Norwegian University of Life Sciences

Preface

This thesis was written at the Norwegian University of Life Sciences, Department of Plant and Environmental Sciences (IPM). My field work was carried out in the beautiful beech forest in Larvik (Vestfold County, Norway) in the spring and summer of 2012. The laboratory work was carried out at Bioforsk Plant Health in Ås. The field work was supported by the Norwegian Genetic Resource Centre, the municipality of Larvik, the county governor of Vestfold, and the Norwegian Public Road Administration.

Acknowledgements

I owe my greatest thanks to my inspiring and supportive supervisor Venche Talgø at Bioforsk Plant Health. Thank you for always taking your time to answer all my questions, for patiently reading through my thesis, and for always making me laugh.

To my supervisors at UMB/Bioforsk, Arne Stensvand and May Bente Brurberg, thank you for good advices and for spending time to read through my thesis and for giving me constructive feedback.

I am also grateful to Trude Slørstad and Grete Lund for their helpful instructions in the lab, to Maria Luz Herrero and Solveig Haukeland for enlightening me about oomycetes and slugs, to Willy Couanon and Louisa Kitchingman (the Food and Environmental Research Agency, UK) for assisting me on some of my field work in Larvik, and to all the helpful staff at Bioforsk Plant Health. I would also take the opportunity to thank my friends and family for their endless support.

Norwegian University of Life Sciences

Ås, 16.12.2013

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Abstract

During the past fifteen years there has been a widespread decline of mature European beech (*Fagus sylvatica*) in Europe and North America. The trees have shown typical symptoms of *Phytophthora* infection: root- and collar rot, bleeding cankers on the stem, and crown dieback. The isolation of *P. cambivora* from a beech tree and *P. plurivora* from a stream in Norway's largest beech forest in Larvik in 2011, led to an extensive *Phytophthora* survey in 2012. Every tree in the beech forest in Larvik with a circumference above 20 cm was examined for bleeding cankers. Samples from the leading edge of the cankers were collected from selected trees, and water and soil were baited with *Rhododendron* leaves. Isolation was carried out on *Phytophthora* selective agar. The survey resulted in 54 trees with bleeding cankers on the stem. Two localities had a denser concentration of diseased trees than the rest of the forest, with 16 of 329 trees (4.9 %) and 12 of 680 trees (1.8 %). Four *Phytophthora* species were recovered in the survey and identified on the basis of morphology and the Internal Transcribed Spacer (ITS) region of ribosomal DNA. The survey yielded *P. cambivora* from beech, *P. plurivora* from soil and water, and *P. gonapodyides* and *P. lacustris* from water. All four species were pathogenic to beech seedlings in a pathogenicity test performed in 2013. In addition, experiments with Spanish slugs (*Arion vulgaris*) were conducted to examine whether slugs could act as vectors of *Phytophthora* spp. Results showed that hyphae were viable after passage through the digestive system of Spanish slugs. However, attempts to infect beech seedlings with slugs that had fed on *Phytophthora*, did not succeed.

Sammendrag

I løpet av de siste femten årene har det vært det vært observert store skader på bøk (*Fagus sylvatica*) i Europa og Nord-Amerika. Trærne viser tydelige symptom på angrep av *Phytophthora*: råte på røtter og stamme, blødende sår på stammen og glissen krone. Isoleringen av *P. cambivora* fra et bøketre og *P. plurivora* fra en bekk i Norges største bøkeskog i Larvik i 2011, førte til en grundig kartlegging av *Phytophthora* i 2012. Samtlige bøketrær i bøkeskogen i Larvik med en omkrets på minimum 20 cm ble undersøkt for blødende sår på stammen. Prøver av overgangen mellom sykt og friskt vev ble samlet fra utvalgte trær, og vann og jord ble baitet med *Rhododendron*-blader. Isoleringen ble utført på *Phytophthora* selektiv agar. Kartleggingen resulterte i funnet av 54 trær med blødende sår på stammen. To lokaliteter hadde en høyere konsentrasjon av syke trær enn resten av skogen med 16 av 329 (4.9 %) og 12 av 680 trær (1.8 %). Fire *Phytophthora* arter ble funnet i kartleggingen som ble identifisert på bakgrunn av morforlogi og sekvensering av «Internal Transcribed Spacer» (ITS) området på ribosomalt DNA. Kartleggingen førte til funnet av *P. cambivora* på bøk, *P. plurivora* i jord og vann, og *P. gonapodyides* og *P. lacustris* i vann. Alle fire arter var patogene på bøk i et smitteforsøk utført i 2013. I tillegg til kartleggingen, ble det utført eksperimenter med brunskogsnegl (*Arion vulgaris*) for å undersøke hvorvidt sneglen kan opptre som en vektor for *Phytophthora* spp. Resultatet viste at hyfer var levende etter transport gjennom fordøyelsessystemet til sneglen. Forsøk på å smitte små bøketrær med *Phytophthora* ved hjelp av snegler lyktes til gjengjeld ikke.

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1. Introduction

1.1. European beech

European beech (*Fagus sylvatica* L.) is a deciduous tree in the beech family (*Fagaceae*) (Coombes 2000). It is widely distributed in Central and Western Europe (Bolte *et al*. 2007), and throughout the north-eastern parts of USA (Houston 2004).

Beech is an economically important tree species. The timber is strong and versatile, and commonly used for furniture, parquet, flooring and plywood (Evans 1984). Beech is also used for smoking meat and cheese (Gómez-Ruiz *et al.* 2006; Guillén & Ibargoitia 1996), and also in the beer brewing industry for barrels for ageing beer (Anonymous 2013a).

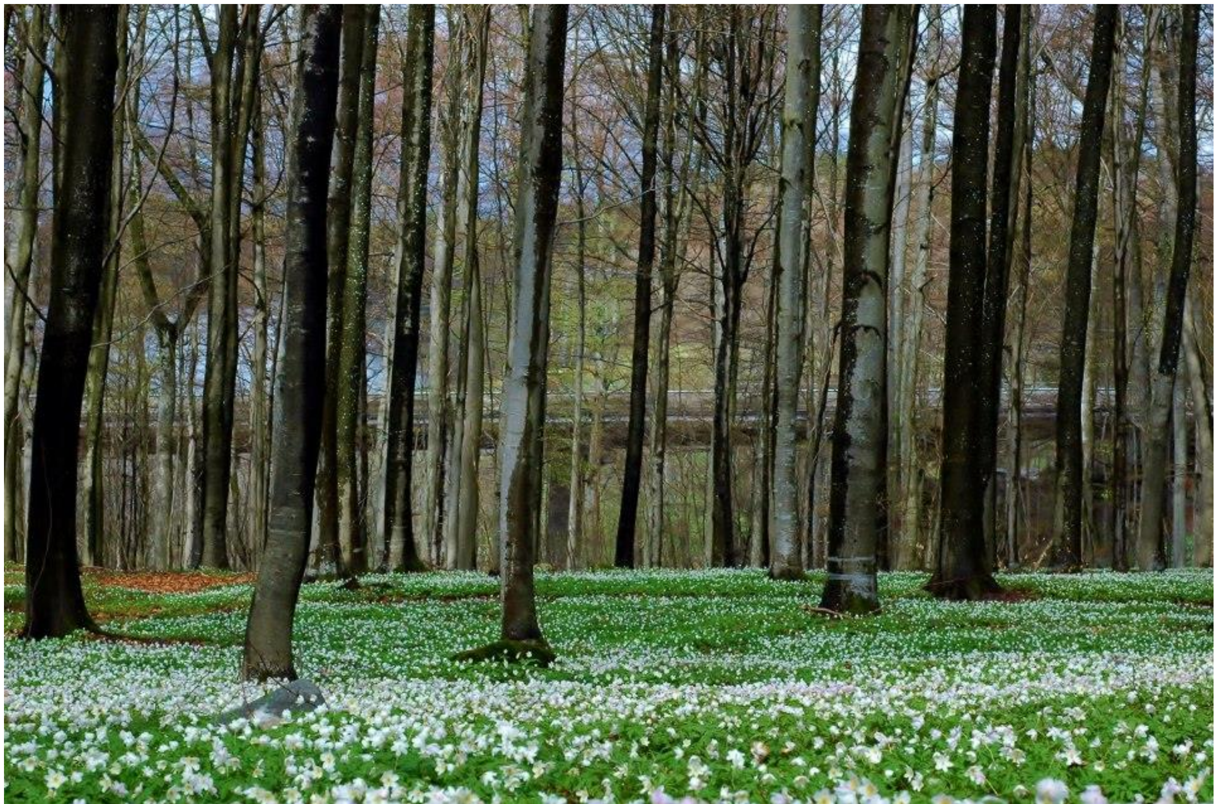

Fig. 1 A beech forest in Larvik, Vestfold County, Norway. Thousands of wood anemones (*Anemone nemorosa* L.) are covering the ground in the spring. Photo: Kari H. Telfer

Beech trees can live for 250 - 300 years, and may reach heights above 40 meters under good conditions (Coombes 2000). It is one of the major tree species in European deciduous forests due to its ability to adapt to different habitats and to changing environmental conditions

(Packham *et al.* 2012). It is also widely used as an ornamental tree in gardens, parks, and semi-natural environments. Beech trees may provide habitats for several species of fungi and animals. In beech tree stands in Norway, several endangered species have been observed, including the fungi *Dentipellis fragilis* (Pers.) Donk, *Inonotus nodulosus* (Fr.) P. Karst. and *Hygrophorus penarius* Fr., the lichen *Usnea florida* (L.) F. H. Wigg, and the moss *Metzgeria fruticulosa* (Dicks.) A. Evans (Aarrestad *et al.* 2001). Beech nuts are an important food source for many wildlife species, and were also formerly used to feed domestic pigs and turkeys (White & More 2005).

During the last few years several beech forests in Germany, Ukraine and Slovakia have been added to UNESCO's World Heritage List because *"…they represent an outstanding example of undisturbed, complex temperate forests and exhibit the most complete and comprehensive ecological patterns and processes of pure stands of European beech across a variety of environmental conditions. They contain an invaluable genetic reservoir of beech and many species associated and dependent on these forest habitats."* (UNESCO 2011)

Fig. 2 Map of Northern Europe pointing out Norway (orange color) and the city of Larvik (black dot) in Vestfold County.

In Norway, beech is distributed in the outer Oslo fjord area, in Grimstad in the south of Norway, and in Seim on the west coast north of Bergen (Aarrestad *et al*. 2001). The latter being the world's northernmost beech stand. The largest forest stand of beech in Norway is situated in Larvik municipality in Vestfold County (Figs 1 and 2). The forest covers an area of approximately 30 hectares. It is situated along a moraine ridge (Bjune *et al*. 2013) and has been considered a landscape protection area since 1980 (Anonymous 2012).

The forest has been referred to as a public park since 1821, and is of great importance to the citizens of Larvik. It is widely used for recreation, concerts and celebrations (Anonymous 2012). There are more than 60 burial mounds in the forest which originate from the younger iron age, and an antiquity path that is

estimated to be one thousand years old (Bjørvik 2007). In 2006, the beech forest was included in the Gea Norvegica Geopark supported by UNESCO (Anonymous 2013b).

1.2. The genus *Phytophthora*

During the past fifteen years, there has been a great decline of beech in Europe and in USA. In the beginning, researchers had difficulties identifying what caused the decline, because of rapid colonization by secondary invaders, e.g. species of the fungal genera *Armillaria*, *Fomes*, *Inonotus* and *Kretzschmaria*, and different wood boring insects (Jung *et al.* 2005). Eventually, the causal agents proved to be different species of the microorganism *Phytophthora* (Jung *et al.* 2005).

Phytophthora was originally classified as fungi because of similarities in features and behavior with true fungi, but is now considered a genus within the phylum Oomycota, the water molds, which belongs to the eukaryotic kingdom Stramenopila (Agrios 2005). The name *Phytophthora* means "plant destroyer" from the two Greek words phytón (plant) and phthorá (destroyer) (Deacon 2005).

There are more than 120 described species of *Phytophthora* in the world, and the number increases every month (Kroon *et al.* 2012; Scott *et al.* 2013). The most well-known species is probably *P. infestans* (Mont.) de Bary causing late blight in potato and tomato all over the world, and being responsible for the Irish potato famine in the 1840s (Agrios 2005). Most of the known *Phytophthora* species are pathogenic to plants, and they cause devastating diseases on crops, forests and ornamentals worldwide, leading to enormous economic and environmental losses (Agrios 2005). E.g. late blight in potato is estimated to cost the global potato industry US$ 6.7 billion every year (USAblight 2013).

Introduced species of *Phytophthora* are responsible for severe damages due to little resistance in the endemic plants. One example is *P. cinnamomi* Rands which probably has its origin in Papua New Guinea (Zentmyer 1988), but is now spread worldwide where it has caused extensive damage to native plant species for more than 150 years (Tainter & Baker 1996). In Australia alone, there are more than 1000 native plant taxa which are known to be susceptible to infection (Cahill *et al.* 2008). *P. cinnamomi* also cause disease on other economically important crops around the world like avocado, macadamia, pineapple, walnut, cherry, and peach (Agrios 2005; Erwin & Ribeiro 1996).

Phytophthora species cause different diseases on a broad range of hosts. Typical diseases are root rot, damping-off, foliar and twig blight, and rots of collar, crown, tubers, corms, buds and fruits (Agrios 2005). Some species, like *P. infestans* and *P. lateralis* Tucker & Milbrath, cause disease on a very limited number of hosts, while others, like *P. cinnamomi* and *P. ramorum* Werres, De Cock & Man in 't Veld, have numerous hosts (Erwin & Ribeiro 1996; Grünwald *et al.* 2008).

1.2.1. Morphology of *Phytophthora*

Both true fungi and *Phytophthora* have filamentous hyphae with apical growth, they reproduce by spores, and they have similar strategies of infecting plants (Deacon 2005). Still, there are several characteristics that differs: the hypha of *Phytophthora* are coenocytic unlike most fungal hypha, the cell wall consists of cellulose and betaglucan instead of chitin, the nuclei of *Phytophthora* are diploid, and the cell membrane consists of plant sterol and not ergosterol, which is the characteristic fungal sterol (Deacon 2005).

Phytophthora is a microorganism that consists of non-septate, hyaline, elongated mycelium. It can reproduce both sexually and asexually. Asexual reproduction involves the formation of a sporangium with several nuclei. Each nucleus becomes a biflagellate, reniform shaped zoospore which is released when the tip of the sporangium breaks down. In some species, e.g. *P. infestans* and *P. ramorum*, the whole sporangium can be dispersed by wind (Hardham 2007). The sporangia are variable in size and shape depending on the species, but usually subspherical, ovoid, limoniform, pyriform, or obpyriform (Ribeiro 1978). In some species, the sporangia have one or two papilla, and other species lack this feature completely. Sporangiophores bear the sporangia, and in some species the sporangiophore can continue to grow through empty old sporangia (internal and extended proliferation), in other species it can emerge from beneath old sporangia (external proliferation), or a new sporangia can develop inside the old sporangia (internal, nested proliferation) (Erwin & Ribeiro 1996).

Another kind of asexual structure is the chlamydospore which is produced in some *Phytophthora* species, e.g. *P. ramorum*, for survival under unfavorable conditions (Erwin & Ribeiro 1996). When the conditions are suitable, the chlamydospores germinate and form sporangia.

Sexual reproduction occurs when the female sex organ, called oogonium, fuse with the male sex organ, the antheridium, to form an oospore (Deacon 2006). Some *Phytophthora* species are self-fertile, known as homothallic. Others, the heterothallic species, require fertilization between two mating types, called A1 and A2, to form oospores (Parke & Eberhart 2013). Like the chlamydospore, the oospore serves as a survival structure during poor conditions. A dormant period is usually needed for the oospore to be able to germinate. The germination results in either a diploid hyphae or a sporangium that can release diploid zoospores (Deacon 2005).

The primary role of the one-celled zoospores is short distance dispersal. The spores may move actively a few millimeters in water by means of their flagella. Studies have shown that they can swim for a few minutes to several hours (Ribeiro 1978). Zoospores are attracted to root exudates chemotactically. When they reach a host plant, they stop swimming and adhere to the host cell by secreting an adhesive material (Hardham & Gubler 1990). Then they shed their flagella, encyst, and produce germ tubes for infection of host cells (Ribeiro 1978).

1.2.2. Common pathways of *Phytophthora*

The movement of plants by human activities is generally accepted as the main contributor to the spread of plant pathogens (Brasier 2008). On a global scale, international plant trade facilitates the spread of plant pathogens from one part of the world to another and increases the risk of establishment of new pathogens in nurseries and natural ecosystems (Brasier 2008). Invasive pathogens can cause enormous damage to endemic plant species due to the lack of co-evolution between pathogen and host which leads to limited resistance in the host (Hansen 2008).

On shorter distances, *Phytophthora* can be spread passively by the movement of growth media, organic matter, irrigation water, and soil on footwear, animals, tires, tools and equipment (Hansen *et al.* 2000; Scott *et al.* 2013; Webber & Rose 2008). The different types of *Phytophthora* spores can be spread by wind, rain splash, streams and soil water (Davidson *et al.* 2005; Hansen 2008). Different studies have also shown that slugs, snails, insects and birds can act as vectors of *Phytophthora* (El-Hamalawi & Menge 1996; Evans 1973; Keast & Walsh 1979; Konam & Guest 2004).

1.2.3. *Phytophthora* – a threat to tree species around the world

Phytophthora species cause widespread mortality of forest trees worldwide, and therefore pose a major threat to biodiversity and sustainability of forest ecosystems. Three examples of *Phytophthora* species responsible for major epidemics are *P. pinifolia* Alv. Durán, Gryzenh. & M.J. Wingf., *P. ramorum* and *P. cinnamomi*. *P. pinifolia* has infected large areas of the Monterey pine tree (*Pinus radiata* D. Don) in Chile. Infected areas increased from 3300 hectars in 2004 to 54 000 hectares in 2006, but have now decreased due to management strategies and environmental conditions (Ahumada *et al.* 2013). In western parts of North America, *P. ramorum* is responsible for the "sudden oak death". Since the late 1990s it has caused the death of millions of tan oak trees (*Notholithocarpus densiflorus* (Hook. & Arn.) Manos, Cannon & S.H.Oh) and coast live oak (*Quercus agrifolia* Née) (Frankel 2012). *P. cinnamomi* is assumed to be responsible for the widespread death of American chestnut trees (*Castanea dentata* (Marsh.) Borkh.) in the mid-1800s prior to the chestnut blight epidemic (Jung *et al.* 2013; Tainter & Baker 1996). In the early 1900s, *P. cinnamomi* caused Littleleaf Disease on shortleaf pine (*Pinus echinata* Mill.) which destroyed more than 2 million hectares of forest in the US (Tainter & Baker 1996). Later, it was introduced to Australia where it killed 20 % of the eucalyptus trees (*Eucalyptus marginata* Donn ex Sm.) (Podger 1972).

In Europe, *Phytophthora* species are responsible for the dieback of many important tree species. Some examples are root and collar rot of alder trees (*Alnus* spp.) caused by subspecies of *P. alni* Brasier & S.A.Kirk (Jung *et al.* 2013), widespread mortality of Japanese larch (*Larix kaempferi* (Lamb.) Carr.) in the U.K caused by *P. ramorum* (Brasier & Webber 2010), oak decline (*Quercus* spp.) caused by *P. cinnamomi* and *P. quercina* T. Jung in Mediterranean climate regions and *P. quercina*, *P. cambivora* (Petri) Buisman and *P. plurivora* T. Jung & T.I. Burgess in temperate areas, and ink disease of chestnut (*Castanea sativa* Mill.) caused by *P. cambivora* in south-east Europe and *P. cinnamomi* in Western Europe (Jung *et al.* 2013).

Decline of mature beech trees have occasionally been reported for several decades (Bisiach *et al.* 1980; Day 1938), but in the last ten to fifteen years beech decline have increased rapidly throughout Europe and north-eastern USA (Jung *et al*. 2005; Jung 2009). The trees show typical *Phytophthora* symptoms, including bleeding cankers, either at the base (collar rot) or higher up on the stem (aerial bleeding cankers), a distinct line between healthy (whitish) and

diseased (reddish) tissue underneath the bark, chlorotic foliage, crown dieback, root rot, and eventually death of the whole tree (Jung *et al*. 2005).

Seventeen species of *Phytophthora* have been isolated from declining beech trees in Europe and North America, and pathogenicity tests to fulfill Koch's postulates have been performed with the majority of the detected species (Table 1). The most common species in Europe are *P. cambivora* and *P. plurivora* (Jung *et al.* 2005). Jung & Burgess (2009) claimed that these two species are the two most threatening *Phytophthora* species in European natural and semi-natural environments because of their aggressiveness and wide host range.

Table 1 *Phytophthora* species associated with beech (*Fagus sylvatica*) in Europe and North America, including references and Koch's postulates.

***Phytophthora* spp.**	**Reference**	**Koch's postulate**
cactorum	Jung *et al.* (2005)	Vettraino *et al.* (2008)
cambivora	Day (1938)	Day (1939)
cinnamomi	Stamps *et al*. (1990)	Day (1939)
citricola I	Jung & Burgess (2009)	Weiland *et al.* (2010)
gonapodyides	Jung *et al.* (2005)	Jung & Blaschke (1996)
inflata[3]	Jung *et al*. (2005)	Jung *et al.* (2005)
inundata	Not found	Brasier & Jung (2003)
kernoviae	Brasier *et al.* (2004)	Brasier *et al.* (2005)
lacustris	Not found	Nechwatal *et al.* (2012)
megasperma	Jung (2009)[1]	-
plurivora[4]	Jung & Blaschke (1996)	Jung & Blaschke (1996)
pseudosyringae	Motta *et al.* (2003)	Jung *et al.* (2003)

psychrophila	Jung (2009)[2]	-
quercina	Jung (2009)[1]	-
ramorum	Brasier *et al.* (2004)	Brasier *et al*. (2002)
syringae	Day (1938)	Day (1939)
taxon 'Pg chlamydo'	Jung (2009)[2]	-
uligonosa	Jung (2009)	Brasier & Jung (2003)
undulata	Jung & Blaschke (1996)[2]	-

[1]Found in nurseries; [2]From soil surrounding beech; [3]Possibly similar to *P. plurivora* according to Jung & Burgess (2009); [4]Referred to as *P. citricola* until 2009 (Jung & Burgess 2009)

Until 2009, there were no reports on *Phytophthora* on beech in Norway, but during 2009 bleeding cankers caused by *P. cambivora* were observed on beech in Bergen (Talgø *et al*. 2010). Later, *P. plurivora* was isolated from bleeding cankers on beech in Stavanger and from a stream running through the beech forest in Larvik (Talgø *et al.* 2012). In 2011, *P.cambivora* was also isolated from a beech tree in Larvik (Talgø *et al*. 2012).

1.3. Other diseases and pests on beech

According to the literature, there are several fungi, insects and vertebrates attacking beech trees, but most of them are not considered economically important.

The beeswax bracket fungi (*Ganoderma pfeifferi* Bres.) may kill mature beech trees by decaying roots, stems and branches, while the artist's conk (*G. applanatum* (Pers.) Pat.) and the southern bracket (*G. adspersum* (Schulzer) Donk) cause butt and branch rot of old beech trees (Evans 1984). Giant polypore (*Meripilus giganteus* (Pers.) P. Karst.), also a bracket fungus, causes white rot and decays the roots of beech, and the ascomycete brittle cinder

(*Kretzschmaria deusta* (Hoffm.) P.M.D. Martin) (Fig. 3) attacks the stem base and mature roots which leave the tree liable to wind throw (Thomson & Skov 2011). Tinder polypore (*Fomes fomentarius* (L.) Fr.), dryad's saddle (*Polyporus squamosus* (Huds.) Fr.) and golden scaly cap (*Pholiota adiposa* (Batsch) P. Kumm.) attack mature trees, often through wounds (Solheim 2010; Thomson & Skov 2011). Different species of the armillaria root rot mushroom (*Armillaria* spp.) and *Neonectria* spp. attack weakened trees (Fig. 4) (Thomson & Skov 2011).

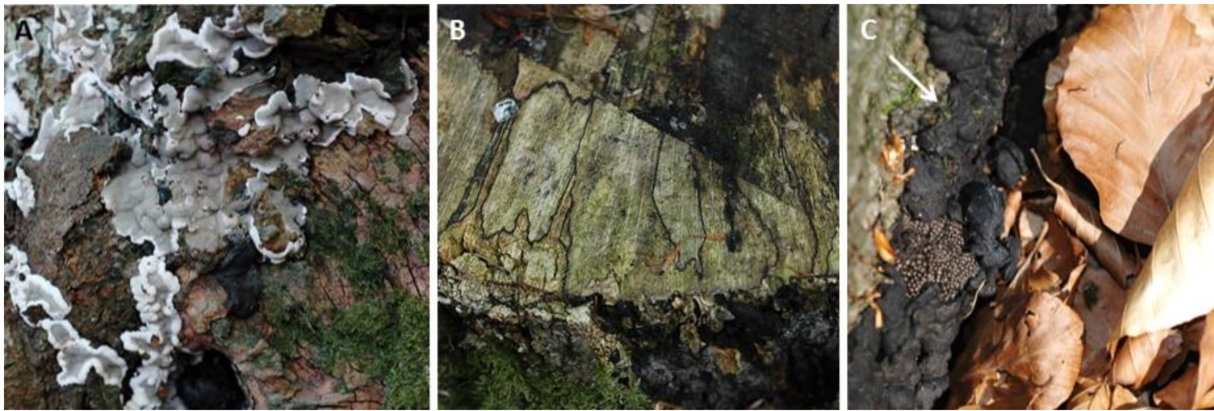

Fig. 3 Brittle cinder (*Kretschmaria deusta*) growing on beech trees (*Fagus sylvatica*). **A**, the anamorph stage; **B**, beech stump with black demarcation lines produced by *K. deusta*; **C**, the teleomorph stage (white arrow). Photos: Kari H. Telfer

Beech trees are also attacked by defoliating insects [*Rhynchaenus fagi* (L.), *Phyllobius viridicollis* (Fabricius) and *Phalera bucephala* (L.)], gall making insects [*Hartigiola annulipes* (Hartig) and *Mikiola fagi* (Hartig)], aphids and scale insects [*Phyllaphis fagi* (L.) and *Cryptococcus fagisuga* (Lindinger)] (Evans 1984), and wood-boring insects [*Agrilus bilineatus* (Weber), *A. viridis* (L.), *Taphrorychus bicolor* (Herbst.) and *Trypodendron domesticum* (L.)] (Jung *et al.* 2005).

According to Packham *et al.* (2012), beech bark is exposed to browsing by red deer (*Cervus elaphus* L.) and roe deer (*Capreolus capreolus* L.), and roots can be damage by wild boar (*Sus scrofa* L.). Browsing on bark and roots cause reduction in sapling and seedling growth.

The most serious damaging agents of the above mentioned species are *Neonectria* spp. and beech scale (*C. fagisuga*). The combination of insect, fungus and abiotic factors have been known since 1934 to be the causal agents of beech bark disease (BBD) (Ehrlich 1934; Houston 1994). The beech scale feeds on the thin outer bark of beech trees, leaving the bark drying and cracking, which subsequently allows *Neonectria* species to infect the tree (Ehrlich

1934). Typical symptoms are a white waxy secrete from the beech scale, and bark necrosis and crater-like scars from the *Neonectria* infection (Tainter & Baker 1996). The crown becomes thin with chlorotic foliage, and if the fungus girdles the tree, it dies (Tainter & Baker 1996). *N. ditissima* (Tul. & C. Tul.) Samuels & Rossman and *N. faginata* (M.L. Lohman, A.M.J. Watson & Ayers) Castl. & Rossman are the most common *Neonectria* species on beech (Cale *et al.* 2012).

Fig. 4 A, young beech tree (*Fagus sylvatica*) with crater like scars caused by *Neonectria*; **B**, *Neonectria* sp. growing in a bleeding canker caused by *Phytophthora*; **C**, *Neonectria* sp. growing on the bark of a declining beech tree. Photos: Kari H. Telfer

In Norway, *Neonectria* spp., scale insects (Talgø *et al.*, unpublished data), brittle cinder (Telfer & Talgø 2013), and tinder polypore (Solheim 2010) have been observed on mature beech trees, mainly in the Larvik area.

1.4. Hypotheses and aims of the study

The increasing number of declining beech trees in Europe (e.g. Jung 2009; Jung *et al.* 2005) and the discovery of *Phytophthora* spp. in the beech forest in Larvik in 2011, led to great concern about the prospects of the forest in Larvik, and also the prospects of a small beech stand in a park in Oslo where bleeding cankers had been reported. The municipality of Larvik and the county governor of Vestfold wanted a thorough survey of *Phytophthora* in the beech forest in Larvik before preparing a management plan for the forest.

Another concern was Norwegian Public Road Administration's (NPRA) plan to build a tunnel for the highway (E18) running next to the beech forest. The tunnel will allow the beech forest to be extended from its present location to the shore of the Farris Lake, like it used to be before the current highway was built. NPRA will transport excessive soil from the tunnel work to another part of Larvik in 2014, and this presents a great threat to native plant species in the selected area if the soil contains *Phytophthora* spp.

The Farris Lake is the main water supply for private households in the Larvik area (Anonymous 2013c). Water contaminated with *Phytophthora* spp. may spread the pathogens to private gardens through irrigation.

These concerns led to the following hypotheses: (1) *Phytophthora* is widely distributed throughout the beech forest, (2) *Phytophthora* is present in soil and water in the construction area of NPRA, (3) *Phytophthora* is present in the drinking water from the Farris Lake, and (4) *Phytophthora* species isolated from beech bark, soil and water are pathogenic to beech.

The aim of this study was to map the distribution of *Phytophthora* on beech trees, in soil and in water in the Larvik area, and to perform a pathogenicity test with possible *Phytophthora* species obtained during the survey.

Preliminary results from the survey have been published in trade journals (Telfer *et al.* 2013a; Telfer *et al.* 2013b) and at a national conference in Norway (Telfer *et al.* 2013c).

Slugs, snails and insects are known from the literature to be possible vectors of *Phytophthora* (e.g. El-Hamalawi & Menge 1996; Alvarez *et al.* 2009). In the beech forest in Larvik, a large number of the invasive Spanish slugs (*Arion vulgaris* Moquin-Tandon) were observed, some of them migrating up and down the trunk of the trees (personal observation) (Fig. 9 A). These observations led to the following hypotheses: (5) *Phytophthora* hyphae and spores may survive after passing through the slug's digestive system, (6) *Phytophthora* may be transmitted from slugs to beech trees, and (7) slugs found close to beech trees with *Phytophthora* symptoms contain *Phytophthora*.

The aim of the latter study was to investigate the Spanish slug's potential as a vector for dispersing *Phytophthora*.

2. Materials and methods

2.1. Field survey, isolation and identification of *Phytophthora*

Field survey. To test the hypothesis that *Phytophthora* spp. is widely distributed in the beech forest in Larvik (Fig. 1), a field survey was conducted in the spring and summer of 2012. A limited area of the forest was surveyed again in 2013. All beech trees in the forest with a circumference above 20 cm were examined for the presence of bleeding cankers on the stem. Trees were examined from the stem base to approximately 1 m up the stem. The coordinates for all diseased trees were recorded on a GPS (Garmin GPSmap 60CSx, Kansas, USA). The circumferences of all trees with bleeding cankers were measured at breast height. Two areas in the forest had a higher concentration of trees with bleeding cankers than the rest of the forest, and in these two areas all the trees were counted to be able to quantify the damage. Seven trees with bleeding cankers were tested with lateral flow devices (LFD) (Pocket Diagnostic®, Forsite Diagnostics Ltd, York, UK), a field kit that can test symptomatic tissue for the presence of *Phytophthora* spp. The reported, symptomatic beech trees in Oslo were also survived for *Phytophthora*.

Direct isolation of *Phytophthora*. Direct isolation was performed from beech trees with bleeding cankers in Larvik and Oslo. Cambium samples covering the border between diseased and healthy tissue (the leading edge) were cut off from symptomatic trees and placed in plastic flasks containing autoclaved pond water that had been frozen and still partly contained solid ice. The flasks were brought back to the laboratory at Bioforsk. The samples were cut in 0.2 – 0.3 × 1 – 3 cm segments and placed in 9 cm Petri dishes (5 – 7 segments per dish) containing *Phytophthora* selective agar with hymexazol (P_{10}ARPH, Appendix I) to prevent the growth of true fungi, bacteria and *Pythium* spp. The plates were incubated in day light at room temperature (± 20°C). After a few days of growth, hyphae resembling *Phytophthora* spp. were transferred to 9 cm Petri dishes with potato dextrose agar (PDA, Appendix I) to achieve pure cultures.

Indirect isolation of *Phytophthora* from water. This was done by baiting with *Rhododendron* leaves according to the protocol of Sutton *et al.* (2009). Leaves from *Rhododendron* 'Cunningham's White' and pieces of polystyrene, serving as floating devices,

were placed in 14 small polyester bags (3 *Rhododendron* leaves and 1 piece of polystyrene in each bag). The baiting bags were placed in ditch water, streams (Fig. 5 A), rivers and lakes in the area surrounding the beech forest.

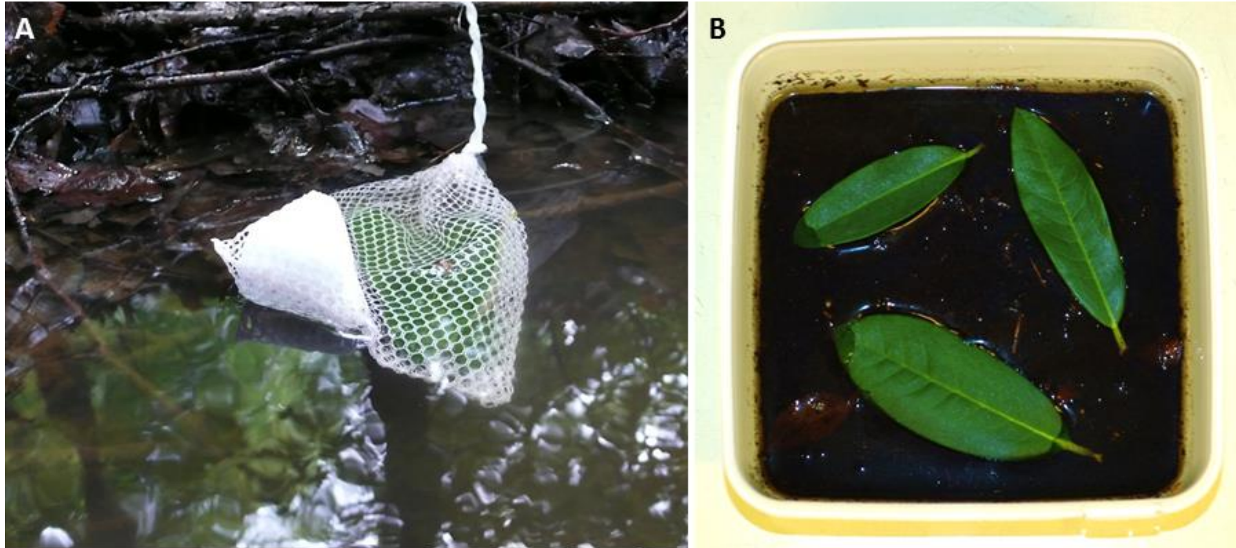

Fig. 5 Baiting with *Rhododendron* 'Cunningham's White' leaves. **A**, baiting in ditch water in the beech (*Fagus sylvatica*) forest in Larvik. Three leaves were placed in a polyester bag together with polystyrene serving as a floating device; **B**, baiting on soil mixed with sterile water. Photo: Venche Talgø (left) and Kari H. Telfer (right)

To test the hypothesis that *Phytophthora* is present in the drinking water from the Farris Lake, three baiting bags were placed in running water inside Gopledal waterworks (water intake is located 200 meters from the shore at a depth of 40 meters). Each bag was placed in a three liter bucket underneath a tap with unfiltered water (3756 liters per day), filtered water (1600 liters per day) and filtered water with added chlorine (1289 liters per day), respectively.

After four days all the bags were brought back to the laboratory and stored at 3°C. After three days the *Rhododendron* leaves were washed in tap water, cut in 0.5 × 0.5 cm pieces covering the leading edge of spots, and placed on P_{10}ARPH agar plates, six pieces on each plate. All plates were incubated in day light at room temperature (± 20°C). After four days, hyphae resembling *Phytophthora* were transferred to acidified potato dextrose agar (PDAS, Appendix I) to minimize bacterial growth, and incubated in day light at room temperature (± 20°C).

Indirect isolation of *Phytophthora* from soil. *Rhododendron* leaves were used to detect *Phytophthora* from soil according to Orlikowski *et al.* (2011). Ten soil samples were taken from ten different locations in the NPRA construction area. Approximately 0.5 liter of soil per

sample was put in a plastic bag and brought back to the laboratory. The soil samples were then placed in plastic boxes, flooded with distilled water and left for particles to settle overnight. Four healthy leaves of *Rhododendron* 'Cunningham's White' were placed on the water surface of each box the next day (Fig. 5 B). The boxes were covered with lids, but not air sealed, and placed at room temperature (± 20°C). After seven days, the *Rhododendron* leaves were washed in tap water, blot-dried with paper towels and cut into approximately 0.5 × 0.5 cm pieces covering the leading edge of spots. Six pieces were placed on plates of P_{10}ARPH agar and incubated in day light at room temperature (± 20°C). After two days, hyphae resembling *Phytophthora* were transferred to PDA to achieve pure cultures.

Identification of isolates. The isolates were morphologically identified to *Phytophthora* spp. and DNA from the pure cultures that resembled *Phytophthora* were extracted, their Internal Transcribed Spacer (ITS) region in the rDNA were amplified by PCR using ITS1 and ITS4 primers, and the PCR products were submitted for sequencing at GATC (Germany). Raw sequences were trimmed and assembled and used for identification of the isolates based on searches in public databases (GenBank and *Phytophthora* Database).

2.2. Morphology, growth rates and optimal temperatures

Temperature - growth relationships. Radial growth rates of one selected isolate from each of four different *Phytophthora* species were measured on PDA. Five mm plugs of 18 days old cultures on PDA were placed in the center of 9 cm PDA plates and incubated at room temperature (± 20°C) for 24 hours to make sure the plugs were attached to the agar with hyphal growth. Three replicates per isolate were incubated at 5, 10, 15, 20, 25, 30, and 35°C in the dark. Radial growth rate was recorded along two lines intersecting the center of the colony at day 3, 7, 10, and at the end of the experiment, according to the protocol of Hall (1993). Measurements ended when the fastest growing culture of each isolate was approximately 1 cm from the edge of the Petri dish, that means at its optimum temperature (Fig. 6). Average growth (mm/day) for each temperature and standard errors (S.E) were calculated (Excel, Microsoft Corp., Seattle, WA) for all four *Phytophthora* species.

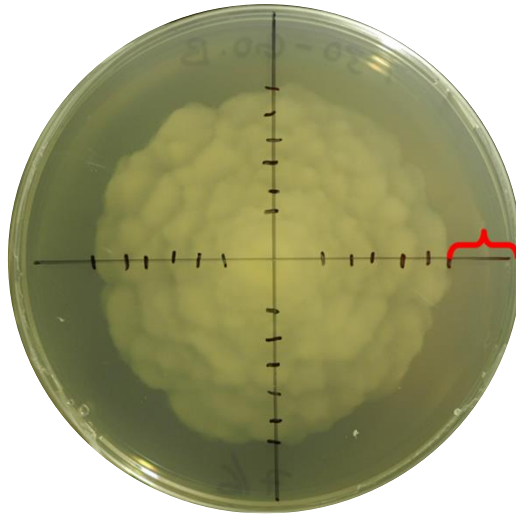

Fig. 6 The temperature-growth experiments were stopped when the fastest growing culture of each *Phytophthora* species had grown to approximately 1 cm from the Petri dish edge (see red curly bracket) at its optimal temperature. Photo: Kari H. Telfer

Colony morphology. Five mm agar plugs from one selected isolate from each of four different *Phytophthora* species were transferred to 9 cm Petri dishes with V8 juice agar (Appendix I) and PDA. The plates were incubated in day light at room temperature (± 20°C). Colony morphology was assessed from 7- and 10-day-old cultures, respectively.

Morphology of sporangia. Sporangia from different cultures were generally produced by cutting a 1 cm^3 square off the growing edge of 4-day-old colonies grown on V8 agar at room temperature (± 20°C). The agar pieces were placed in separate 9 cm Petri dishes, flooded with autoclaved pond water, incubated in the dark at 15°C for 24 - 36 hours (Talgø *et al.* 2007), and examined daily in the microscope for sporangia formation.

Sporangia from a culture from one of a the isolate (*P. cambivora*) were produced as described above, except that a 0.1 × 0.1 cm piece of a *Rhododendron* 'Cunningham's White' leaf was placed in the Petri dish together with the agar piece. Sporangia growing out of the leaf were used for measurements.

Length, width and other characteristic features were measured on 25 randomly selected sporangia per isolate using a light microscope (Leica DM2000) at 400x magnification, a microscope camera (Leica DFC320) and specialized computer software used for photography and measurements (Leica application, version 2.8.1). Length : width ratio was calculated based on mean values for each isolate.

Morphology of oogonia, antheridia and oospores. Homothallic species produced oogonia, antheridia and oospores in single culture on V8 agar incubated in day light at room temperature (± 20°C). The diameter of oogonia and oospores, and the oospore wall thickness were measured using the above described equipment.

Formation of sexual organs and determination of mating types of heterothallic species were attempted by pairing them with tester strains of *P. cryptogea* Pethybr. & Laff. (Werres *et al.* 2001) on carrot piece agar (CPA, Appendix I). Two isolates were also paired with an A2 mating type of *P. cambivora.* The pairing was done by placing a 5 mm agar plug of the isolate to be tested and the tester isolate approximately 3 cm apart on a 9 cm Petri dish with CPA. Mating type A1 (*P. cryptogea* isolate BBA 65909) and A2 (*P. cryptogea* isolate BBA 63651) of the tester strains were also paired as a control. The plates were incubated in day light at room temperature (± 20°C) and checked for gametangial formation regularly for 5 weeks.

2.3. Pathogenicity test

Pathogenicity tests with one isolate of four species of *Phytophthora* were performed in February 2013 on 55 potted beech seedlings dug up in a beech stand in Nordskogen (Ås municipality, Norway). The seedlings varied in height from 30 - 80 cm when potted. The seedlings were cultivated in 3.5 (the youngest seedlings) and 5 liter containers (older seedlings). Before the inoculation the tallest seedlings were pruned back to a height of approximately 50 cm to fit under the lamps. Light intensity varied from 63 to 195 µmol $m^{-2}s^{-1}$.

Agar containing 24-day-old cultures were placed in the growth medium close to the roots of 24 seedlings (six seedlings per *Phytophthora* isolate) according to the protocol of Talgø *et al.* (2006). Twenty four seedlings were inoculated with map pins (six seedlings per *Phytophthora* isolate) according to the procedure of Talgø & Stensvand (2013). Briefly explained, four map pins were used to make holes in the bark on four sides of the stem base. Then the map pins were used to pick up inoculum from agar plates with 24-days-old cultures, and inserted back into the holes. The map pins were left in the stem throughout the experimental period. Seven seedlings served as control plants of which three were inoculated with sterile PDA in the growth medium and three were inoculated with sterile map pins in the stem. One seedling did not get any treatment.

The seedlings were incubated in a growth room with fluorescent light at a mean temperature of 23°C (minimum 19°C and maximum 26°C) and 16 h day and 8 h night. The seedlings were monitored weekly. Twenty weeks after inoculation (July 2013) one seedling from each treatment (n = 10) was examined for lesions on stem and roots, and the pathogens were re-isolated. Reisolation of the pathogens was done by washing the roots in tap water, cutting off parts of the roots covering the leading edge and placing the root bits on 9 cm Petri dishes with P_5ARPH (Appendix I) agar. Reisolation from the map pin inoculation was performed using the above-mentioned "direct isolation" method. All plates were incubated at room temperature. Pure cultures were obtained by transferring hyphal tips from dishes with P_5ARPH agar onto PDA. The remaining 45 seedlings were kept under the same conditions for another 10 weeks. Thirty weeks after inoculation, in September 2013, all seedlings were examined for lesions on roots and stem and *Phytophthora* was re-isolated on P_5ARPH. The stems with map pins were kept at 3°C for 11 days before the lesion length was measured.

Origin of the isolates used, including isolate numbers, are given under Results.

Statistical analysis. Differences in lesion lengths caused by the four *Phytophthora* species were determined by an analysis of variance (ANOVA) using the general linear model (GLM) procedure in Minitab 16 (Minitab Inc, Coventry, UK). Tukey's test was used to determine which groups' means were significantly different from each other. Differences were significant if $P \leq 0.05$. Microsoft Excel 2010 (Microsoft Corp., Seattle, USA) was used to prepare a bar diagram.

2.4. Potential role of slugs as vectors of *Phytophthora* spp.

To test the hypothesis that Spanish slugs can act as vectors of *Phytophthora*, slugs fed on colonies of *Phytophthora* spp. Faeces from the slugs were then examined for oospores and viable hyphae.

Phytophthora fed slugs were placed on beech seedlings for 18 days, and the seedlings were monitored for *Phytophthora* symptoms for 6 months.

Slugs were collected from the beech forest in Larvik and placed on PARPH agar. Faeces were then examined for *Phytophthora* hyphae.

The two species *P. plurivora* and *P. cambivora* were the chosen *Phytophthora* species for the slug experiment because they are known from literature to be highly aggressive to beech (Day 1939; Jung *et al.* 2005; Jung 2009).

2.4.1. Passage of hyphae and oospores through the digestive system of slugs

Viability of *Phytophthora* after passage through slugs. Fifteen Spanish slugs hatched in the laboratory were put in a plastic container (20 × 15 × 10 cm) with a moist paper napkin to prevent the slugs from drying up. The slugs were starved for 4 days to make them hungry and to ensure that the digestive system was empty. Faeces from this period were collected and kept in microtubes for DNA analysis.

Two plastic containers (35 × 25 × 10 cm) covered with PDAS and P_{10}ARPH, respectively, were each inoculated with 6 agar plugs of *P. cambivora*. One plastic container (35 × 25 × 10 cm) covered with V8 agar was inoculated with 6 agar plugs of *P. plurivora* (Fig. 7). *P. cambivora* was allowed to grow for 15 days to be able to cover most of the agar in the two boxes. *P. plurivora* grew for 19 days to ensure that the agar was covered with hyphae and to ensure oospores production. Five Spanish slugs (≈1 gram each) were placed in each of the three boxes together with 9 cm plastic containers placed upside down in each box as shelter for the slugs. A hole was cut on the side of each container as an entrance for the slugs.

Fig. 7 Plastic box containing V8 juice agar, *Phytophthora plurivora* and a 9 cm plastic container as shelter for the Spanish slugs (*Arion vulgaris*). Photo: Kari H. Telfer

Slug soap (Antischneck-Gel, Neudorff ®, GmbH, Emmerthal, Germany) was swabbed along the edges of each box to obstruct the slugs from escaping. The boxes were stored in a growth room at 16°C, 80 % RH, and 10 h day and 14 h night. After 2 days of feeding, faeces from the *P. cambivora* feeding slugs were carefully sampled from the sides of the boxes and placed in microtubes for DNA testing. Faeces from slugs feeding on *P. plurivora* were plated on to P_{10}ARPH agar. After 5 days, hyphae that grew out from the faeces were transferred to V8 to achieve pure cultures and to stimulate oospore production.

After six days of feeding, the 15 slugs from the three containers were transferred to 15 separate Petri dishes with P_{10}ARPH agar, and kept for 24 hours at the same conditions, after which the slugs were removed and the plates examined for faeces. The plates with faeces were incubated at room temperature (± 20°C) and monitored regularly for growth of hyphae resembling *Phytophthora* hyphae. Hyphae growing out of the faeces were transferred first to PDAS and later to V8 agar to be able to compare the colony morphology to the original isolates that were used to feed the slugs. Microscopy slides with hyphae and oospores were made and assessed in the microscope.

Isolation of DNA from slug faeces. Microtubes with samples of faeces collected prior to the experiment and after two days of feeding on *P. cambivora* were and kept in liquid nitrogen before and after disruption of the faeces. One 5 mm stainless steel bead (Qiagen GmbH, Hilden, Germany) was used to disrupt the faeces in a mixer mill (Retsch MM301, Haan, Germany) for 1 minute at 30 Hz. To extract DNA from the faeces steps 2 – 11 in the DNeasy® Plant Mini Kit (Qiagen Inc., Valencia, CA, USA) protocol from April 2012 (Appendix II) were followed. Briefly explained, after disruption, the cells were lysed, RNA was cleaved by the enzyme RNase A, and cell debris, polysaccharides and proteins were removed during several washing steps. DNA was bound to a special membrane before it was eluted in a low salt buffer.

Amplification of DNA. Polymerase chain reaction (PCR) is a sensitive method used to amplify small DNA segments that can be used for further testing (Klug *et al.* 2007). Both ends of the target sequence must be known to be able to perform PCR. Short pieces of single-stranded DNA that are complementary to these ends, called forward and reverse primers, must be added together with PCR-buffer, nucleotides (single units of the bases A, T, G and C, known as dNTPs), bovine serum albumin (BSA), a heat stable DNA polymerase (Taq) and

sterile distilled water (sdH_2O) to synthesize new DNA. The synthesis is driven by temperature manipulations in three steps: denaturation (separation of the double-stranded DNA into single-stranded DNA) at 94 – 95°C, annealing (primers bind to the two ends of the DNA template) at e.g. 58°C depending on the primer set, and extension (Taq polymerase synthesizes new DNA by adding dNTPs to the two template strands) at 72°C. These steps are repeated in cycles to obtain the desired number of DNA copies. After each cycle, the amount of DNA strands is doubled. To ensure that any remaining single stranded DNA is fully extended, the temperature is held at 70–74°C for 5 – 15 minutes.

Amplification of DNA from slug faeces. The samples of DNA from slug faeces were diluted 10 times (18 µl H_2O and 2 µl DNA) to remove possible inhibitors. Both the diluted and undiluted samples of DNA were used as templates in the PCR reactions.

Genus specific *Phytophthora* primers (Table 2) were used to detect any possible *Phytophthora* DNA in the slug faeces sampled prior to the feeding on *Phytophthora*. Specific *P. cambivora* primers (Table 2) were used in the faeces collected after two days of feeding on *P. cambivora*. To verify that the DNA in the samples could be amplified, a PCR test designed to detect all fungi, and which also detects most plants (May Bente Brurberg at Bioforsk, Norwegian Institute for Agricultural and Environmental Research, Ås, personal communication), by targeting the ribosomal ITS regions with primers ITS 1 and 4 (Table 2), were used.

Table 2 Forward and reverse primers used to amplify DNA in faeces from Spanish slug (*Arion vulgaris*) feeding on *Phytophthora* spp. Bp = base pair. ITS = internal transcribed spacer.

Target	Forward primer	Reverse primer	Sequence size (bp)	Reference
Phytophthora spp.	Yph1F	Yph2R	470	Schena *et al.* (2006)
P. cambivora	Ycam3R	Ycam3R	183	Schena *et al.* (2008)
ITS region	ITS1	ITS4	Variable sizes	White *et al.* (1990)

Twenty-three µl of Master Mix (14.8 µl sdH_2O; 2.5 µl PCR-buffer; 2.0 µl dNTPs; 2.5 µl BSA; 0.5 µl forward primer; 0.5 µl reverse primer; 0.125 µl Taq polymerase) and 2 µl DNA

were pipetted into small PCR tubes. Two µl of sterilized water was used as a negative control. Two µl of *P. ramorum* DNA was used as a positive control.

The following thermal cycling parameters were used for the general *Phytophthora* primers and the *P. cambivora* primers: initial denaturation for 2 minutes at 95°C followed by 35 cycles of 30 seconds 94°C, 45 seconds at 58°C, and 30 seconds at 72°C. After the final PCR cycle the machine held 72°C for 10 minutes, and finally kept cool at 4°C until gel electrophoresis. The following parameters were used for the ITS primers: initial denaturation for 5 minutes at 94°C followed by 35 cycles of: 30 seconds at 94°C, 30 seconds at 55°C and 1 minute at 72°C. Finally, elongation at 72°C for 7 minutes, and cooling at 4°C.

Gel electrophoresis. Gel electrophoresis is a method used to separate DNA fragments of various lengths on a gel matrix. The size of the DNA fragments can be determined by the use of ladders which are DNA fragments of known sizes (Klug *et al.* 2007).

A gel was prepared with agarose (1 %), Tris/Borate/EDTA (TBE) buffer and ethidium bromide (one drop per 50 ml TBE buffer) and with the appropriate number of wells. Ethidium bromide was used to visualize DNA under UV light. The agarose gel was put in an electrophoresis tank (BIO RAD Sub-cell® GT, Hercules, California), and PCR products, loading buffer and a 100 base pair ladder were pipetted into wells prepared in the gel. The power supply (BIO RAD Power Pac 300, Hercules, California) was set on 90 V until the fragments were seperated. The gel was visualized under UV light (BIO-RAD ChemiDoc™ XRS+, Hercules, California).

2.4.2. Transmission of *Phytophthora* to beech seedlings

Possible transmission of *P. cambivora* and *P. plurivora* by Spanish slugs to small beech seedlings were investigated by letting 5 slugs feed on *P. cambivora* (61-day-old culture grown on PDAS) and 5 slugs feed on *P. plurivora* (45-day-old culture grown on V8) for 2 days. The slugs had been starved for 4 days prior to the *Phytophthora* feeding. After the feeding period, the 5 slugs (≈ 2 gram each) feeding on *P. cambivora* were placed in a 70 liter plastic container together with a beech seedling and a small 9 cm plant pot as shelter for the slugs. The 5 slugs (≈ 2 gram each) feeding on *P. plurivora* were placed in a similar container with beech seedling and plant pot. Faeces were collected from both groups of slugs to check for hyphal

growth. The two containers were covered with plastic bags, supported by plant sticks (Fig. 8), to increase the humidity and to confine the slugs, and placed in a growth room at 16°C (10 h day/14 h night). After 18 days, the slugs were removed.

Fig. 8 Two plastic containers each containing 1 beech seedling (*Fagus sylvatica*) and 5 slugs (*Aron vulgaris*) that had fed on either *Phytophthora cambivora* and *P. plurivora*. Both containers were covered with plastic bags supported by plant sticks. Photo: Kari H. Telfer

The trees were kept at 16°C for 6 months and monitored regularly for *Phytophthora* symptoms. At the end of the experiment the soil from both containers was flooded with sterilized water and baited with *Rhododendron* 'Cunningham's White' leaves using the same technique as described above in section 2.1.

2.4.3. Slugs collected in the beech forest in Larvik.

Twenty Spanish slugs, 10 woodlouses (*Oniscidea*) and 2 earthworms (*Lumbricidae*) from the beech forest in Larvik were collected and placed on P_5ARPH agar in 9 cm Petri plates or plastic containers (25 × 20 × 8 cm) (Fig. 9 B and C). Containers and plates were brought back to the laboratory and kept at room temperature (± 20°C) for 24 hours. The P_5ARPH agar was examined for growth resembling *Phytophthora,* and faeces were collected from the containers and grown on P_5ARPH agar.

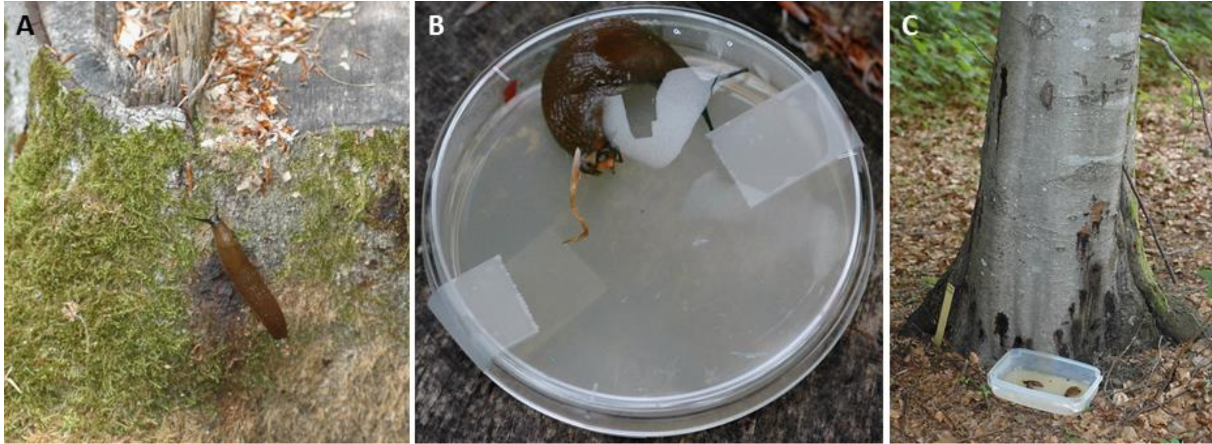

Fig. 9 A, a Spanish slug (*Arion vulgaris*) on a beech (*Fagus sylvatica*) stump with bleeding canker; **B**, a Spanish slug collected from a *Phytophthora* infected beech tree placed in a Petri dish with *Phytophthora* selective agar (P_5ARPH); **C**, two slugs collected around a beech tree infected with *Phytophthora* and placed in a plastic container with P_5ARPH agar. Photos: Kari H. Telfer

3. Results

3.1. *Phytophthora* survey, isolation and identification of the pathogen

Survey of beech trees. The survey in 2012 resulted in the discovery of 54 trees with bleeding cankers (Table 3). Most cankers were observed at the base of the stem (0.1 – 2 meters above ground), but one tree had symptoms 7 meters above ground. The exudates from the cankers had a deep red color (Fig. 10). Below the bark there was a distinct line between healthy (whitish color) and diseased (reddish) tissue (Fig 11 A). Several trees had cracked bark (Fig. 11 B), and many of trees with bleeding cankers had sparse crowns, dieback of branches (Fig. 12 B) and yellow foliage.

The circumference of the trees with bleeding cankers ranged from 40 cm to 310 cm (Table 3), but most trees (88 %) had a circumference above 100 cm. Seven trees were tested with LFD tests, and all seven trees tested positive for *Phytophthora* spp. (Fig. 13).

Fig. 10 Bleeding cankers caused by *Phytophthora* spp. on the trunk of mature beech trees (*Fagus sylvatica*) in Larvik. Photos: Kari H. Telfer

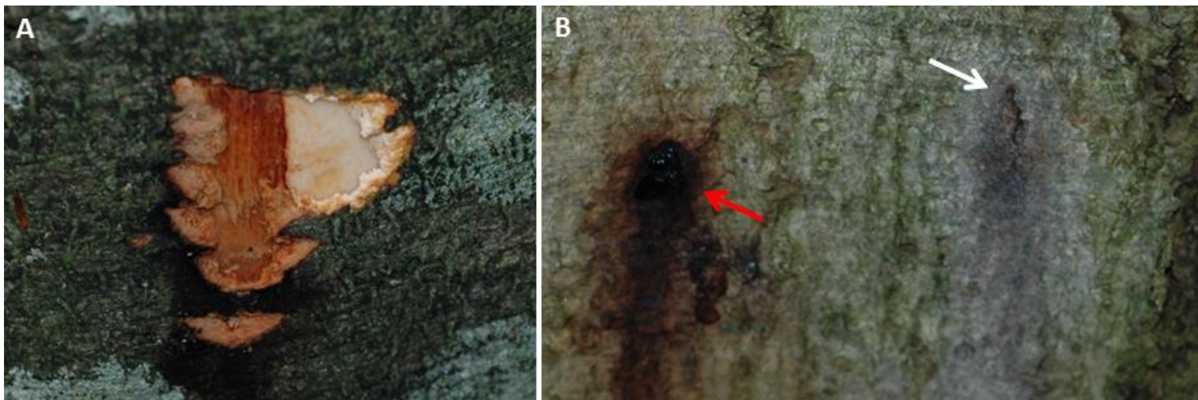

Fig. 11 Stems of mature, declining beech trees (*Fagus sylvativa*) in Larvik infested with *Phytophthora* spp. **A**, under the bark there was a distinct line between healthy (whitish) and diseased (reddish) tissue, the so-called leading edge. **B**, bleeding canker (red arrow) and canker with light red color and cracked bark (white arrow). Photos: Kari H. Telfer

Fig. 12 The crowns of beech trees (*Fagus sylvatica*) either dead or dying of *Phytophthora* infection in the beech forest in Larvik. **A**, dead beech trees standing close to trees with bleeding cankers; **B**, beech tree observed in 2013 with many bleeding cankers on the stem base, sparse crown and epicormic shoots; **C**, a dead beech tree observed in 2013. The same tree was alive in 2011 and 2012, but with bleeding cankers on the stem. Photos: Kari H. Telfer

Table 3 Result of the *Phytophthora* survey performed in the beech (*Fagus sylvatica*) forest in Larvik (Vestfold County, Norway) in 2012. Each coordinate represents a beech tree with bleeding cankers. Information about circumference (cm) at breast height and locality in the forest is provided for each tree**.** Bark samples were taken from 17 trees. Result of DNA-sequencing and testing with lateral flow devices (LFD) are shown in the last column including isolate numbers. (-) = isolation of beech bark on *Phytophthora* selective medium (P_{10}ARPH) did not yield *Phytophthora* spp.; (+) = LFD tested positive for *Phytophthora* spp.

Coordinates	Circumference (cm) at breast height	Locality	Isolated species confirmed by DNA-analysis and/or LFD test
59° 3'36.01"N 10° 1'30.47"E	99	Wet area	(-)
59° 3'36.59"N 10° 1'32.69"E	65	Wet area	(-)
59° 3'34.78"N 10° 1'30.79"E	275	Close to restaurant	(-)
59° 3'35.75"N 10° 1'34.24"E	291	Wet area	***Phytophthora cambivora***
59° 3'35.97"N 10° 1'34.82"E	105	Wet area	(-)
59° 3'36.27"N 10° 1'35.96"E	154	Wet area	(-)
59° 3'37.67"N 10° 1'33.28"E	208	Wet area	Not sampled
59° 3'37.61"N 10° 1'34.98"E	81	Wet area	Not sampled
59° 3'37.68"N 10° 1'35.88"E	40	Wet area	Not sampled
59° 3'25.24"N 10° 1'19.05"E	208	Swamp	Not sampled
59° 3'24.16"N 10° 1'21.75"E	106	Swamp	(-)
59° 3'29.43"N 10° 1'25.08"E	160	Information is lacking	*Mortierella pulchella*
Information is lacing	300	Information is lacking	Not sampled
59° 3'28.78"N 10° 1'27.59"E	155	Information is lacking	Not sampled
59° 3'29.43"N 10° 1'27.56"E	200	Information is lacking	Not sampled
59° 3'39.24"N 10° 1'27.41"E	210	Close to E18	Not sampled
59° 3'33.67"N 10° 1'20.75"E	63	Information is lacking	Not sampled
59° 3'32.81"N 10° 1'25.59"E	230	Information is lacking	Not sampled
59° 3'31.55"N 10° 1'29.01"E	104 and 117	The tree behind the scene.	***Phytophthora cambivora*** (+) **(isolate number 250 154)**
59° 3'29.36"N 10° 1'18.38"E	145	Information is lacking	Not sampled
59° 3'35.17"N 10° 1'20.55"E	134	Close to E18	(+)
59° 3'35.03"N 10° 1'17.57"E	240	Close to E18	Not sampled
59° 3'35.00"N 10° 1'18.21"E	160	Close to E18	Not sampled
59° 3'16.98"N 10° 1'14.35"E	170	Close to path	Not sampled
59° 3'23.76"N 10° 1'26.17"E	100	Swamp	Not sampled
59° 3'21.88"N 10° 1'26.23"E	220	Close to path	(+)
59° 3'20.86"N 10° 1'27.54"E	65	Close to path	(+)
59° 3'19.79"N 10° 1'26.76"E	190	Close to path	Not sampled
59° 3'20.50"N 10° 1'24.07"E	160	Close to path	***Phytophthora cambivora*** (+)
59° 3'17.55"N 10° 1'13.94"E	155	Close to path	Not sampled
59° 3'18.22"N 10° 1'14.94"E	190	Close to path	Not sampled
59° 3'18.13"N 10° 1'13.19"E	310	Close to path	(-) (+)
59° 3'20.48"N 10° 1'19.70"E	105	Close to path	Not sampled
59° 3'22.53"N 10° 1'22.27"E	170	Close to path	Not sampled
59° 3'16.35"N 10° 1'10.52"E	160	Close to path	Not sampled

59° 3'29.26"N 10° 1'26.09"E	112	Swamp	(+)
59° 3'27.92"N 10° 1'14.51"E	150	Information is lacking	Not sampled
59° 3'39.37"N 10° 1'35.28"E	200	Close to stream	Not sampled
59° 3'37.27"N 10° 1'38.14"E	270	Information is lacking	Not sampled
59° 3'36.22"N 10° 1'33.96"E	165	Wet area	Not sampled
59° 3'36.27"N 10° 1'32.92"E	145	Wet area	Not sampled
59° 3'36.54"N 10° 1'33.27"E	223	Wet area	Not sampled
59° 3'35.92"N 10° 1'31.99"E	185	Wet area	Not sampled
59° 3'29.67"N 10° 1'24.52"E	160	Swamp	Not sampled
59° 3'28.83"N 10° 1'22.99"E	190	Swamp	Not sampled
59° 3'26.19"N 10° 1'27.79"E	188	Swamp	Not sampled
59° 3'24.20"N 10° 1'22.52"E	100	Swamp	Not sampled
59° 3'23.85"N 10° 1'25.41"E	69	Swamp	Not sampled
59° 3'44.56"N 10° 1'29.48"E	185	Beech stand in construction area	***Phytophthora cambivora***
59° 3'45.46"N 10° 1'30.45"E	152	Beech stand in construction area	Not sampled
59° 3'45.52"N 10° 1'30.83"E	135	Beech stand in construction area	(-)
59° 3'44.42"N 10° 1'28.33"E	150	Beech stand in construction area	Not sampled
59° 3'53.04"N 10° 1'2.65"E	185	Beech stand in construction area	Not sampled
59° 3'47.92"N 10° 1'0.76"E	145	Beech stand in construction area	Not sampled

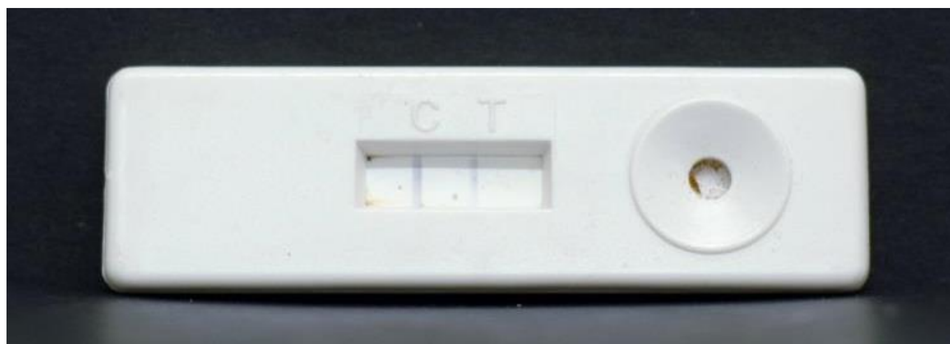

Fig. 13 Lateral Flow Device (LFD) used to check for the presence of *Phytophthora* spp. C = control, T = test. This LFD test came up with both a control line and a test line, thus positive for *Phytophthora* spp. Photo: Kari H. Telfer

Two areas had a higher concentration of trees with bleeding cankers in the 2012 survey than the rest of the forest, with 16 of 329 (4.9 %) and 12 of 680 (1.8 %) (Fig. 14). The remaining 26 trees were mostly situated along a frequently used path on top of a ridge.

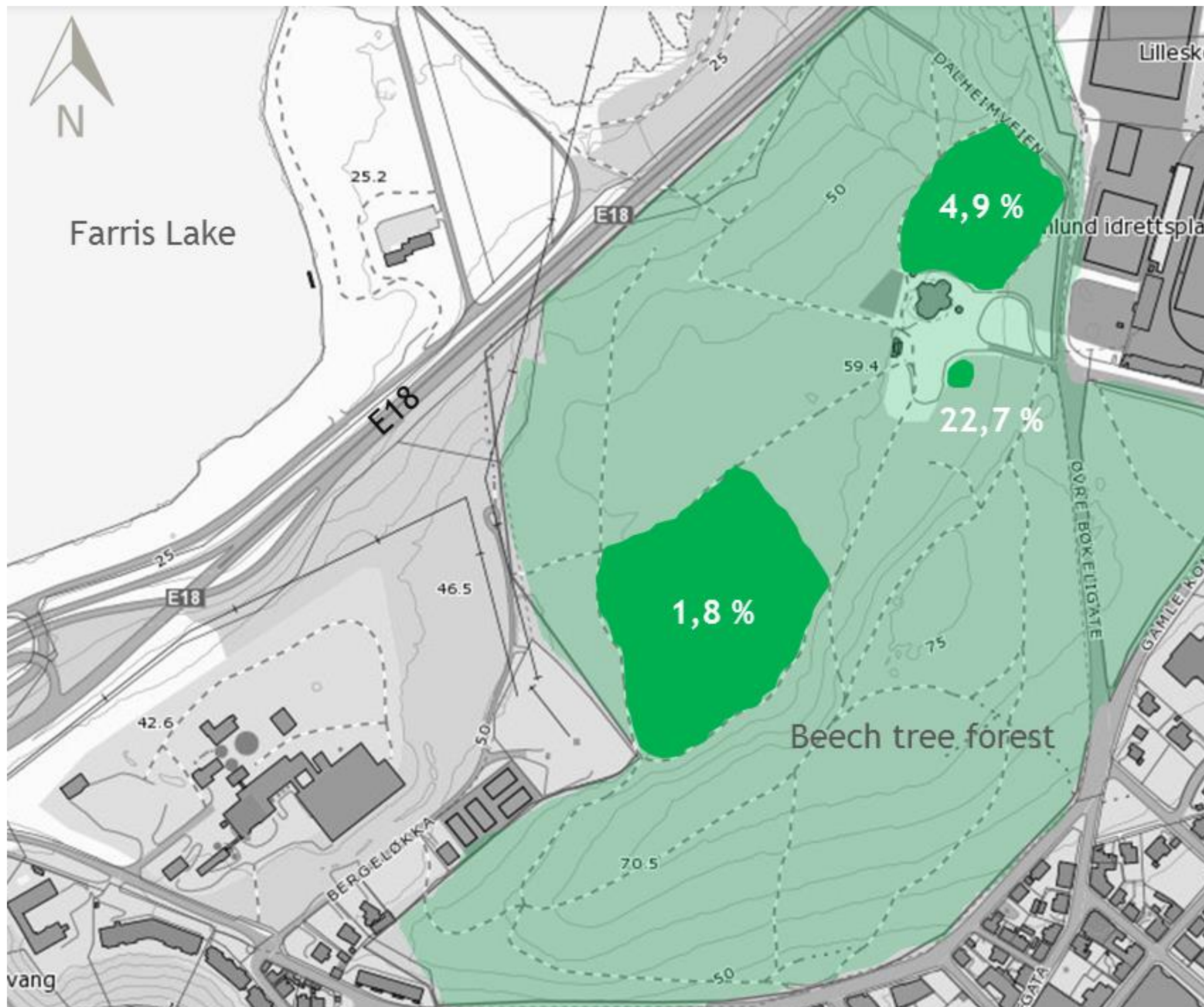

Fig. 14 Light green color mark the beech forest (*Fagus sylvatica*) in Larvik (Vestfold County, Norway). Two areas had a higher concentration of trees with bleeding cankers than the rest of the forest (marked with dark green) in the 2012 survey, with 4.9 % and 1.8 %, respectively. The survey in 2013 revealed a new area with high concentration of beech trees with bleeding cankers (22.7 %). Map source: Geoport

On a short visit to the beech forest in Larvik in October 2013, several trees that were asymptomatic in 2012 had developed bleeding cankers on the stem. In a limited area, 5 out of 22 trees (22.7 % indicated in Fig. 14) had both bleeding cankers and thin crowns in the 2013 survey.

Direct sampling. Samples of symptomatic tissue were collected from 11 beech trees in the beech forest and from 2 beech trees in the NPRA construction area. The DNA-sequencing revealed *P. cambivora* from 3 trees and the zygomycete *Mortierella pulchella* Linnem. from one tree in the beech forest, and *P. cambivora* from one tree in the construction area (Table 3). Isolation from the remaining 9 trees yielded different hyphae, but none of them resembled *Phytophthora* spp. One beech tree in Oslo was sampled which yielded *P. plurivora* (isolate no. 250 151).

Baiting soil. *P. plurivora* was isolated from 1 out of 10 soil samples from the construction area of NPRA (Table 4). The rest of the samples had no growth resembling *Phytophthora* spp.

Table 4 Table shows result of baiting in water and soil with leaves of *Rhododendron* 'Cunningham's White' in Larvik (Vestfold County, Norway) in 2012. Coordinates, sample source, and locality description is provided for each sample. *Rhododendron* leaves with spots were placed on *Phytophthora* selective medium (P_{10}ARPH) and hyphae resembling *Phytophthora* were sequenced. The results of the DNA-sequencing are shown in the last column including isolate numbers. (-) = baiting did not yield hyphae resembling *Phytophthora* spp.

Geographical location	Source	Locality	Species confirmed by DNA-analysis
N59^{o}.03.620 E010^{o}.01.294	Soil	Construction area	(-)
N59^{o}.03.437 E010^{o}.01.228	Soil	Construction area	(-)
N59^{o}.03.761 E010^{o}.01.382	Soil	Construction area	(-)
N59^{o}.03.756 E010^{o}.01.458	Soil	Beech stand in construction area	(-)
N59^{o}.03.587 E010^{o}.01.214	Soil	Construction area	(-)
N59^{o}.03.738 E010^{o}.01.470	Soil	Beech stand in construction area	(-)
N59^{o}.03.768 E010^{o}.01.496	Soil	Beech stand in construction area	(-)
N59^{o}.03.404 E010^{o}.00.354	Soil	Construction area	(-)
N59^{o}.03.368 E010^{o}.01.184	Soil	Construction area	***Phytophthora plurivora***
N59^{o}.03.743 E010^{o}.01.491	Soil	Beech stand in construction area	(-)
N59^{o}.03.717 E010^{o}.01.148	Water	Farris Lake close to construction area	(-)
N59^{o}.03.306 E010^{o}.00.711	Water	Farris River	***Phytophthora lacustris*** **(isolate number 250 160)**
N59^{o}.03.065 E010^{o}.01.237	Water	Farris River	***Phytophthora lacustris***
-	Water	Gopledal waterworks (unfiltered water)	(-)
-	Water	Gopledal waterworks (filtered water)	(-)
-	Water	Gopledal waterworks (filtered water with chlorine)	(-)
N59^{o}.04.459 E010^{o}.01.082	Water	Farris Lake, close to Gopledal waterworks	***Phytophthora gonapodyides***
N59^{o}.04.749 E010^{o}.01.616	Water	Stream running out in Dambukta (part of Farris Lake)	***Phytophthora plurivora***
N59^{o}.04.997 E010^{o}.03.246	Water	Location for deposition of soil from construction area	(-)
N59^{o}.03.852 E010^{o}.01.263	Water	Farris Lake, close to construction area	***Phytophthora gonapodyides*** **(isolate 250 157)**
N59^{o}.03.440 E010^{o}.01.456	Water	Ditch water in the beech tree forest	*Pythium undulatum*
N59^{o}.03.598 E010^{o}.01.327	Water	Ditch water in the beech tree forest	*Pythium anandrum*
N59^{o}.03.593 E010^{o}.01.536	Water	Ditch water in the beech tree forest	***Phytophthora plurivora***
N59^{o}.03.410 E010^{o}.01.415	Water	Ditch water in the beech tree forest	*Pythium undulatum*

Baiting in water. Six of the 14 baits, yielded *Phytophthora* spp. (Table 4). DNA-sequencing revealed *P. plurivora* from ditch water inside the beech forest and from a stream running in to the Farris Lake, *P. lacustris* Brasier, Cacciola, Nechw., T. Jung & Bakonyi from two locations in the Farris River, and *P. gonapodyides* (H.E. Petersen) Buisman from two locations in the Farris Lake. The leaves from the 8 remaining locations developed several lesions, but none of them yielded hyphae resembling *Phytophthora* spp. Two *Pythium* species, *Pythium undulatum* H.E. Petersen and *Pythium anandrum* Drechsler was isolated from *Rhododendron* leaves used as bait in ditch water inside the beech forest. The leaves used as bait inside Gopledal waterworks were asymptomatic.

3.2. Morphology, growth rates and cardinal temperatures

In addition to DNA sequencing, one isolate of the each *Phytophthora* species isolated in the 2012 survey (Table 3 and 4), were identified on the basis of growth-temperature response, colony morphology, and characteristics of sporangia, hyphae, antheridia, oogonia, and oospores according to Erwin & Ribeiro (1996), Jung & Burgess (2009) and Nechwatal *et al.* (2012).

Temperature-growth relationships. Growth (mm per day) for *P. plurivora* (isolate 250 151), *P. cambivora* (isolate 250 154), *P. gonapodyides* (isolate 250 157) and *P. lacustris* (isolate 250 160) can be seen at different temperatures in Fig. 15.

Mean daily growth rate on PDA at optimum temperature for *P. plurivora*, *P. cambivora*, *P. gonapodyides* and *P. lacustris* were 4.5, 2.7, 2.4 and 2.4 mm, respectively. Optimal temperature for mycelial growth was 30°C for *P. lacustris* and 25°C for the other three species.

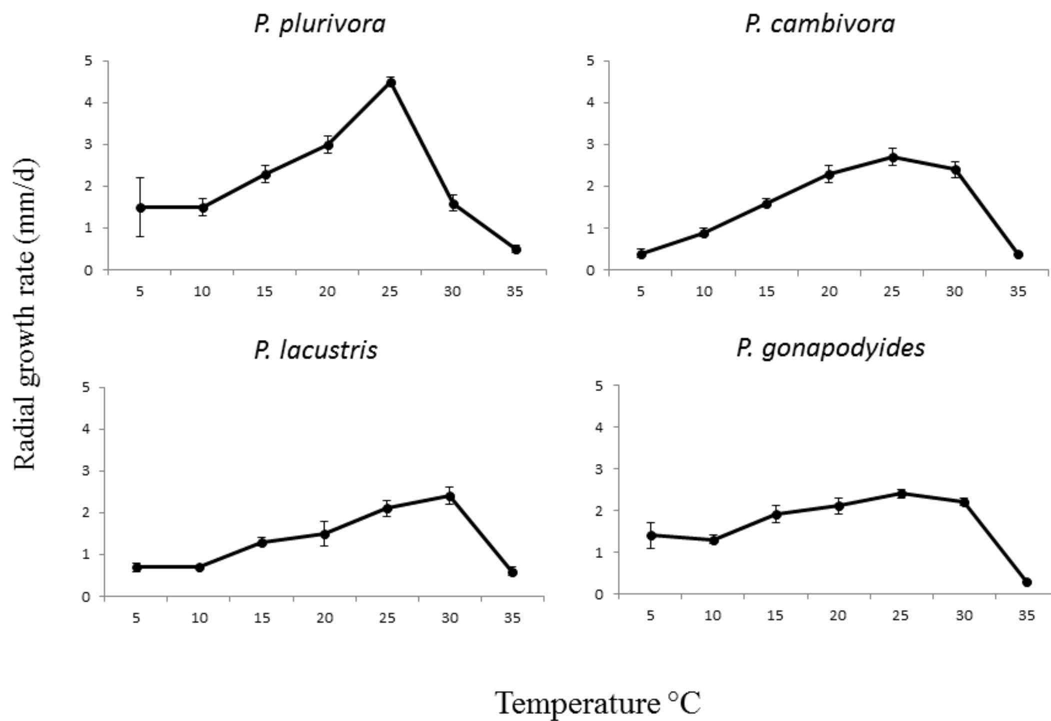

Fig. 15 Radial growth rates (mean and standard errors of three replicates at each temperature) of *Phytophthora plurivora* (isolate 250 151), *P. cambivora* (isolate 250 154), *P. gonapodyides* (isolate 250 157), and *P. lacustris* (250 160) grown on potato dextrose agar at different temperatures.

Morphological characteristics. Colony morphology of *P. plurivora* (isolate 250 151) and *P. cambivora* (isolate 250 154), *P. gonapodyides* (isolate 250 157) and *P. lacustris* (250 160) grown on PDA for 10 days and V8 for 7 days are shown in Fig. 16.

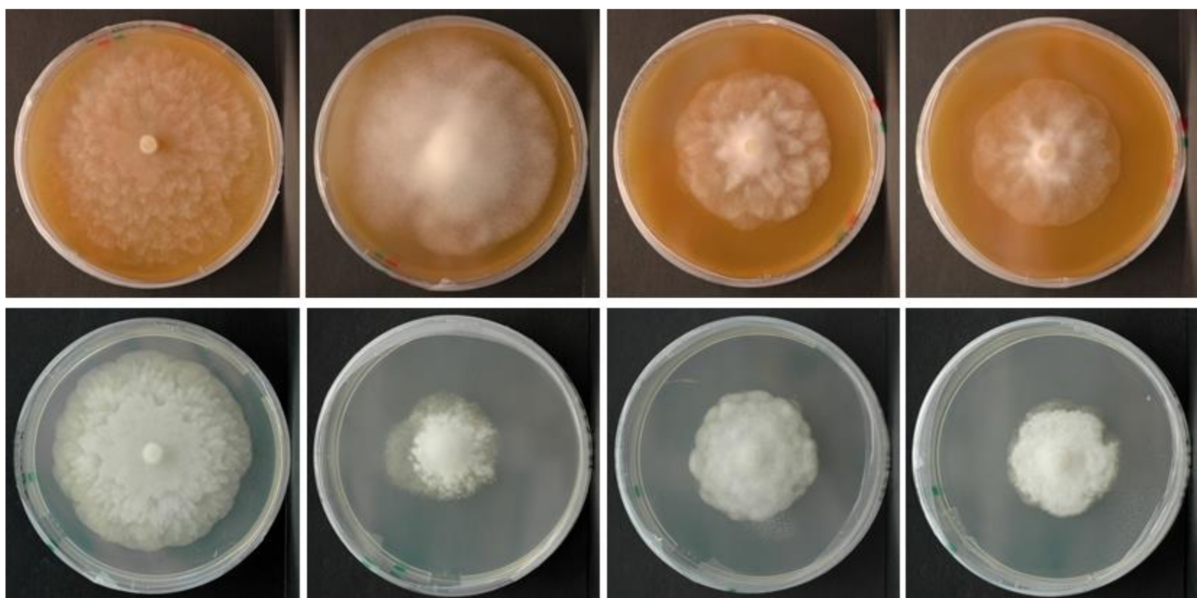

Fig. 16 Colony morphology of (from left to right) *Phytophthora plurivora* (isolate 250 151), *P. cambivora* (isolate 250 154), *P. gonapodyides* (isolate 250 157) and *P. lacustris* (250 160) on (from top to bottom) V8 juice agar after 7 days growths and potato dextrose agar after 10 days growth at 20°C. Photos: Kari H. Telfer

Morphology of sporangia, oogonia, antheridia, oospores, hyphal swellings and hyphae of *P. plurivora* (isolate 250 151) and *P. cambivora* (isolate 250 154), *P. gonapodyides* (isolate 250 157) and *P. lacustris* (250 160) are shown in Fig. 17 – 20.

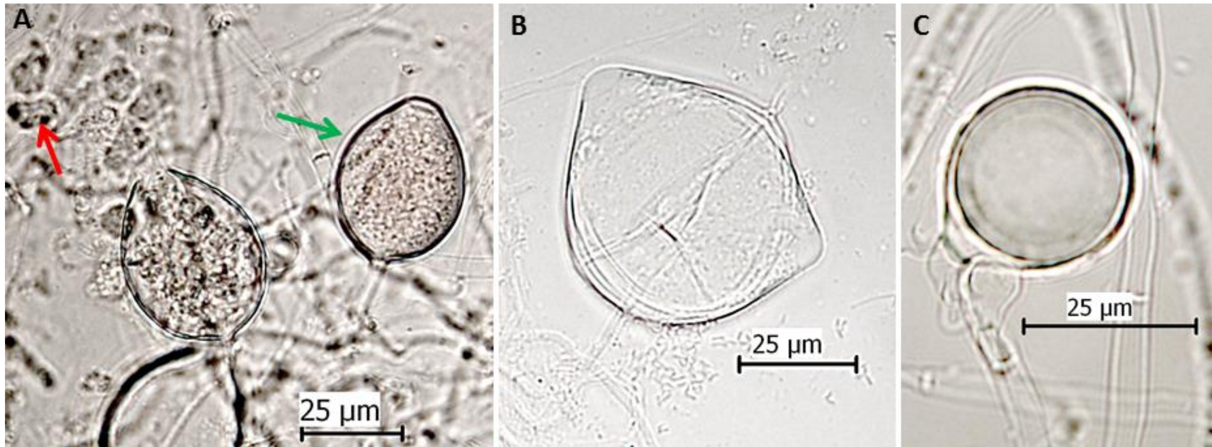

Fig. 17 Structures of *Phytophthora plurivora* (isolate 250 151) on V8 juice agar with autoclaved pond water added. **A**, releasing of zoospores (red arrow pointing out one of the zoospores) from sporangium and ovoid semi-papillate sporangium (green arrow); **B**, bipapillate sporangium; **D**, oogonium with plerotic oospore and paragynous antheridium. Photos: Kari H. Telfer

Fig. 18 Morphology of *Phytophthora cambivora* (isolate 250 154) growing on leaves of *Rhododendron* 'Cunningham's White' floating in autoclaved pond water. **A**, ovoid non-papillate sporangium; **B**, internal, nested proliferation; **C**, nested and extended, internal proliferation; **D**, coralloid mycelium. Photos: Kari H. Telfer

Fig. 19 Structures of *Phytophthora gonapodyides* (isolate 250 157) after flooding colonies growing on V8 juice agar with autoclaved pond water. **A**, obpyriform, nonpapillate sporangium; **B**, internal, nested proliferation; **C**, internal, extended proliferation; **D**, release of zoospores (red arrow). Photos: Kari H. Telfer

P. plurivora, *P. cambivora* and *P. lacustris* had ovoid sporangia (Fig. 17 A, 18 A and 20 A), while the sporangia of *P. gonapodyides* were mostly obpyriform shaped (Fig. 19 A). *P. plurivora* was the only species that produced bipapillate sporangia (Fig. 17 B).

P. cambivora, *P. gonapodyides* and *P. lacustris* produced new sporangia by both nested and extended internal proliferation (Fig. 18 B and C, 19 B and C, and 20 B and C). *P. lacustris* was the only species that produced hyphal swellings (Fig. 20 D).

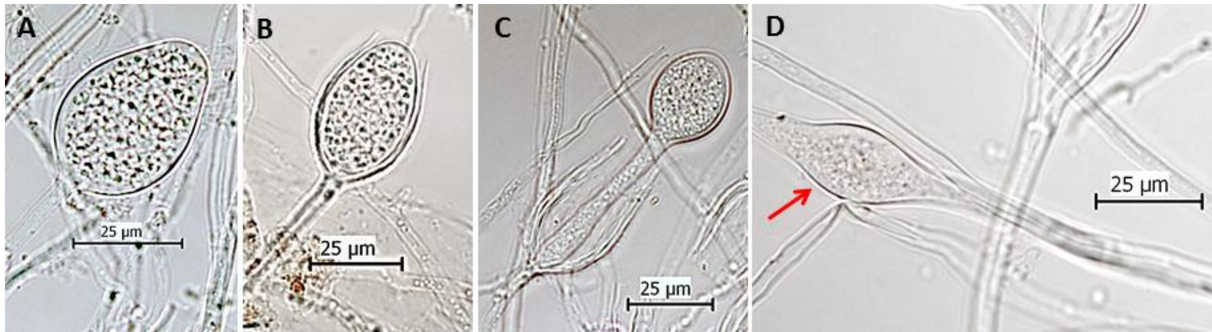

Fig. 20 Structures of *Phytophthora lacustris* after flooding colonies growing on V8 juice agar with autoclaved pond water. **A**, ovoid non-papillate sporangium; **B**, internal, nested proliferation; **C**, extended, internal proliferation; **D**, ellipsoid hyphal swelling (red arrow). Photos: Kari H. Telfer

P. plurivora produced paragynous antheridia, oogonia and oospores in single culture (Fig. 17 C). Smooth-walled oogonia and amphigynous antheridia were produced when *P. cryptogea* A1 and A2 were paired as a control (Fig. 21 B). Only smooth-walled oogonia of the *P. cryptogea* type were observed when *P. cambivora* was paired with *P. cryptogea* mating type A1 (isolate BBA 65909) (Fig. 21 B). The attempt to cross *P. cryptogea*, A1 and A2, and *P. cambivora* (isolate 250 154) mating type A2 with *P. gonapodyides* and *P. lacustris* did not lead to the formation of antheridia and oogonia.

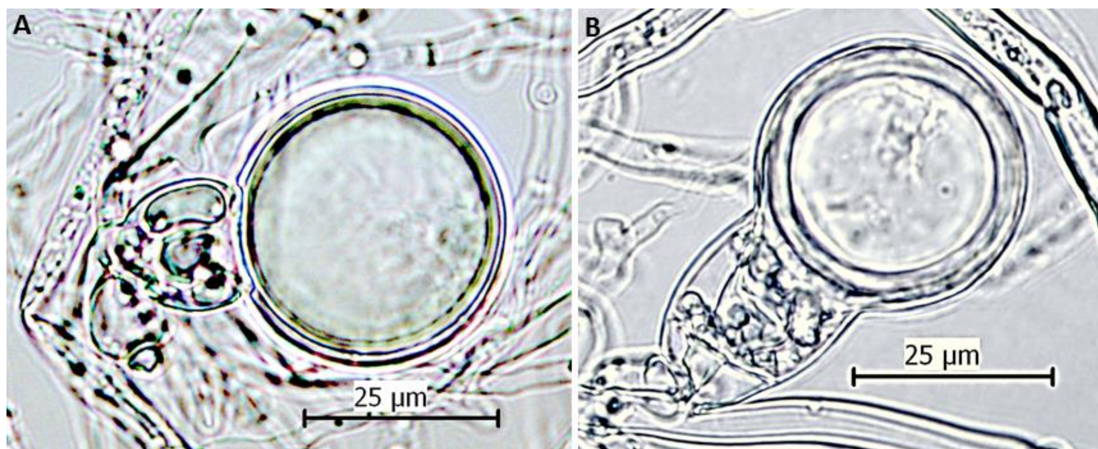

Fig. 21 *Phytophthora cryptogea*. **A**, oogonium and amphigynous antheridium produced after a crossing between *P. cryptogea* isolate BBA65909 (A1) and *P. cryptogea* isolate BBA 63651 (A2); **B**, oogonium and amphigynous antheridium produced after a crossing between *P. cryptogea* isolate BBA 65909 (A1) and *P. cambivora* isolate 250 154 (A2). Photos: Kari H. Telfer

Morphological characteristics and optimum temperatures of the four *Phytophthora* species are presented in Table 5.

Table 5 Morphological characters and dimensions (µm) of spores of one isolate of each of the following species: *Phytophthora plurivora*, *P. cambivora*, *P. gonapodyides*, and *P. lacustris*. The information provided in the table is based on results from this study.

	P. plurivora	*P. cambivora*	*P. gonapodyides*	*P. lacustris*
Isolate number	250 151	250 154	250 157	250 160
Host/source	Beech tree (Oslo)	Beech tree (Larvik)	Farris Lake	Farris River
Colony pattern				
PDA	Chrysanthemum (**Fig. 16**)	Cottony (**Fig. 16**)	Chrysanthemum (**Fig. 16**)	Irregular (**Fig. 16**)
V8	Chrysanthemum (**Fig. 16**)	Cottony (**Fig. 16**)	Chrysanthemum (**Fig. 16**)	Chrysanthemun-like pattern with less defined lobes. (**Fig. 16**)
Hyphal swellings	Not observed	Not observed	Not observed	Ellipsoid (**Fig. 20 D**)
Sporangia	Semi-papillate (**Fig. 17 A**)	Non-papillate (**Fig. 18 A**)	Non-papillate (**Fig. 19 A**)	Non-papillate (**Fig. 20 A**)
length x width (µm)	37.3 - (49.3) - 73.8 × 28.8 - (37.3) - 47.2 (n = 25)	37.9 - (47.7) - 63.5 × 26.3 - (32.4) - 41.4 (n = 25)	33.4 - (44.2) - 54.0 × 22.0 - (29.7) - 37.2 (n = 25)	36.8 - (50.2) - 64.2 × 22.8 - (34,1) - 53.2 (n = 25)
l/w ratio	1.32	1.47	1.49	1.47
Oogonia	Subglobose/globose	Not produced [1] (**Fig. 21 B**)	Not produced	-
Diameter (µm)	22.7 – (27.2) – 30.3 (n = 25) (**Fig. 17 D**)			
Oospore		Not produced [1] (**Fig. 21 B**)	Not produced	-
Aplerotic oospores	80 %			
Diameter (µm)	20.9 – (24.3) – 26.3 (n = 25)			
Wall thickness (µm)	2.4 – (3.5) – 4.5 (n = 25)			

Antheridia	Paragynous (**Fig. 17 D**)	Not produced[1] (**Fig. 21 B**)	Not produced	-
Sex	Homothallic	Heterothallic	-	-
Mating type	-	A2	Could not be determined in this study	Could not be determined in this study

[1] When paired with an A1 tester strain of *Phytophthora cryptogea* (isolate BBA65909) oogonia of the *P. cryptogea* type were formed.

3.3. Pathogenicity test on beech seedlings

The *P. plurivora* isolate (isolate 250 151) that were used in the inoculation tests had been isolated from beech in Oslo. The *P. cambivora* isolate (isolate 250 154) were isolated from a beech tree in Larvik. *P. gonapodyides* (isolate 250 157) and *P. lacustris* (isolate 250 160) both originated from bait tests with *Rhododendron* leaves in waterways in Larvik.

In the map pin inoculation none of the seedlings developed bleeding cankers 30 weeks after inoculation. Instead, the seedlings inoculated with *P. plurivora* (Fig. 22 A and B), *P. gonapodyides* (Fig. 24 A and B) and *P. lacustris* (Fig. 25 A) developed a sunken area around the inoculation site. The seedlings inoculated with *P. cambivora* and the control plants developed callus around the map pin (Fig. 23 A and 26 A). Two seedlings inoculated with *P. plurivora* died in March and May, respectively. *P. plurivora* was re-isolated from both stems.

When the stems were cut open, all the *Phytophthora* inoculated trees had oblong, narrow reddish colored lesions (Fig. 22 C, 23 B, 24 C and 25 B). *P. plurivora*, *P. gonapodyides* and *P. lacustris* were successfully re-isolated from lesions on the stems 20 weeks after inoculation. *P. cambivora* was re-isolated 30 weeks after inoculation.

The lesion lengths on the stems were measured 30 weeks after inoculation. The mean lesion length on the seedlings inoculated with *P. plurivora*, *P. cambivora*, *P. gonapodyides* and *P. lacustris* were 17, 9, 12, and 12 mm, respectively (Fig. 27).

Fig. 22 Results from pathogenicity tests with beech (*Fagus sylvatica*) inoculated with *Phytophthora plurivora*. **A**, **B**, deformed trunks and sunken areas around the inoculation site 30 weeks after inoculation; **C**, lesions developed inside the trunk 30 weeks after inoculation with map pins; **D**, infected root 20 weeks after agar inoculation; **E**, good root development (left) where the stems had been inoculated with map pins, poor root development (right) where seedlings had been inoculated with mycelium on agar in the root zone, 30 weeks after inoculation. Photos: Kari H. Telfer

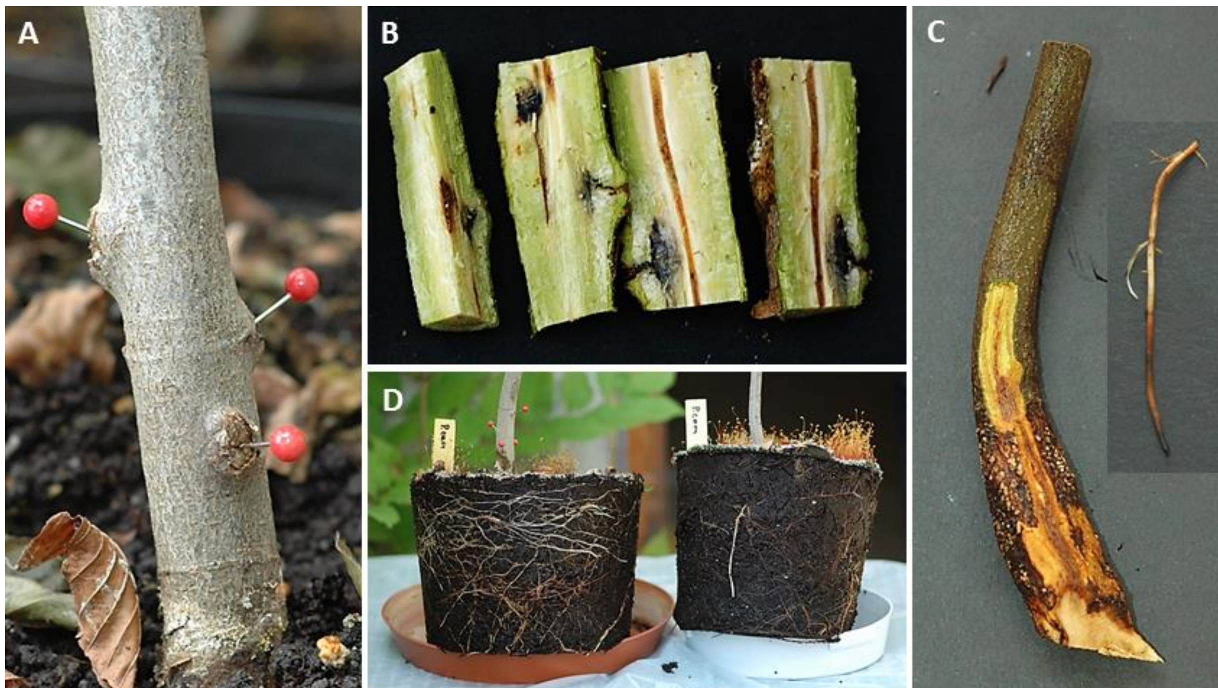

Fig. 23 Results from pathogenicity test with beech (*Fagus sylvatica*) inoculated with *Phytophthora cambivora*. **A**, no lesions developed on the outside when the trunk was inoculated with map pins; there were only some callus formation around the inoculation site 30 weeks after inoculation; **B**, lesions inside the trunk 30 weeks after inoculation with map pins; **C**, infected main root 30 weeks after agar inoculation. Inserted picture shows smaller root with infection; **D**, good root development (left) where the stems had been inoculated with map pins, poor root development (right) where seedlings had been inoculated with mycelium on agar in the root zone 30 weeks after inoculation. Photos: Kari H. Telfer

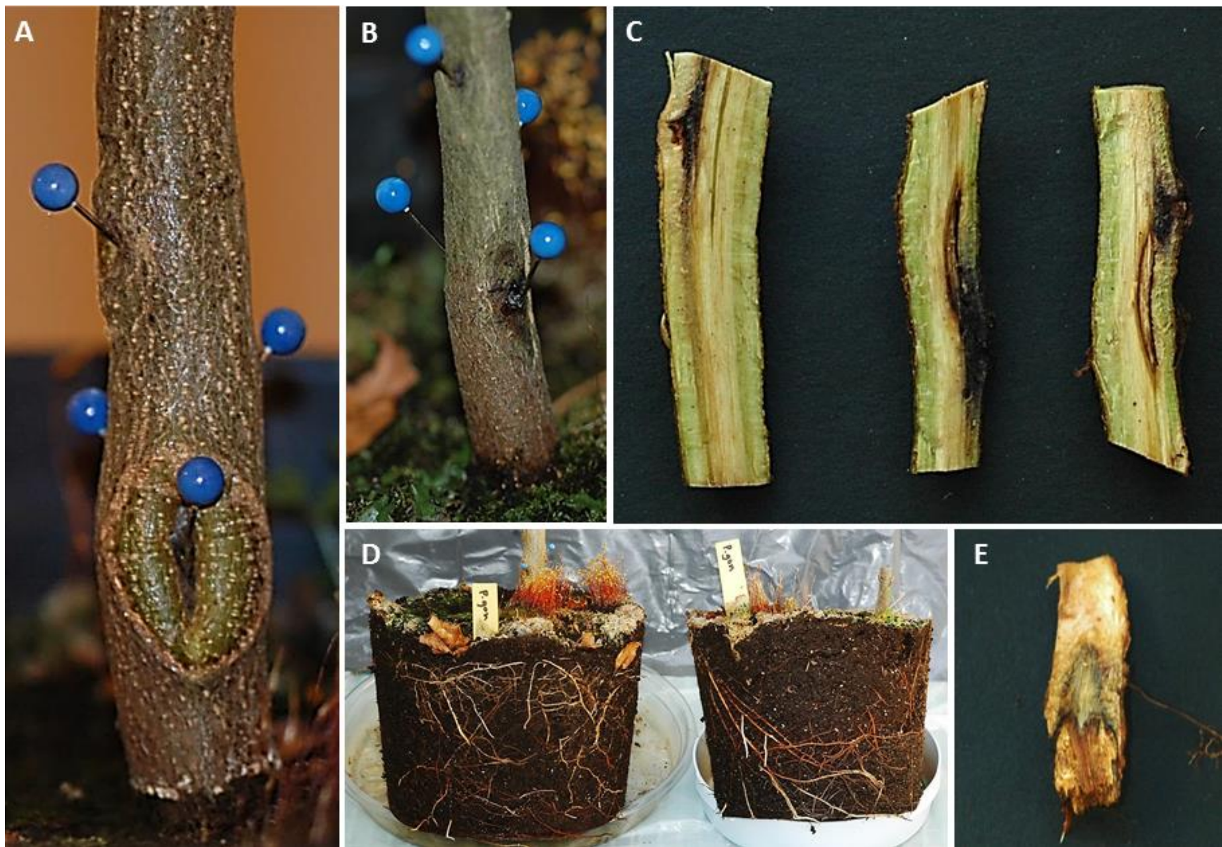

Fig. 24 Results from pathogenicity tests with beech (*Fagus sylvatica*) inoculated with *Phytophthora gonapodyides*. **A**, callus formation on trunk inoculated with map pins 30 weeks after inoculation; **B**, sunken area around the inoculation site 30 weeks after inoculation; **C**, lesions inside the trunk after inoculation with map pins 30 weeks after inoculation; **D**, good root development (left) where the stems had been inoculated with map pins, poor root development (right) where seedlings had been inoculated with mycelium on agar in the root zone 30 weeks after inoculation; **E**, discoloration in main root 20 weeks after agar inoculation. Photos: Kari H. Telfer

The control plants and the map pin inoculated seedlings had well-developed roots 30 weeks after inoculation compared to the seedlings that were inoculated with mycelium in the root zone (Fig. 22 E, 23 D, 24 D, 25 C and 26 C). Agar inoculation with all four *Phytophthora* species resulted in extensive dieback of fine and thicker roots. Discoloration was observed on several roots in all four groups. Discolored lesions on main roots (Fig. 22 D, 23 C, 24 E and 25 D and E) were used for re-isolation of the pathogen. *P. plurivora* and *P. gonapodyides* were successfully re-isolated from symptomatic tissue 20 weeks after inoculation. *P. lacustris* and *P. cambivora* were re-isolated from roots 30 weeks after inoculation.

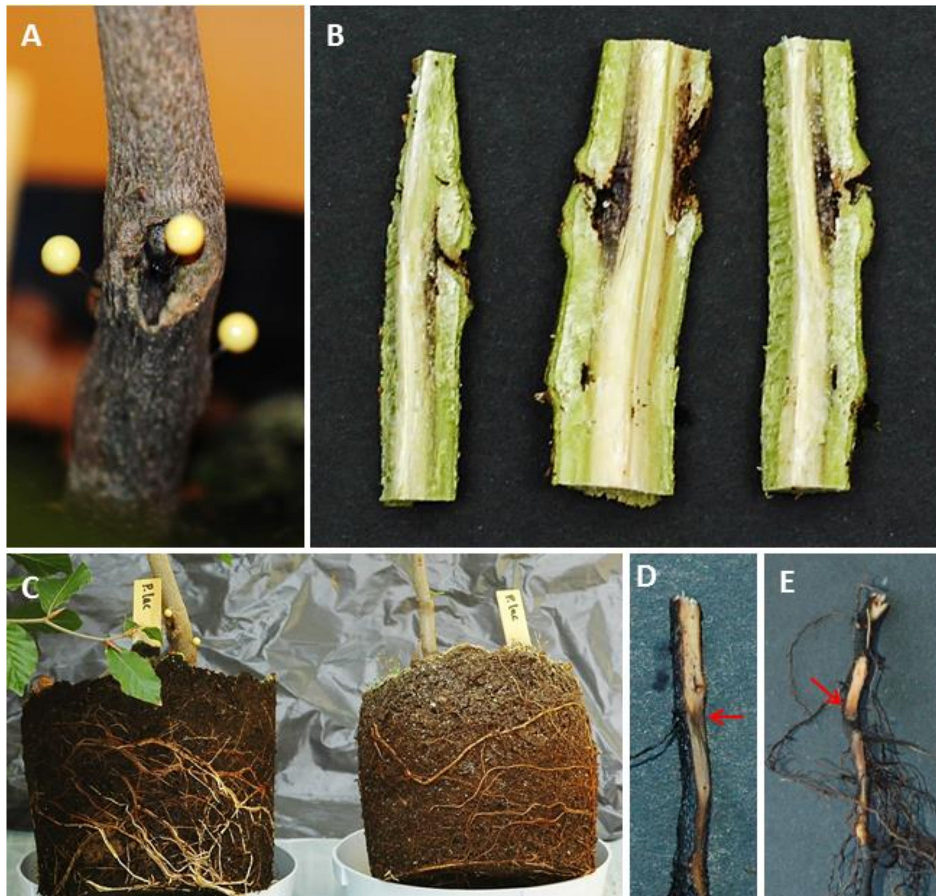

Fig. 25 Results from pathogenicity tests with beech (*Fagus sylvatica*) inoculated with *Phytophthora lacustris*. **A**, sunken area where trunk has been map pin inoculated with *P. lacustris* 30 weeks after inoculation; **B**, lesions inside the trunk 30 weeks after inoculation with map pins; **C**, good root development (left) where the stems had been inoculated with map pins, poor root development (right) where seedlings had been inoculated with mycelium on agar in the root zone, 30 weeks after inoculation; **D**, **E,** roots with *Phytophthora* symptoms (red arrows) 30 weeks after agar inoculation. Photos: Kari H. Telfer

Fig. 26 Beech seedlings (*Fagus sylvatica*) used as control plants in a pathogenicity test with four different *Phytophthora* species. **A**, Some callus formation around the inoculation site 30 weeks after inoculation with sterile map pins; **B**, no lesions inside the trunk 30 weeks after inoculation with sterile map pins; **C**, healthy root 30 weeks after inoculations with sterile agar. Photos: Kari H. Telfer

There was a significant difference in lesion length between *P. plurivora* and *P. cambivora* (P = 0.05) 30 weeks after inoculation (Fig. 27). However, there was no difference between *P. plurivora*, *P. gonapodyides* and *P. lacustris*, or between *P. cambivora*, *P. gonapodyides* and *P. lacustris* (Fig. 27).

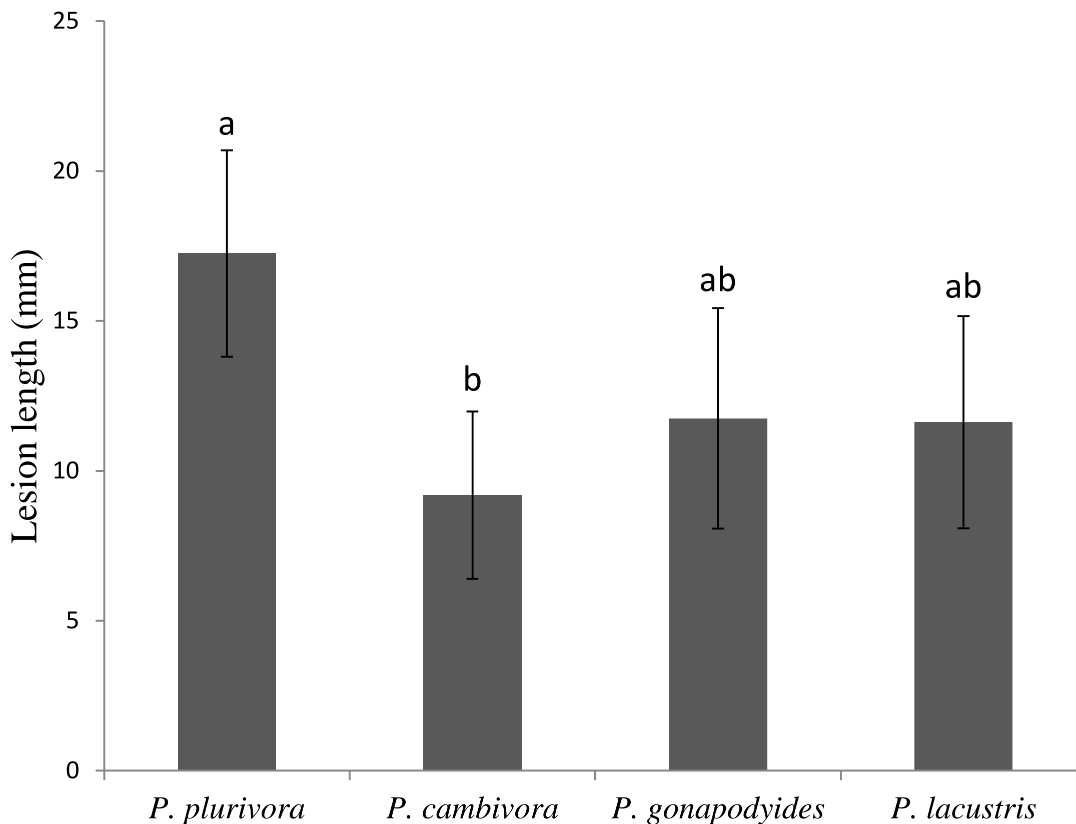

Fig. 27 Mean lesion lengths (mm) caused by *Phytophthora plurivora*, *P. cambivora*, *P. gonapodyides* and *P. lacustris* on map pin inoculated beech seedlings (*Fagus sylvatica*) 30 weeks after inoculation (P = 0.05). Error bars represent standard error of the mean of each group of inoculated plants. Different letters indicate significant difference at 95% confidence interval.

3.4. Potential role of slugs as vectors of *Phytophthora*

Passage of hyphae through slugs. No *Phytophthora* could be detected in the faeces before the start of the experiment (Fig. 28 and 29). PCR ran with ITS-primers yielded bands in all samples (Fig. 28 B and 29 B). The positive control (*P. ramorum*) got visible bands close to 500 base pairs (Fig. 28 A and 29 A).

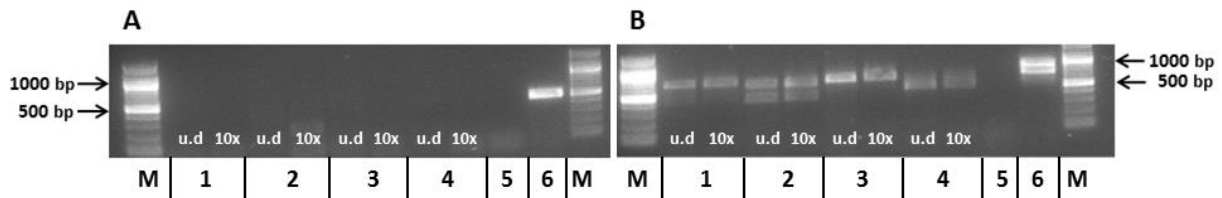

Fig. 28 Agarose gel with amplification products from DNA extracted from slug faeces collected from slugs prior to feeding on *Phytophthora cambivora*. Lane M: 100 base pair (bp) ladders; lanes 1 – 4, slug faeces, undiluted (u.d) and diluted 10 times (10x); lane 5, negative control (sterilized water); lane 6, positive control (*Phytopthora ramorum*). **A**, amplified DNA obtained with general *Phytophthora* primers (Yph1F and Yph2R). DNA fragment was 470 bp. **B**, amplified DNA obtained with primer pair ITS1 and ITS4.

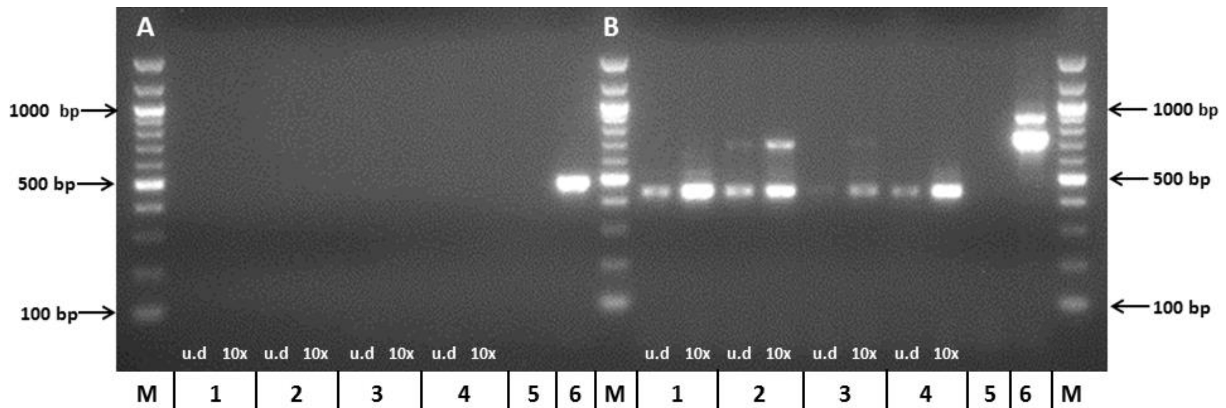

Fig. 29 Agarose gel with amplification products from DNA extracted from slug faeces collected from slugs prior to feeding on *Phytophthora plurivora* Amplification of DNA extracted from slug faeces. Lane M: 100 base pair (bp) ladders; lanes 1 – 4, slug faeces, undiluted (u.d.) and diluted 10 times (10 x); lane 5, negative control (water); lane 6, positive control (*Phytopthora ramorum*). **A**, amplified DNA obtained with general *Phytophthora* primers (Yph1F and Yph2R). The DNA segment was 470 bp. **B**, amplified DNA obtained with primer pair ITS1 and ITS4.

Six samples of faeces were sampled from the slugs after two days of feeding on *P. cambivora* mycelium. A *P. cambivora* specific PCR test confirmed that all 6 samples contained DNA of *P. cambivora* (Fig. 30).

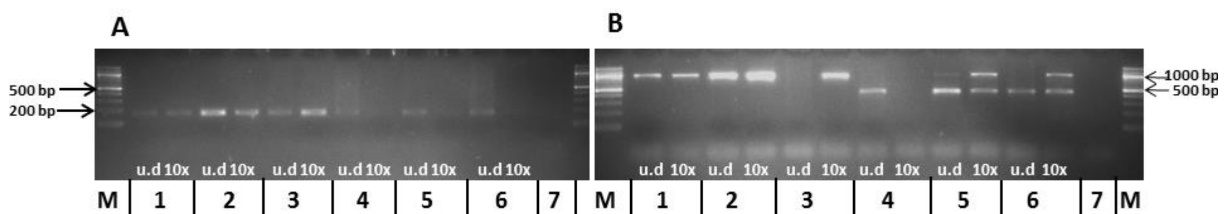

Fig. 30 Agarose gel with amplification products from DNA extracted from slug faeces collected from Spanish slugs (*Arion vulgaris*) after 2 days of feeding on *Phytophthora cambivora*. Lane M: 100 base pair (bp) ladders; lanes 1 – 6, slug faeces, undiluted (u.d) and diluted 10 times (10 x); lane 7, negative control (water); **A**, amplified DNA obtained with *Phytophthora cambivora* primers (Ycam4F and Ycam3R). The DNA segment was 183 bp. **B**, amplified DNA obtained with primer pair ITS1 and ITS4.

Faeces from slugs that had fed on *P. plurivora* mycelium for 2 days were plated on P_{10}ARPH agar. When transferred to V8 agar, a lot of antheridia, oogonia and oospores resembling *P. plurivora* were produced. Colony morphology on V8 also resembled that of *P. plurivora*.

After 6 days of feeding on *Phytophthora* spp. the slugs were placed on Petri dishes with P_{10}ARPH agar. Fig. 31 A shows hyphae growing from the faeces of slugs that had been feeding on *P. cambivora*, and when these hyphae were transferred to PDA (Fig. 31 C), the growth resembled that of *P. cambivora*.

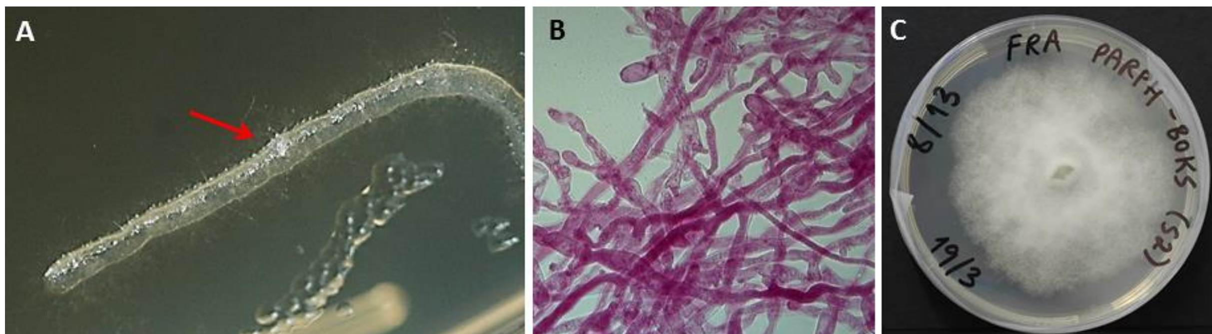

Fig. 31 Passage of *Phytophthora cambivora* through the digestive system of a Spanish slug (*Arion vulgaris*) **A**, hyphae (red arrow) growing from Spanish slug faeces on selective *Phytophthora* agar (P_5ARPH). **B**, coenocytic hyphae growing in Spanish slug faeces, artificially dyed with lactofuchsin. **C**, colony morphology resembling *P. cambivora* on potato dextrose agar originating from hyphae transferred from Spanish slug faeces.

Fig. 32 A shows hyphae growing out of faeces produced by slugs that had been feeding on *P. plurivora* mycelium. Oospores were observed in the faeces (Fig. 32 B), and when hyphae were transferred from P_5ARPH to PDA (Fig. 32 C), the growth resembled that of *P. plurivora*.

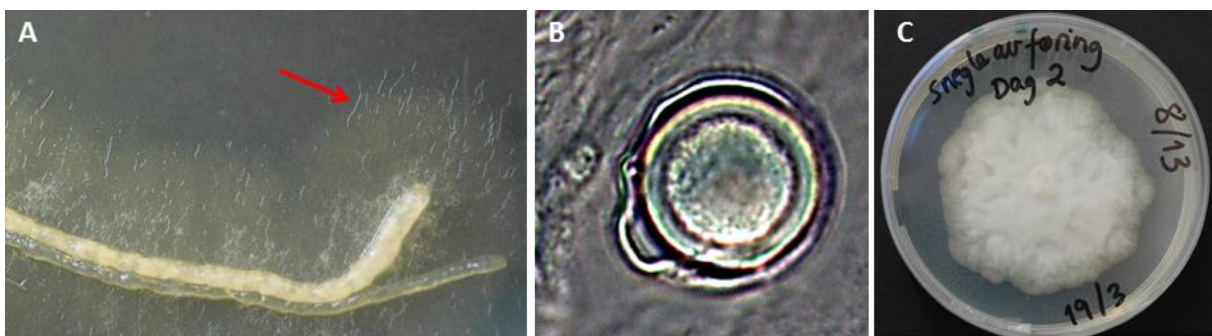

Fig. 32 Passage of *Phytophthora plurivora* through the digestive system of a Spanish slug (*Arion vulgaris*) **A**, hyphae (red arrow) growing from Spanish slug faeces on selective *Phytophthora* agar (P_5ARPH). **B**, oospore found in Spanish slug faeces. **C**, colony morphology resembling *P. plurivora* on potato dextrose agar. Hyphae transferred from Spanish slug faeces.

Transmission of *Phytophthora* with slugs to beech seedlings. Faeces collected after the starving period contained viable *Phytophthora* hyphae. The attempt to infect beech seedlings

with *Phytophthora* using slugs as vectors did not succeed. The trees showed no visible symptoms after 6 months. At the end of the experiment, the soil was baited with *Rhododendron* leaves. The leaves got a few spots, but when the leaves were transferred to P_5ARPH agar, there were no hyphal growth resembling *Phytophthora* spp.

Slugs collected in the beech forest in Larvik. The slugs, woodlouses and earthworms collected from the beech forest in Larvik contained different hyphae and bacteria which continued to grow when placed on P_5ARPH agar. None of the hyphae resembled *Phytophthora* spp.

4. Discussion

The results presented in this study show that *P. cambivora* is widely distributed on beech trees in the beech forest in Larvik. The frequency of trees with bleeding cankers was higher in two areas with very wet soil compared to the rest of the forest. Three *Phytophthora* species were recovered from soil and water inside the beech forest and in the construction area of NPRA. However, there were no findings of *Phytophthora* in the drinking water provided for private households in Larvik. In total, four *Phytophthora* were isolated from symptomatic tissue, soil and water, and all four species were pathogenic to roots and stems of beech seedlings. A small-scale survey of beech in a park in Oslo revealed *P. plurivora* from the leading edge of a bleeding canker.

The experiments regarding slugs as vectors of *P. plurivora* and *P. cambivora* clearly showed that *Phytophthora* hyphae are viable after passage through the digestive system of Spanish slugs. The attempt to infect beech seedlings with slugs as vectors did not succeed, and slugs, earthworms and woodlouses collected in the beech forest did not contain *Phytophthora* hyphae when placed on *Phytophthora* selective agar.

Field survey. Beech trees with bleeding cankers were found at several locations in the beech forest in Larvik. *P. cambivora* was recovered from symptomatic tissue and *P. plurivora* from *Rhododendron* leaves used as bait in ditch water. Positive LFD tests also confirmed the presence of *Phytophthora* spp. These findings support the hypothesis that *Phytophthora* spp. are widely distributed in the forest in Larvik.

Two areas with wet soil had a much higher concentration of trees with bleeding cankers than the rest of the forest. This observation can be explained by the beech trees low tolerance to waterlogging (Packham *et al.* 2012) and the rapid spread of zoospores in soil water and subsequent infection of roots (Erwin & Ribeiro 1996). Increased mortality of beech trees in waterlogged areas have been reported several times from different locations during the last decade, e.g. by Hartmann & Blank (1998) and Jung (2009). Jung (2009) has also indicated a correlation between extreme climatological conditions and *Phytophthora* epidemics, and the predicted climate change could therefore be an indirect threat to beech forests.

The 2012 survey revealed 54 trees with bleeding cankers. However, the actual number of infected trees may have been considerably higher. Several observations support this theory. One tree had small bleeding cankers in 2011, but was asymptomatic when the survey was done in 2012. Another tree had a canker where the dark reddish colored exudates had almost faded away in 2012 (Fig. 11 B), maybe due to rainfall. Similar cankers could easily have been overlooked in the survey. Other trees had lots of cracks and shed bark, but no bleeding cankers. Jung (2009) described similar observations in Germany where 1-year old *Phytophthora* cankers started to crack and 2-year old cankers where almost shed. However, there may be several explanations for cracking of the bark, including frost and sun scald. Many trees were colonized with brittle cinder (Fig. 3), a fungus that is often regarded a secondary invader on beech, especially in lesions produced by *Phytophthora* spp. (Jung 2009; Jung *et al.* 2005). Trees with cracks, shed bark, and infection by brittle cinder, but no visible bleeding cankers, were therefore not included in the 2012 survey. Five trees in a group of 22 that had no visual symptoms in 2012, had developed bleeding cankers in 2013, which indicate that trees may be infected for a longer period without showing symptoms (Thomas Jung, *Phytophthora* Research and Consultancy, Brannenburg, Deutschland, personal communication, 2012). All these observations may indicate that *Phytophthora* is more widespread than the 2012 survey revealed.

Another interesting observation was a beech tree with active bleeding cankers and sparse crown observed in 2011 (Venche Talgø at Bioforsk, Norwegian Institute for Agricultural and Environmental Research, Ås, personal communication), which, by October 2013, was completely dead (Fig. 12 C). Based on Jung's (2009) remarks about the cracking and shedding of bark of 1- and 2-year-old cankers, it seems plausible that the active bleeding cankers seen in 2011 were new. If so, the duration from the first sign of bleeding cankers till

the tree dies could be as short as two years. Rapid death of mature trees was also observed by Jung *et al.* (2005), though the exact duration was not mentioned.

Several trees with bleeding cankers were growing within 1 meter of a frequently used path. Further away from the path there were no trees with visible symptoms. This observation may indicate that the disease had been spread via human activity or animal vectors. A study on human vectors disseminating *Phytophthora* spp. was done in England by Webber & Rose (2008). They collected 400 soil samples from walker's boots in an area were *P. ramorum* (air borne species) and *P. kernoviae* (soil borne species) were widespread. Thirty percent of the samples contained both soil and airborne *Phytophthora* spp. which shows that humans can act as vectors. However, the ability of boot-carried inoculum to initiate infection in a *Phytophthora* free area is yet unknown. Another example is *P. lateralis*, an aggressive species on Port-Orford-Cedar (*Chamaecyparis lawsoniana*). This pathogen is claimed to be spread by humans on boots and vehicles, which have led to both permanent and seasonal closure of many roads in national forests (Hansen *et al.* 2000). Routinely washing of equipment and vehicles has also been done to prevent the spreading of *P. lateralis* from infested to uninfested areas (Hansen *et al.* 2000).

Neonectria spp., typical wound parasites, were observed in Larvik on several young (Fig. 4 A) and mature trees (Fig. 4 B and C) (Talgø *et al.* unpublished data). In previous studies, drought stress, species of *Neonectria* and scale insects were claimed to be the most important factors of beech bark disease (BBD) (Ehrlich 1934). However, in a study by Jung (2009) almost no scale insects were observed, but rather many mature trees with both bleeding cankers and *Neonectria*. Jung described cankers where *Phytophthora* was isolated from the leading edge of the canker and *Neonectria* was isolated 1-2 cm behind. Jung presented a hypothesis stating that *Phytophthora* species are a primary pathogen and that they play an important role of BBD. Observations done in the beech forest in Larvik, support Jung's hypothesis. Almost no scale insects were seen, but *Neonectria* fruiting bodies were observed on several trees with bleeding cankers, but not on randomly selected trees without bleeding cankers (Telfer & Talgø, unpublished data).

P. cambivora was the only species recovered from symptomatic tissue in the beech forest in Larvik. This species is well-known to cause root and collar rot on beech in Europe according to e.g. Day (1938), Jung *et al.* (2005) and Orlikowski *et al.* (2006). *P. cambivora* was

originally described by Petri in 1917 and is known to cause ink disease of chestnuts (*Castanea* spp.) in Europe and USA. It is also known to cause root and collar rot on several other deciduous trees, including *Prunus* spp., *Malus* spp. and horse chestnut (*Aesculus hippocastanum* L.) (Erwin & Ribeiro 1996).

P. cambivora is a heterothallic species that produces nonpapillate, ovoid sporangia by both nested and extended internal proliferation. The optimum temperature for growth is 22 - 24°C. The hyphae are often coralloid shaped and the growth on V8 and PDA are described as cottony. Isolate no. 250 154 from a beech tree in Larvik had an optimum temperature and morphological characteristics that corresponded well with the literature (Erwin & Ribeiro 1996; Vannini & Vettraino 2011). The attempt to produce oospores by crossing *P. cambivora* with a tester of *P. cryptogea* strain led to the production of amphigynous antheridia and oogonia with a smooth surface (Fig. 21 B). Since most *P. cambivora* oogonia are bullate (Vannini & Vettraino 2011) the oogonia obtained in the study reported here were therefore probably of the *P. cryptogea* type. The test did, however, reveal that the *P. cambivora* isolate no. 250 154 was of the A2 mating type.

P. plurivora was recovered from ditch water in the beech forest and from the beech tree in the park in Oslo. *P. plurivora*, formally known as *P. citricola* (Erwin & Ribeiro 1996; Jung & Burgess 2009), is known to be a serious pathogen on a broad range of deciduous trees in Europe. In recent years, it has become one of the most important pathogens on beech trees causing root and collar rot (Jung *et al.* 2005; Jung 2009; Jung & Burgess 2009; Milenković *et al.* 2012; Weiland *et al.* 2010).

P. plurivora is described as a homothallic species with paragynous antheridia and subglobose to globose oogonia and an optimum temperature of 25°C for hyphal growth. The sporangia are semi-papillate and can have many different shapes e.g. ovoid, limoniform, obpyriform, and ellipsoid. Bipapillate sporangia are observed frequently. Colony morphology is described as chrysanthemum shaped. The morphological characters and optimum temperature observed in this study correspond well to the original description of the species done by Jung & Burgess (2009).

Close to the beech forest in Larvik, *P. pseudosyringae* T. Jung & Delatour was discovered on wild bilberries (*Vaccinium myrtillus* L.) (Talgø *et al.* 2013). This species is previously isolated from necroses on beech in Germany (Jung *et al.* 2003). Together with *P. cambivora*

and *P. plurivora*, *P. pseudosyringae* could become an additional threat to the beech trees in the Larvik area.

Despite the use of *Phytophthora* selective agar (PARPH) which contains antimicrobial chemicals, including hymexazol inhibiting *Pythium* spp., the zygomycete *Mortierella pulchella* was isolated from a beech tree and the oomycetes *Pythium undulatum* and *Pythium anandrum* were recovered from ditch water in the beech forest. The two *Pythium* species are not considered problematic pathogens on beech, but *P. undulatum* has caused root rot in Noble Fir (*Abies procera* Rehder) and Douglas Fir (*Pseudotsuga menziesii* (Mirb.) Franco) in Germany (Weber *et al.* 2004), and *P. anandrun* has been isolated from fine roots of declining *Quercus* spp. (Jung & Blaschke 1996). *Mortierella pulchella* is known to be a inhabitant of peat (Summerbell 2005), and there is no reports in the literature about this organism beeing pathogenic to plants. Interestingly, Willis & Lambe (1980) found that some species of *Mortierella* inhibited the growth of *P. cinnamomi* when *Azalea* spp. were inoculated with both species.

In the construction area of NPRA for the new E18 road, *P. plurivora* was recovered from soil and *P. gonapodyides* and *P. lacustris* from waterways. These findings prove the second hypothesis stating that *Phytophthora* spp. are present in soil and water in the construction area. Considering the fact that *P. plurivora* is homothallic and therefore produce abundant oospores that can survive for long periods in the soil, the planned moving of soil from the construction area to a forest area could be a potential threat to beech and other host plants where soil will be deposited.

P. gonapodyides was recovered from baiting in two locations in the Farris Lake and *P. lacustris* in the Farris River. *P. gonapodyides* has been known for more than 100 years (Petersen 1909) and has been isolated from soil and water in the rhizosphere and from necrotic lesions from several broadleaved tree species in Europe including beech (Jung 2009); *Quercus* spp., *Salix* spp., and *Alnus* spp. (Brasier *et al.* 1993; Brasier *et al.* 2003; Corcobado *et al.* 2010). Even though the symptoms are similar to that of other *Phytophthora* species (Jung & Blaschke 1996), it is considered a minor pathogen by Erwin & Ribeiro (1996) and only found infrequently in e.g. beech stands (Jung 2009).

According to Erwin & Ribeiro (1996) and Brasier *et al.* (1993), *P. gonapodyides* is known to have nonpapillate, obpyriform to elipsoid sporangia and internal proliferation both by nesting

and by extending the sporangiophore, and an optimum temperature around 25°C. The colonies are chrysanthemum shaped on PDA and V8. In this study, the morphology and optimum temperature of one of the isolates of *P. gonapodyides* (isolate 250 157) recovered from water, corresponded with other reports (Erwin & Ribeiro 1996; Brasier *et al.* 1993).

P. gonapodyides is heterothallic (Erwin & Ribeiro 1996), but many isolates are thought to be sterile. According to Brasier *et al.* (1993), some *P. gonapodyides* isolates produce gametangia when paired to *P. cambivora* mating type A2. The crossing between these two species gave no result in this study even though the *P. cambivora* isolate (no. 250 154) had proven to be A2. Oospores were not produced in the crossing between *P. gonapodyides* and *P. cryptogea* mating types A1 and A2.

P. lacustris was formerly assigned to *P. gonapodyides*, but got the informal name *P.* taxon salixsoil in 2003 (Brasier *et al.* 2003) and was designated *P. lacustris* in 2012 (Nechwatal *et al.* 2012). *P. lacustris* is usually found in riparian habitats (Nechwatal & Mendgen 2006; Nechwatal *et al.* 2012), but have also been isolated from necrotic lesions on ash (*Fraxinus excelsior* L.) (Orlikowski *et al.* 2011), alder (*Alnus glutinosa* L.) and *Prunus* spp., usually after flooding (Nechwatal *et al.* 2012). The pathogenicity test performed in this study and in a study by Nechwatal *et al.* (2012) show that *P. lacustris* is capable of infecting beech.

According to literature, *P. lacustris* is sexually sterile with nonpapillate, ovoid sporangia and nested and extended internal proliferation. It produces ellipsoid hyphal swellings and is chrysanthemum shaped on V8 and with a less defined pattern on PDA at 25°C. The optimum temperature lies between 28 – 33°C. The morphology and optimum temperature of isolate no. 250 159 from this study corresponds well with the original description of the species (Nechwatal *et al.* 2012) The mean length and width measured were slightly larger than the mean size of the 34 isolates measured by Nechwatal *et al.* (2012), but the length to width ratio was almost the same.

According to Nechwatal *et al.* (2012), *P. lacustris* is a ‘silent A1’ which means it can induce gametangial formation in A2 isolates of other heterothallic species. In his study, *P. cambivora* produced oospores when paired to *P. lacustris*. The latter remained sterile. In this study, oospores were not formed in the crossing with *P. cambivora* A2 or with *P. cryptogea* A1 and A2.

Baiting water inside the waterworks gave no lesions on the *Rhododendron* leaves. The hypothesis stating that *Phytophthora* is present in the drinking water was therefore disproved. This result indicates that the intake depth of 40 meters is not a suitable habitat for Oomycetes. Irrigating private gardens with this water seems safe in regard to *Phytophthora*.

Pathogenicity tests. All four species of *Phytophthora* were pathogenic to root and stem of beech. For the species isolated from beech trees, *P. plurivora* (isolate 250 151) and *P. cambivora* (isolate 250 154), Koch's postulates were fulfilled. The results of the pathogenicity test were consistent to previous studies (Jung 2009; Jung & Blaschke 1996; Nechwatal *et al.* 2012).

The soil infestation test resulted in poor root development compared to the seedlings inoculated with map pins and the control plants. Thirty weeks after inoculation, the seedlings had few fine roots left, and several roots had necrotic lesions.

None of the map pin inoculated seedlings developed bleeding cankers on the stem. However, sunken areas around the inoculation sites were observed on seedlings inoculated with *P. plurivora,* (Fig. 22 A and B), *P. gonapodyides* (Fig. 24 A and B) and *P. lacustris* (Fig. 25 A). Sunken, necrotic areas on the stem after inoculation with *Phytophthora* spp. were also reported by Jung & Blaschke (1996).

In the map pin inoculation, *P. plurivora* was more aggressive to beech than *P. cambivora* (Fig. 27). *P. gonapodyides* and *P. lacustris* were almost equally pathogenic to beech, but not significantly different to the other two species. *P. cambivora* is known to be one of the most frequently isolated *Phytophthora* species on beech (Day 1939; Jung 2009; Jung *et al.* 2005; Orlikowski *et al.* 2006) and the most aggressive species in pathogenicity tests (Jung *et al.* 2005; Orlikowski *et al.* 2006). However, in this study and in a study by Nelson *et al.* (2010), *P. plurivora* was more aggressive than *P. cambivora*. Brasier & Kirk (2001) have performed several pathogenicity tests with *Phytophthora* spp. on several woody hosts, including beech, and reported that lesion lengths varied a lot from isolate to isolate and from year to year, which could explain the results in this study.

In this study, the number of isolates and the number of seedlings used in the pathogenicity tests were limited, and the lesion lengths varied a lot between individual seedlings in each of the four groups (data not shown). More isolates per *Phytophthora* species, a higher number of

seedlings and repeated experiments would be required to determine more about which species is the most aggressive.

Slugs as vectors of *Phytphthora*. Most beech trees in Larvik had cankers on the stem base, but one tree had symptoms 7 meters above ground. Jung (2009) observed the same phenomena on beech and managed to isolate the soilborne pathogens *P. cambivora*, *P. gonapodyides* and *P. plurivora* from beech stems 20 meters above ground. Brown & Brasier (2007) claimed that *Phytophthora* can spread in non-symptomatic vessels which could explain how the symptoms could appear at such heights. Movement of animal vectors, such as snails, slugs, and insects, may also explain the symptoms (Jung 2009).

Viability of hyphae. Faeces from Spanish slugs feeding on cultures of *P. cambivora* and *P. plurivora* contained DNA from *Phytophthora* and more importantly, viable hyphal fragments that continued to grow on *Phytophthora* selective agar (Figs 31 A and 32 A). These results strongly support the hypothesis that *Phytophthora* hyphae can survive after passing through the slug's digestive system. Previous studies of snails, slugs and other *Phytophthora* spp. have reached the same conclusion; Turner (1967) showed that *P. palmivora* sporangia survived passing through the digestive system of the giant African snails (*Achatina fulica* Bow.). Parke *et al.* (2008) examined faeces from pacific banana slugs (*Ariolimax columbianus* Gould) microscopically after feeding slugs with *P. ramorum* infested leaves (tanoak, rhododendron, bay) and found that the faces contained numerous chlamydospores of *P. ramorum*. When plated on *Phytophthora* selective agar, chlamydospores were viable (Parke *et al.* 2008).

Oospores passing through the *P. plurivora* fed Spanish slugs in this study were not specifically examined for viability, but germination of oospores has been observed in a similar experiment with *P. erythroseptica* Pethybr. passing through the brown garden snail (*Helix aspersa* O. F. Müller) (Gregg 1957).

Transmission of *Phytophthora* to beech seedlings. Even though faeces from slugs contained viable *Phytophthora*, no visible infection was observed after the slugs had stayed on the beech seedlings for 18 days. This is in contrast to results observed by Alvarez *et al.* (2009), El-Hamalawi & Menge (1996), and Parke *et al.* (2008). After pacific banana slugs were fed *P. ramorum* in a laboratory trial, they were able to infect 2 out of 9 tanoak logs (*N. densiflorus*) (Parke *et al*. 2008). El-Hamalawi & Menge (1996) showed that brown garden snails that had fed on *P. citricola* infected avocado trees (*Persea Americana* Mill.), were able to transmit the

disease and infect avocado trees. Alvarez *et al.* (2009) transferred brown garden snails that had been feeding on *P. citrophthora* to potted clementine trees (*Citrus* clementine Hort ex. Tanaka) in a glasshouse experiment. After 10 days, stems showed discoloration and *P. citropthora* was isolated from symptomatic tissue. The experiment conducted in this study was a pilot test with only two trees, which could explain the lack of results.

Slugs collected in the beech forest in Larvik. Slugs, earthworms and woodlouses from diseased beech trees and the area surrounding the trees, did not result in any *Phytophthora* growth after incubation on *Phytophthora* selective agar. The hypothesis proposed in this study suggesting that slugs collected on or near beech trees with *Phytophthora* symptoms contain *Phytophthora*, could not be verified. Previous studies have shown that insects and snails can contain *Phytophthora* in the field (El-Hamalawi & Menge 1996; Konam & Guest 2004; Turner 1967). A study performed by Alvarez *et al.* (2009) showed that 5 % of 220 brown garden snails collected from citrus trees with *Phytophthora* cankers contained *Phytophthora* (Alvarez *et al.* 2009). A limited number of examined slugs, earthworms and woodlouses in this study could therefore explain the negative result.

Practical considerations.

The beech forest in Larvik is one of the world's northernmost beech forests. It is of great importance locally as a public park, and nationally as the largest beech forest in Norway. Two of the *Phytophthora* species that were recovered from Larvik, *P. plurivora* and *P. cambivora*, are known to be highly aggressive to beech and have contributed to the decline of beech in Europe (Jung *et al.* 2005; Jung 2009; Milenković *et al.* 2012; Orlikowski *et al.* 2006; Talgø *et al.* 2012; Thinggaard 2009; Witzell & Hultberg 2012). It is therefore of great concern that these two species have been found in the beech forest in Larvik.

Another concern is the ongoing discussion about partially replacing Norway spruce (*Picea abies* (L.) H. Karst.) suffering from drought stress with beech in some areas in southern parts of Norway (Madsen *et al.* 2013). This might be a problem because of the widespread distribution of *Phytophthora* infected beech trees (this study; Talgø *et al.* 2012; Talgø *et al.* 2010). The replacement of Norway spruce with beech will require non-infected nursery stock, as well as sound management strategies in *Phytophthora* infected areas.

Based on the results in this study, it is difficult to draw conclusions about the importance of Spanish slugs as vectors of *Phytophthora* spp. However, hyphae proved to be viable after passage through the digestive system, and it would therefore be interesting to continue the work on a larger scale.

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APPENDIXES I-II

I. Agar receipts

Phytophthora selective agar (P_{10}ARPH)*

*Used until 1st of April 2013

Corn meal agar (Difco™)... 17.0 g
Distilled water .... 1000.0 ml
Autoclaved for 20 min at 121°C

Pimaricin.. 0.4 ml
Ampicillin .... 0.25 g
Rifampicin... 0.01 g
Dimethylsulfoxide... 1.0 ml
Pentachloronitrobenzen.. 0.10 g
Hymexazol... 0.05 g

Phytophthora selective agar (P_5ARPH)*

*Used after 1st of April 2013

Corn meal agar (Difco™)... 17.0 g
Distilled water .... 1000.0 ml
Autoclaved for 20 min at 121°C
Pimaricin.. 0.2 ml
Ampicillin .... 0.25 g
Rifampicin... 0.01 g
Dimethylsulfoxide... 1.0 ml
Pentachloronitrobenzen.. 0.05 g
Hymexazol... 0.025 g

Difco™ Potato Dextrose Agar (PDA)
Approximate formula per liter distilled water.
Potato Starch (from infusion).................................... 4.0 g
Dextrose .... 20.0 g
Agar .... 15.0 g

(PDAS)

Tartaric acid (per 1000 ml PDA)................................ 19.0 ml

Tartaric acid 1:10
90 ml distilled water
10 g Tartaric acid
Autoclave for 20 minutes

Carrot piece agar (CPA)

Fresh, shreddet carrots……... 35.0 g

Bacto agar .... 22.0 g

Distilled water .... 1000.0 ml

V8® Juice Agar

Distilled water ……... 1416 ml

V8® juice... 354 ml

$CaCO_3$……………………………………………….. 3.5 g

Agar……………………...………………………... 26.5 g

II. DNA isolation method

DNeasy® Plant Mini Kit from Qiagen

DNeasy® Plant Mini Kit

The DNeasy Plant Mini Kit (cat. nos. 69104 and 69106) can be stored at room temperature (15–25°C) for up to 1 year.

For more information, please refer to the *DNeasy Plant Handbook*, which can be found at www.qiagen.com/handbooks.

For technical assistance, please call toll-free 00800-22-44-6000, or find regional phone numbers at www.qiagen.com/contact.

Notes before starting

- Perform all centrifugation steps at room temperature (15–25°C).
- If necessary, redissolve any precipitates in Buffer AP1 and Buffer AW1 concentrates.
- Add ethanol to Buffer AW1 and Buffer AW2 concentrates.
- Preheat a water bath or heating block to 65°C.

1. Disrupt samples (≤100 mg wet weight or ≤20 mg lyophilized tissue) using the TissueRuptor®, the TissueLyser II, or a mortar and pestle.
2. Add 400 μl Buffer AP1 and 4 μl RNase A. Vortex and incubate for 10 min at 65°C. Invert the tube 2–3 times during incubation.

   Note: Do not mix Buffer AP1 and RNase A before use.
3. Add 130 μl Buffer P3. Mix and incubate for 5 min on ice.
4. **Recommended**: Centrifuge the lysate for 5 min at 20,000 x *g* (14,000 rpm).
5. Pipet the lysate into a QIAshredder spin column placed in a 2 ml collection tube. Centrifuge for 2 min at 20,000 x *g*.
6. Transfer the flow-through into a new tube without disturbing the pellet if present. Add 1.5 volumes of Buffer AW1, and mix by pipetting.

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7. Transfer 650 µl of the mixture into a DNeasy Mini spin column placed in a 2 ml collection tube. Centrifuge for 1 min at ≥6000 x *g* (≥8000 rpm). Discard the flow-through. Repeat this step with the remaining sample.

8. Place the spin column into a new 2 ml collection tube. Add 500 µl Buffer AW2, and centrifuge for 1 min at ≥6000 x *g*. Discard the flow-through.

9. Add another 500 µl Buffer AW2. Centrifuge for 2 min at 20,000 x *g*.

   Note: Remove the spin column from the collection tube carefully so that the column does not come into contact with the flow-through.

10. Transfer the spin column to a new 1.5 ml or 2 ml microcentrifuge tube.

11. Add 100 µl Buffer AE for elution. Incubate for 5 min at room temperature (15–25°C). Centrifuge for 1 min at ≥6000 x *g*.

12. Repeat step 11.

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual.

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