung *et al.*, 2015), *Tilia* spp., *Acer* spp., *A. hippocastanum* (Jung *et al.*, 2009)**,** *Populus* spp. (Brasier, 1999), *Malus* spp., and *Prunus* spp. (Erwin & Ribeiro, 1996).

Sources of error

The isolation of *Phytophthora* was successful in 13 of 63 samples (20.6%). The lack of success in obtaining isolates from all samples may be attributed to a number of factors.

First, sampled trees may not have been infected. *Phytophthora* species are not the only biotic organism causing stem lesions on trees. For example, damages caused by *Neonectria* spp. (the beech bark disease pathogen) closely resemble those of *Phytophthora* (Cale *et al.*, 2015) (Fig. 13). *N. coccinea* was isolated from several trees in this study and those trees may have been visually mistaken for those caused by *Phytophthora*. However, Jung (2009) found that *N. coccinea* is an important secondary agent on trees already infected by *Phytophthora* species and argued that *Phytophthora* is likely a primary agent in the whole beech bark disease complex.

Figure 13. Beech in Bokskogen, from which *Neonectria coccinea* was isolated

Another possible source of error is that *Phytophthora* could have been missed during the sampling of soil, since soil microbial communities often are heterogenic (Rodríguez Molina *et al.*, 2000).

Since most *Phytophthoras* are hemibiotrophic, (i.e. organisms that live off of healthy, moribund or recently dead host tissue) (Oßwald *et al.*, 2014), the age of the lesion seems to be a key factor for successful isolation and culturing of *Phytophthora* from infected tissue. Furthermore, the activity of *Phytophthora* is seasonal (Cooke *et al.*, 2007), and detection of *Phytophthora* through baiting of soil following dry periods can be increasingly difficult (Anderson, 2006). Finally, the selective media contained several chemicals, including hymexazol, which is used to exclude e.g. *Mortierella-,* and *Pythium* species (Martin *et al.*, 2012). Hymexazol has been reported to inhibit growth of some *Phytophthora* species, including *P. cinnamomi*, *P. palmivora* and *P. citrophthora* (Drenth & Sendall, 2001). Though none of these were species were found in this study, it cannot be excluded the possibility that certain chemicals in the selective media could affect the isolation success. Further testing in this regard is needed to optimize the selective media.

Potential impact of forest *Phytophthoras* in southern Sweden

As of today, eight forest *Phytophthora* species have been identified in Swedish forest settings. Since several of those are capable of infecting multiple tree species, including those native to Sweden (Table 3), the potential impact if becoming established in nature could probably be rather extensive. Since many *Phytophthora* species are being spread from nurseries across Europe (Jung *et al.*, 2015), it is likely that more *Phytophthora* species already have, or will be introduced to Sweden in the near future.

Table 3. Known pathogenicity of the forest *Phytophthora* species found in Sweden and the most common tree species in Götaland (% of total volume). S = The *Phytophthora* species can infect the host **species** and/or has been found in soil in close proximity to the host species. S[p] = *B. pendula*, S[r] = *Q. robur*, S[g] = *A. glutinosa*.
G = No data was found regarding susceptibility on tree species level, but members of the host **genera** have proven to be susceptible to the *Phytophthora* species and/or the *Phytophthora* species have been found in soil in close proximity. ALN = *P. alni; CAC = P. cactorum*; CAM = *P. cambivora;* GON = *P. gonapodyides*; PLU = *P. plurivora*; PSE = *P. pseudosyringae*; QUE = *P. quercina*; SYR = *P. syringae.* [1] (Skogsdata, 2015), [2] (Jung *et al.*, 2015; Keča *et al.*, 2015; Redondo *et al.*, 2015a; Orlikowski *et al.*, 2011; Jung & Burgess, 2009; Jung *et al.*, 2009; Lakatos & Szabó, 2009; Jönsson *et al.*, 2003; Thomas *et al.*, 2003; Brasier, 1999; Jung *et al.*, 1996).

Tree species	Volume [1]		*Phytophthora* species [2]							
	(mill. m³sk)	(%)	ALN	CAC	CAM	GON	PLU	PSE	QUE	SYR
Picea abies	420	45.3			S	S	S			
Pinus sylvestris	280	30.2		S			S			
Betula pendula, Betula pubescens	102	11		S[p]		G	S[p]			
Quercus robur, Quercus petraea	36.1	3.9		S	S	S	S	S[r]	S	S[r]
Alnus incana, Alnus glutinosa	24.5	2.6	S	S[g]	S[g]	S[g]	S	S[g]		S[g]
Fagus sylvatica	21.9	2.4		S	S	S	S	S		S
Populus tremula	19.8	2.1		G			G			G
Sum	904.3	97.5								

Impacts of *Phytophthora* to forest ecosystems are difficult to assess fully. Examples range from large-scale destruction of entire ecosystems by highly virulent and invasive *P. cinnamomi* in Australia (Weste, 2003), to more passive presence of forest *Phytophthoras* with little to no affect to tree health (Hansen & Delatour, 1999).

Phytophthora species are primary invaders and can infect healthy hosts (Oßwald *et al.*, 2014; Sturrock *et al.*, 2011). The expression of disease caused by *Phytophthora* species can be visualized through the plant pathological paradigm known as the disease triangle (Scholthof, 2007) which illustrates the interactions between the environment, susceptible host/genotype and the pathogen, to cause disease (Fig. 14).

Whether or not disease is expressed on the tree depends on the interaction between the particular *Phytophthora*-species, i.e. in terms of its virulence, the host tree species susceptibility, (Oßwald *et al.*, 2014; Werres, 1995), and environmental conditions such as temperature, soil moisture and drought that may affect disease development (Redondo *et al.*, 2015a; Oßwald *et al.*, 2014; Martin *et al.*, 2012; Sturrock *et al.*, 2011; Jung, 2009). The cumulative damage to the tree also depends on which secondary agents colonize the trees (Jung, 2009). Assessment of potential impacts are further complicated by the fact that *Phytophthora* species distributions and/or virulence and their respective host interactions may be altered as a consequence of climate change.
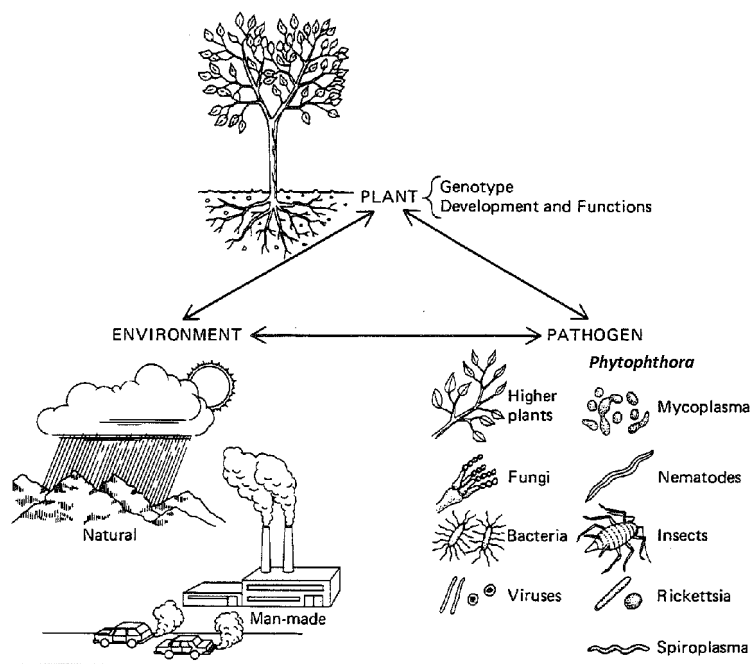

Figure 14. The disease triangle, illustrating disease as an expression of interactions between the host, environment, and pathogen (source: Manion, 1981).

Predicted climate scenarios for Sweden (2071-2100) suggest an increase in mean annual temperatures ranging from 3°C to 7°C (Kjellström *et al.*, 2014). The predictions for precipitation are though more variable and uncertain, but generally precipitation is expected to increase, locally up to 40% (Kjellström *et al.*, 2014). It is also likely that local flooding events and drought will increase because of extreme climate events. In Skåne, mean annual temperatures during the reference period (1961-1990) was -0.6°C in winter and 15.4°C in summer (Ohlsson *et al.*, 2015). Climate scenarios predict increasing winter and summer temperatures of between 3°C to 5°C in Skåne (Ohlsson *et al.*, 2015). The mean annual rainfall for the reference period was 748 mm, which is expected to increase with 15-25% (Ohlsson *et al.*, 2015).

Most *Phytophthora* species can grow at temperatures between 1°C and 35°C (Erwin & Ribeiro, 1996), but their optimal growing temperature often ranges between 15°C and 30 °C. (Martin *et al.*, 2012). Considering the predicted climate scenarios, conditions for *Phytophthora* species will likely improve in the future. A warmer climate will reduce the number of days with temperatures of inactive *Phytophthora* growth, and prolong the season of optimal growing temperatures. A wetter climate will also favor the activity of zoospores, which can move towards new hosts in wet soils only (Judelson & Blanco, 2005).

Management of *Phytophthora*

Spread of *Phytophthora* from nurseries

Nurseries are important sources for the spread of *Phytophthora* (Jung *et al.*, 2015). Current silvicultural practices in Sweden, with 75 % of the regeneration area planted annually (Skogsstyrelsen, 2014), therefore constitutes a considerable risk. If the spread of *Phytophthora* is to be limited, nurseries need to revise their production processes to ensure that only healthy plants are distributed, especially since *Phytophthora* species have the potential to hybridize when brought together in nurseries (Burgess, 2015), and form new species that can potentially be more aggressive than their parent species. An example of this is the hybridization between subspecies of *P. alni*, which gave rise to an even more aggressive subspecies that escaped from the nurseries and led to a widespread dieback of alders throughout Europe (Burgess, 2015). However, preventive measures can be challenging since infected plants can be non-symptomatic, and therefore are often distributed unknowingly to forests, parks and gardens worldwide (Jung *et al.*, 2015).

Preventive measures in healthy areas

There are no efficient treatments for *Phytophthora* (Dalio *et al.*, 2014; Hee *et al.*, 2013). As *Phytophthoras* are unrelated to fungi, most fungicides are ineffective and once established in a forest or a park, *Phytophthora* species are practically impossible to eradicate due to variable survival strategies with e.g. spore types; zoospores which can swim through moist soil towards potential hosts (Hansen, 2015), or resting spores that can lie dormant for several years awaiting suitable conditions for germination (Jung, 2011). Therefore, the most efficient management

strategy of *Phytophthora* is in fact to prevent its introduction, in terms of reducing its probability of successful establishment (Hansen, 2015) and the consequential costs to society (Barber *et al.*, 2013) . For example, costs for diagnosis, inventory and felling of *Phytophthora*-infected beech trees in the 45-hectare Pildammsparken amounted to 400.000-500.000 SEK since 2010 when the symptoms were first noted (Arne Mattson, pers. comm.).

Considering the risks associated with planting of nursery grown seedlings, natural regeneration in healthy stands is an alternative regeneration strategy that could be successful in preventing new establishment on sites. Since most *Phytophthora* species are soil borne (Oßwald *et al.*, 2014), direct seeding could reduce the risk of introduction as it does not involve transportation of soil. However, several *Phytophthora* species, including *P. cinnamomi* and *P. gonapodyides*, are known to cause mortality to seeds (Martín-García *et al.*, 2015). Hence, it seems likely that seeds obtained from infested sites potentially could lead to introduction of *Phytophthora* when sown in other areas. However, more research in this regard is warranted.

Management in infected areas

Considering the risks of hybridization, new introductions of *Phytophthora* to already-infected areas should also be avoided. Furthermore, field workers should be educated in appropriate sanitary management aiming at reducing the risk of spread. Planning of daily work is essential and should start in healthy areas and move towards diseased. Other important measures include disinfection of machines and equipment, supply of information and instructions on how to handle infected trees, ornamental plants, soil and water (e.g. disease free irrigation and removal of standing water).

Immediately after detection, an invasive species is likely present in low densities, which makes the search for the species in a larger areas both time consuming and expensive (Cacho *et al.*, 2010). However, the public may encounter the organism at low densities, which means that public involvement may be a good strategy to facilitate the search of invasive species (Cacho *et al.*, 2010). This could probably be effective for *Phytophthora*, since bleeding stem wounds are rather apparent. In this respect, information boards within urban forests and parks could be useful.

Phosphite ($H_2PO_3^-$), a reduced form of phosphate, is frequently used against a range of different oomycetes, including *Phytophthora* (Gómez-Merino & Trejo-Téllez, 2015). Application of phosphite can give managers time to develop long-term solutions, such as planting resistant tree species. In Australia, common methods of phosphite application in communities infected with *P. cinnamomi* include stem spray, stem injections and aerial spraying of the foliage (Crane & Shearer, 2014). Comparisons between methods has pointed at stem injection as being the most efficient method of application for inhibiting *P. cinnamomi* (Crane & Shearer, 2014). Apparently, its efficacy depends on its concentration in the host tissue, though detailed mechanisms underlying this remains to be explored (Gómez-Merino & Trejo-Téllez, 2015; Dalio *et al.*, 2014). Nevertheless, low concentrations of phosphite facilitates activation of host defenses, while higher concentrations can prevent mycelia growth of *Phytophthora* (Dalio *et al.*, 2014; Jackson *et al.*, 2000). Jackson *et al.* (2000) showed that lesion development was more restricted at higher phosphite concentrations than in lower

concentrations, hence prevention of mycelia growth seems to be more effective than the activation of host defense. However, too high concentrations are toxic to plants (Gómez-Merino & Trejo-Téllez, 2015). If applied correctly, phosphite improves plant root growth, stress tolerance, nutrient uptake and production (Gómez-Merino & Trejo-Téllez, 2015). In accurate concentrations phosphite treatment also reduces the production of oospores, chlamydospores, sporangia and zoospores (Daniel & Guest, 2006). Unfortunately, the production of zoospores cannot be terminated completely (Wilkinson *et al.*, 2001). Hence, even infected areas treated with phosphite may serve as sources for *Phytophthora* dispersal.

Fagus sylvatica is highly susceptible to several *Phytophthora* species. Schlink (2010) found that *F. sylvatica* was unable to recognize attacks of *P. plurivora,* which led to an inactivated defense response during infection. Although phosphite treatment reduces damages caused by *P. plurivora* to *F. sylvatica* (Dalio *et al.*, 2014), there seems to be no general guidelines regarding phosphite application to beech. Nevertheless, a phosphite trial was set up in Pildammsparken including aerial foliage application from a helicopter and punctual application to soil close to infected trees. Preliminary results from this work indicates an increase in tree vitality through increased growth during the first year following application (Anna Levinsson, pers. comm).

Breeding for resistance

Phytophthora-host interactions are driven by natural selection (Oßwald *et al.*, 2014), which opens up for the possibility of breeding for resistance against the pathogen (Hansen, 2008). To minimize extensive damages caused by *Phytophthora*, resistant or less susceptible tree species should therefore be planted in urban forests and parks. Generally, *Sorbus aria*, *S. aucuparia*, *Sorbus intermedia*, *Prunus* spp. and several species of *Salix* and *Populus* are less susceptible, and at the same time suitable for the conditions in southern Sweden (Jung, 2011). As many invasive *Phytophthora* species originate from Asia, Jung (2011) suggests that resistance of Asian tree species should be investigated since it is likely that Asian trees have coevolved with a variety of *Phytophthora* species that are invasive in various parts of Europe.

Conclusion

Five *Phytophthora* species were identified in southern Skåne; *P. cactorum, P. cambivora, P. plurivora* and *P. syringae* and the first records of *P. gonapodyides* as the causal agent of stem wounds on *F. sylvatica* in Sweden. Inoculation tests confirmed pathogenicity of *P. gonapodyides* on beech causing markedly longer stem lesions compared to just wounding or control. Globalization has facilitated the spread of a wide range of invasive pathogens and pests worldwide, including *Phytophthora* species (Pautasso *et al.*, 2015).

In a globalized world, restricting the flow of goods is not a realistic option, focus should rather be at controlling the source of disease. Therefore, efforts preventing spread of disease from nurseries are essential. This problem should be addressed as soon as possible to avoid further environmental damage. In the meantime, science has a critical role to play, to further examine both the distribution of various pathogens and their effects to their environment, thereby facilitating the adoption of suitable management strategies in practice.

Today the information regarding tolerance and resistance to *Phytophthora* among woody plants at the molecular level is rather limited (Oßwald *et al.*, 2014). This would be essential as a first step for developing resistant tree species in order to minimize losses. Pathogen-host interactions, including identification of highly susceptible hosts as well as less susceptible hosts that may provide connectivity to the pathogens (Pautasso *et al.*, 2015), should be investigated.

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Appendix I. Agar recipes

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

Basal medium ingredients	Amount
Vegetable juice	50 ml
Deionized water	950 ml
Agar	15 g

Amendments	Amount
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

* Antifungal properties, ** Antibacterial properties, *** Solvent

Method
1. Mix vegetable juice, deionized water and agar
2. Autoclave at 121°C for 15 minutes
3. Let the medium cool down a bit before placing in water bath
4. Cool down the media in water bath ($\sim$ 50°C)
5. Dissolve rifampicin in a test tube with DMSO
6. Dissolve the remaining amendments in test tubes with 10 ml sterile water; vortex
7. Add the amendments into the 50°C medium
8. Rinse all amendment tubes with sterile water and add to the medium
9. Mix the media slowly with a magnetic stirrer
10. Pour into Petri dishes
11. Cool the plates in room temperature
12. Store in plastic bags in a dark refrigerator

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Potato Dextrose Agar (PDA)

Ingredients:
Potato dextrose agar (Oxoid CM139) 39 g
Distilled water 1000 ml

Method
1. Soak potato dextrose agar in a small amount of the water
2. Boil the remaining water
3. Add the potato dextrose agar, stir constantly
4. Autoclave at 121°C for 15 minutes
5. Mix with a magnetic stirrer
6. Pour into Petri dishes
7. Cool the plates in room temperature

Malt Extract Broth

Ingredients:
20 g malt extract
1000 ml deionized water

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

Appendix II. Maps of Pildammsparken and Bokskogen

Sampled, and infected trees in Pildammsparken

Sampled, and infected trees in Bokskogen in Torup