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Phytophthora in Finnish nurseries

Anna Rytönen

Department of Forest Ecology
Faculty of Agriculture and Forestry
University of Helsinki

Academic dissertation

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Author: Anna Rytkönen

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Supervisors:

Doc. Arja Lilja

Finnish Forest Research Institute, Vantaa Unit, Finland

Prof. Jarkko Hantula

Finnish Forest Research Institute, Vantaa Unit, Finland

Pre-Examiners:

Dr. rer. silv. Thomas Jung

Phytophthora Research and Consultancy, Brannenburg, Deutschland/

Centre for Phytophthora Sciences and Management (CPSM), Murdoch University, Australia

Dr. Mauritz Vestberg

Plant Production Research, MTT Agrifood Research Finland

Opponent:

Prof. Everett Hansen

Department of Botany and Plant Pathology, Oregon State University, USA

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ABSTRACT

International trade and travel have facilitated the spread of harmful organisms around the world. Human-mediated movement of plants and plant products is now generally accepted to be the primary mode of introduction of pathogens and pests. Species of *Phytophthora*, a genus of plant pathogens, are commonly spread in this way and have caused severe epidemics in silviculture, horticulture and natural systems all over the world.

The objective of the study was to gather information on the occurrence of *Phytophthora* spp. in Finnish nurseries. Furthermore, the aim was to produce information for risk assessments for these *Phytophthora* spp. and establish molecular means for their detection. *Phytophthora cactorum* was found to persist in natural waterbodies and results suggest that irrigation water might be a source of inoculum for the disease in nurseries. In addition to *P. cactorum*, isolates from ornamental *Rhododendron* in nurseries yielded three species new to Finland: *P. ramorum*, *P. plurivora* and *P. pini*. The only species with quarantine status, *P. ramorum*, was able to persist in the nursery in spite of an annual European Commission sanitation protocol. *Phytophthora plurivora* and the closely related *P. pini* had more hosts among Nordic tree species and other plants abundant in Finnish nurseries and forest ecosystems. They were also found to have higher infectivity rates compared to *P. ramorum* and *P. cactorum*. All four species survived two weeks in -5 °C, and thus soil survival of these *Phytophthoras* in Finland is likely under current climatic conditions.

The most common tree species in Finnish nurseries, *Picea abies*, was highly susceptible to *P. plurivora* and *P. pini* in pathogenicity trials. In a histological examination of *P. abies* shoot tissues inoculated with *P. plurivora* zoospores, fast necrotrophic growth was observed in nearly all tissues of the fresh shoot. The production of propagules in *P. abies* shoot tissue was only weakly indicated.

In this study, a PCR–DGGE technique was developed for simultaneous detection and identification of *Phytophthora* spp. It reliably detected *Phytophthora* in plant tissues and could discriminate most test species as well as indicate instances of multiple-species infections. It proved to be a useful detection and identification tool either applied alone or in concert with traditional isolation culture techniques. The limitations of the method are also discussed.

In conclusion, all of the introduced species of *Phytophthora* had properties that promote a high risk of establishment and spread in Finland. The efficient transport of *Phytophthora* via commercial traffic and favorable conditions due to expected climate change will increase the incidence and establishment of *Phytophthora* in new areas. Thus, it is probable that pathogens of this genus will be introduced and become established in Finland and other Nordic countries unless efficient phytosanitary control becomes standard practice in the international plant trade.

Keywords: *Phytophthora*, pathogenicity, survival, molecular detection, DGGE

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Vantaa, November 2011
Anna Rytönen

LIST OF ORIGINAL ARTICLES

The thesis is based on the following articles, which are referred to by their Roman numerals (I–IV) in the text. Articles I–II are reproduced with the kind permission from the publishers, while the study III and IV are the author versions of the submitted manuscripts.

- I Rytkönen, A., Lilja, A., Petäistö R.-L. and Hantula, J. 2008. Irrigation water and *Phytophthora cactorum* in a forest nursery. *Scandinavian Journal of Forest Research*, 23: 404-411. doi: 10.1080/02827580802419034
- II Rytkönen, A., Lilja, A. and Hantula, J. 2010. New PCR-DGGE method for in planta detection and identification of *Phytophthora* species. *Forest Pathology*, in press. doi: 10.1111/j.1439-0329.2011.00716.x
- III Rytkönen, A., Lilja, A., Vercauteren, A., Sirkiä, S., Parikka, P., Soukainen, M. and Hantula, J. 2011. Identity and potential pathogenicity of *Phytophthora* species found on symptomatic rhododendron plants in a Finnish nursery. *Canadian Journal of Forest Research*, submitted MS.
- IV Rytkönen, A., Lilja, A., Sirkiä, S., Werres, S. and Hantula, J. 2011. Infectivity, survival and pathology of Finnish strains of *Phytophthora plurivora* and *P. pini* in Norway Spruce (*Picea abies* L.). *Plant Disease*, submitted MS.

AUTHOR'S CONTRIBUTION

- I Anna Rytkönen planned the work with J. Hantula and A. Lilja. Microbe isolations were done by A. Lilja and R.-L. Petäistö. Rytkönen was responsible for characterisation of the isolates except for the pathogenicities, as well as all of the laboratory work. Statistical analyses were done by Rytkönen together with A. Lilja. Rytkönen had the main responsibility for the writing process. The coauthors participated in the writing and discussion of the results.
- II Anna Rytkönen planned the work with J. Hantula and was responsible for the laboratory work. Rytkönen interpreted the results and had the main responsibility for the writing process. The coauthors participated in the discussion of results.
- III Anna Rytkönen planned the work with A. Lilja. Rytkönen was responsible for sampling and microbe isolations in collaboration with M. Soukainen. Rytkönen did the laboratory work in collaboration with A. Vercauteren and microscopic work with A. Lilja. Rytkönen conducted the pathogenicity trials in collaboration with P. Parikka and A. Lilja, and was responsible for the survival tests as well as the statistical analyses in collaboration with S. Sirkiä. Rytkönen had the main responsibility for the writing process. The coauthors participated in the writing and discussion of the results.
- IV Anna Rytkönen planned the work with A. Lilja. Rytkönen was responsible for inoculations and temperature trials. Rytkönen planned and conducted the histological examinations together with S. Werres, and statistical analyses in collaboration with S. Sirkiä. Rytkönen interpreted the results, wrote the manuscript, and received comments from coauthors.

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1 INTRODUCTION

1.2 Genus *Phytophthora* as plant pathogens

Typical features of Phytophthora

Phytophthora (derived from Greek: “plant-destroyer”) is a genus of Oomycetes (water molds) that are not true fungi despite filamentous hyphal growth and reproduction by spores. They are more closely related to brown algae, and belong to the Chromalveolata, which in the most recent classification of protists is regarded as one of the six major groups within the Eukaryota (Adl et al. 2005). The Oomycetes mainly differ from fungi by having primarily diploid hyphae and cell walls composed of cellulose and betaglycans instead of chitin. The genus *Phytophthora* consists mainly of species that are parasitic to various plant hosts. Some of the species are host specific at the species or generic scale whereas many others appear to be host generalists (Erwin & Ribeiro 1996). They are well known for causing enormous economic losses to farmers, gardeners and foresters worldwide. *Phytophthora* spreads mainly through the movement of infected plants, soil, growth media and water, although there are species that are capable of aerial transmission. The infective propagules are asexual biflagellate zoospores, which are produced in sporangia and spread via aquatic media, interstitial soil moisture, raindrop splashes and wind-driven rain. Consequently, damages often occur under wet climatic conditions and poorly drained soils (Sanogo 2006, Xie et al. 1999, Rhoades et al. 2003, Ma et al. 2008). Some of the most devastating *Phytophthora* diseases occur in periodically wet and dry environments by synergistic interaction between fine root losses by *Phytophthora* and severe droughts (Jung et al. 1996, 2000, Balci & Halmschlagler 2003, Newhook & Podger 1972). *Phytophthora* spp. persist during unfavorable conditions as thick-walled asexual chlamydospores or as sexual oospores, which are produced when a female oogonium is fertilized by an antheridium. In heterothallic *Phytophthora* species, two individuals representing different mating types are required for oospore production.

Endemic *Phytophthoras* in their native ecosystems have coevolved with their hosts, and coexist while not usually causing severe diseases. Disease problems most often arise when the pathogen is introduced to a new habitat. In that respect, species of *Phytophthora* are well suited to transfer via human activities. They can cause major problems to plants in nurseries and natural ecosystems where plants have little resistance against the alien pathogen and where it has few if any natural enemies. The scale of problems that can be caused by introduced *Phytophthora* is perhaps best illustrated by the case of *P. cinnamomi* Rands, which has a long history of ecological destruction. *P. cinnamomi* is believed to have destroyed the American *Castanea dentata* (Marshall) Borkh. prior to the blight that caused their near extinction in the early 1900s. Around the same time, *P. cinnamomi* was implicated as the cause of Littleleaf Disease in *Pinus echinata* Miller, which destroyed over 2 million hectares of forest in the southeastern US (Tainter & Baker 1996). The devastation was also due to reckless land use in the 1700s, after which the natural populations of *P. echinata* were mostly growing on poor and eroded soils. The introduction of *P. cinnamomi* to Western Australia in the 1920s (Podger 1972), most likely due to the nursery trade, caused an epidemic dieback of *Eucalyptus marginata* Donn ex Sm. (Newhook & Podger 1972). As a result of a separate introduction, Eucalypt ecosystems in Victoria are also experiencing dieback caused by *P. cinnamomi* (Marks & Smith 1991). Today, *P. cinnamomi* is considered one of the most serious environmental threats to the continent. Many of the native plants in the understory of forest ecosystems and on heathlands, some of which are endangered species, have proven to be even

more susceptible than *Eucalyptus* spp. and the disease has advanced quickly with ruinous effect (Hardham 2005, Weste 1994). In Western Australia, at least 54% of the native flora are susceptible to *P. cinnamomi* (Shearer et al. 2004, Shearer & Dillon 1995, 1996). In Southern Europe, *P. cinnamomi* causes wide spread decline of *Quercus suber* L. and *Q. ilex* L. (Brasier et al. 1993, Brasier 1996, Robin et al. 1998, Moreira & Martins 2005), root rot in *Juglans regia* L. (Belisario et al. 2006) and in combination with *P. cambivora* (Petri) Buisman, the devastating ink disease of *Castanea sativa* Mill. (Vettrano et al. 2005).

In contrast to the wide host range of *P. cinnamomi*, *P. lateralis* Tucker & Milbrath is host-specific to the endemic *Chamaecyparis lawsoniana* (Murr.) Parl. (Port-Orford-Cedar) in Northern California and Oregon. *C. lawsoniana* is a valuable and commercially harvested conifer which, since the 1920s, has been dying from root and collar infections by *P. lateralis*, an alien pathogen introduced most likely by the nursery trade and translocated in the landscape via human activity (Hansen et al. 2000, Hansen 2008). Its origin is suggested to be Asian (Brasier et al. 2010). In Europe, *P. lateralis* was first detected in hedges in France and in nurseries in the Netherlands in 2005. In 2010, the pathogen arrived in Scotland where it has caused the death of established hedgerows of *C. lawsoniana* at an alarming speed at four separate locations (Sarah Green, personal communication). Infections at these sites are currently being treated.

1.2 New species of *Phytophthora* in nurseries and natural ecosystems

Nursery plants and seedlings are vulnerable to agricultural diseases including those caused by *Phytophthora*. They are commonly found in horticultural as well as forest tree nurseries all over the world, and the species diversity seems to have increased in recent years (MacDonald et al. 1994, Themann et al. 2002, Jung & Blaschke 2004, Davidson et al. 2006, Schwingle et al. 2007, Hong et al. 2008, Jung 2009, Moralejo et al. 2009a, Yakabe et al. 2009, Hulvey et al. 2010, Goss et al. 2011). They cause root and collar rot, dieback, damping off, aerial bark lesions on stems as well as twig and leaf blights on various plant species.

In recent years, multiple new species of *Phytophthora* have been described, most of which have been found through studies and surveys of diseases in forests or semi-natural ecosystems, eg. alder dieback in Europe (*P. alni* C. Brasier & S.A. Kirk, *P. polonica* M. Belbahri, E. Moralejo & F. Lefort) (Brasier et al. 2004a, Belbahri et al. 2006), declining oak stands in Europe (*P. europaea* E.M. Hansen & T. Jung, *P. gallica* T. Jung & J. Nechwatal, *P. pseudosyringae* T. Jung & Delatour, *P. psychrophila* T. Jung & E.M. Hansen, *P. uliginosa* T. Jung & E.M. Hansen and *P. quercina* T. Jung) and the US (*P. ramorum* De Cock & Man in 't Veld, *P. nemorosa* E.M. Hansen & Reeser and *P. siskiyouensis* Reeser & E.M. Hansen), and declining native ecosystems in Argentina (*P. austrocedrae*; Greslebin et al. 2007) and Australia (*P. multivora* P.M. Scott & T. Jung, *P. elongata* A. Rea, M. Stukely & T. Jung, *P. gibbosa* T. Jung, M.J.C. Stukely & T.I. Burgess, *P. gregata* T. Jung, M.J.C. Stukely & T.I. Burgess, *P. litoralis* T. Jung, M.J.C. Stukely & T.I. Burgess, *P. thermophila* T. Jung, M.J.C. Stukely & T.I. Burgess and *P. fluvialis* T. Jung & T. I. Burgess) (Jung et al. 1999, Werres et al. 2001, Jung et al. 2002, Rizzo et al. 2002, Jung et al. 2003, Reeser et al. 2007, Jung & Nechwatal 2008, Scott et al. 2009, Rea et al. 2010, Crous et al. 2011, Jung et al. 2011, Reeser et al. 2011), as well as dieback in forest plantations in New Zealand (*P. captiosa* M. A. Dick & Dobbie and *P. fallax* Dobbie & M. A. Dick) (Dick et al. 2006) and Southafrica (*P. alticola* Maseko, Coutinho & M.J Wingf., *P. frigida* Maseko, Coutinho & M.J Wingf. and *P. capensis* C.M. Bezuidenhout, S. Denman, A. McLeod & S.A. Kirk) (Maseko et al. 2007, Bezuidenhout et al. 2010).

The increasing taxonomic diversity in the genus might partly be due to increased proficiency of modern detection methods, since traditional isolation and identification of pathogenic *Phytophthora* has been relatively difficult. Improvements in molecular methods have also revealed cryptic species. For example, the morphospecies *P. citricola* Sawada has been shown to consist of multiple morphologically similar but genetically distinct species, some of which have recently received a formal description. These include *P. capensis*, *P. menzei*, *P. multivora* and *P. plurivora* (Hong et al. 2009, Jung & Burgess 2009, Scott et al. 2009, Bezuidenhout et al. 2010), as well as the recently resurrected *P. pini* (Hong et al. 2011). Also the morphospecies *P. megasperma* Drechsler *sensu lato* has been shown to contain multiple distinct species (Hansen & Hamm 1983, Hamm & Hansen 1991, Cooke et al. 2000). After the first molecular phylogeny of *Phytophthora* based on internal transcribed spacer (ITS) 1 sequences by Cooke et al. (2000), a re-evaluation of the polyphyletic *P. megasperma* as well as the whole of ITS Clade 6 was needed. Since then, many distinct, undescribed taxa have been found within the clade, resulting in description of many new species (Brasier et al. 2003a, b, Hansen et al. 2009, Duran et al. 2008, Jung et al. 2011).

More efficient detection methods have also revealed previously unknown communities of *Phytophthora* species in forest soils and streams which are more or less undisturbed (Jung et al. 1996, Hansen & Delatour 1999, Hong et al. 2008, Reeser et al. 2011). Some of these forest soil *Phytophthoras* are probably indigenous and can also be found in healthy forest stands. In Europe, these indigenous *Phytophthora* spp. most likely contribute to oak decline which is mainly caused by invasive *Phytophthora* species (Hansen & Delatour 1999, Jung et al. 2000). For the most part, new species are increasingly being recognized when they are exported via human activity from their natural ecosystems where they do not necessarily cause serious damage to their host plants, and introduced to natural ecosystems and agricultural or forestry systems in new geographical areas where disease typically occurs.

One of the most notorious among the recently described species of *Phytophthora* is *P. ramorum*. It has been associated with twig blight of nursery *Rhododendron* in Germany and the Netherlands since the 1990s and was described in 2001 (Werres et al. 2001). Later, the same species was identified as the causal agent of Sudden Oak Death (SOD) of *Quercus* spp. and *Notholithocarpus densiflorus* (Hook. & Arn.) Manos, Cannon & S.H. Oh in California, and is now also present in Oregon and Southwestern Canada (Rizzo et al. 2002, Rizzo & Garbelotto 2003, Davidson et al. 2005, Rizzo et al. 2005, Hansen et al. 2008, Kliejunas 2010) (Fig 1.). Due to its caducous sporangia *P. ramorum* is adapted to an aerial lifestyle, and in addition to causing lethal canker on oak stems, it also infects the leaves of various other plants in the understory (Davidson et al. 2005). The leaf hosts, most importantly *Umbellularia californica* (Hook. & Arn.) Nutt., enable the build-up of an enormous inoculum and subsequent spread of sporangia to stems of oaks, which then rapidly succumb due to the multitude of cankers. *Phytophthora ramorum* has so far been detected in several European countries (EPPO 2011), mainly in nurseries, gardens and parks where it occurs most commonly on *Rhododendron*, *Viburnum* and *Camellia*. In the UK and the Netherlands, *P. ramorum* has also been isolated from mature trees, but before 2009, always in close proximity to understory *Rhododendron* (Brasier et al. 2004b, 2004c). However, in 2009, *P. ramorum* was reported for the first time from a semi-natural environment in Europe causing widespread dieback and mortality of introduced *Larix kaempferi* (Lanb.) Carriere (Japanese larch) in southwest England. The pathogen was subsequently detected in 2010 on the same tree species in Wales, Northern Ireland and the Republic of Ireland (Brasier & Webber 2010). It has also been found on *L. decidua* Mill. (European larch) in an area with infected *L. kaempferi* trees nearby (Forestry Commission 2011). These represent the first cases of extensive damage to conifers and plantation trees in

areas without an infected population of *Rhododendron* nearby. As the understory vegetation sustains the pathogen and allows efficient proliferation (sporulation) in an epidemic of SOD, the appearance of “sudden larch death” represents a new life strategy of *P. ramorum*, because it is shown to sporulate efficiently high in the tree canopy. Isolates of *P. ramorum* examined to date comprise three distinct lineages: EU1, NA1 and NA2 (Ivors et al. 2004, Ivors et al. 2006, Grünwald et al. 2009). The European populations of *P. ramorum* consist solely of EU1 genotypes and represent predominantly A1 mating type (Grünwald et al. 2009, Vercauteren et al. 2011, Werres & De Merlier 2003). NA1 consists of only a single genotype, which is responsible for SOD and is the most widespread genotype in US nurseries (Ivors et al. 2006). NA2 isolates have been found in relatively few US nurseries, but it is the most common lineage in Canadian ornamentals (Goss et al. 2011).

In 2004, an unusual needle and twig blight appeared in Chilean plantations of *Pinus radiata* D. Don, which form the basis of a major industry. By the end of 2006, the damage had expanded to approximately 60,000 ha and the disease (“Daño Foliar del Pino”, DFP) was recognized as the most serious problem to have affected pine forestry in the country. The causal agent was described in 2008 as *P. pinifolia* A. Durán, Gryzenh. & M.J. Wingf. (Duran et al. 2008). It affects trees of all ages and causes severe needle loss resulting in rapid death of seedlings. It is the first *Phytophthora* known to be associated with needles and shoots of *Pinus*. It is also the only member of the species-rich soilborne *Phytophthora* ITS Clade 6 which has caducous sporangia enabling the aerial life style. Genetic analyses indicate a clonal population (Duran et al. 2010), thus it is very likely that the impact of *P. pinifolia* is due to a recently introduced pathogen finding a new and susceptible host in *P. radiata*.

International plant trade and global climate change

Movement of living plants and plant products by human activity is now generally accepted to be the primary mode of introduction of exotic pathogens and pests. The expansion of the international horticultural trade as well as its structural change have played a major part in the world-wide movement of plant pathogens (Brasier 2008, Dehnen-Schmutz et al. 2010). Phytosanitary concerns related to the expanding world trade were realized in the 1950s, when the International Plant Protection Convention (IPPC) of the The Food and Agriculture Organization of the United Nations (FAO) and the World Trade Organisation (WTO) set up international protocols to control the process of trade. The aim of the protocols was to reduce the likelihood of accidental pathogen introductions. The Sanitary and Phytosanitary Agreement (SPS) of the WTO entered into force in 1995 as the basis for plant health regulations in the EU today, where protocols are applied in most member states, including Finland. Harmful invasive pathogens are referred to in Finnish Law; in acts regulating the controlling of plant diseases and pests, which are the plant protection act (702/2003), the seedling material act (1205/1994), the seed trade act (728/2000) and the act for control of insect and fungal disease damage in forests (263/1991). However, these agreements and legislation have not been adequate in stopping invasions of exotic pathogens. Brasier (2008) suggested that the current SPS protocols are flawed, since they aim purely to minimize any disruption to trade and they consist of lists of named harmful organisms excluding potential or unknown pathogens. Also, the focus on fungal and other microbial species instead of genotypes, which can vary in their disease-inducing properties, is problematic. Purely visual inspections and failure to implement regulations worldwide are also of concern. Nevertheless, protocols of pest risk analyses remain a valuable means of predicting the impact of specific and well-known organisms.

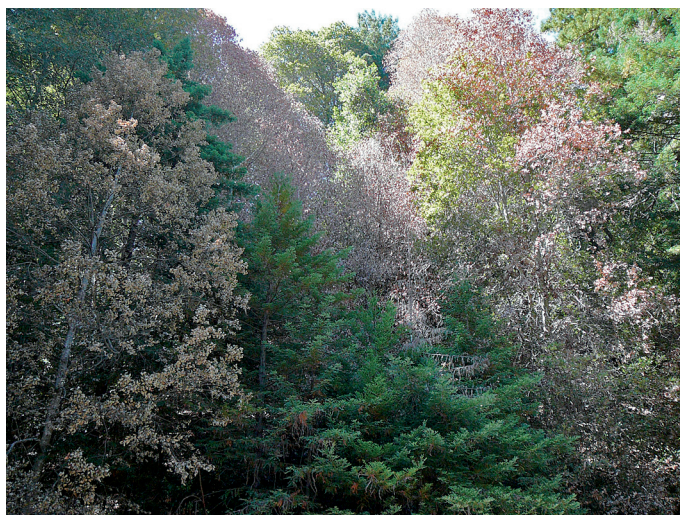


Fig 1. *Lithocarpus densiflorus* and *Quercus* spp. are particularly vulnerable to Sudden Oak Death caused by *Phytophthora ramorum* in California. (A. Rytkönen).

Increasing global temperatures and greenhouse gases predicted by the Intergovernmental Panel on Climate Change (IPCC) will most likely change weather patterns all over the world. As plant disease epidemics are dependent on environmental conditions, changes in climatic variables will most likely affect the incidence and impact of disease. Changes in precipitation and temperature can affect pathogen-host interactions directly or indirectly by stressing host plants. For example, in areas where rainfall is predicted to diminish or become periodic, drought stress will make plants more susceptible to disease. Expected changes will also affect the life cycles and biological synchronicity of perennial plants and pathogens. In addition, a mean temperature increase is expected to promote the establishment of introduced species into new geographical areas (Brasier 1996, Venette & Cohen 2006, Venette 2009, Roos et al. 2011, Sturrock et al. 2011). The distribution and impact of *P. cinnamomi* in Europe with increasing temperature has been modeled by Brasier (1996). In addition, it was recently shown that the epidemic beech decline in Central Europe is caused by the interaction between climatic extremes and *Phytophthora* infections (Jung 2009).

Due to issues concerning the global plant trade, in addition to increased travel and climate change, the risk of introduction and establishment of new plant diseases in Finnish nurseries and natural ecosystems is increasing. Similar concerns in other European countries led to the establishment of the European Cooperation in Science and Technology (COST) Action FP0801 “Established and Emerging *Phytophthora*: Increasing Threats to Woodland and Forest Ecosystems” in 2008. Finland is involved in this project, the aim of which is to increase understanding of the biology and ecology of *Phytophthora* species with the potential to damage European forestry. Resulting knowledge will be used in the development of effective control and management protocols for problems caused by *Phytophthora* in European forests and woodlands.

Risk analyses

Usually, new severe and sudden disease episodes are caused by newly escaped or introduced pathogens. When such a disease episode is recognized in some part of the world, a decision concerning the regulation of the causal agent is based on Pest Risk Analysis (PRA). PRA

can also be initially triggered by the identification of an introduction pathway such as an imported commodity that may allow a pest or pathogen means of entry. Indeed, commodity risk assessments and if necessary total closures of identified pathways for the introduction of potentially harmful pathogens are increasingly needed in the future, because the species-by-species approach of listed organisms (which most often have already entered Europe before they were added to the list) has not provided sufficient means to ensure biosecurity.

The PRA procedure of a specific organism includes evaluation of biological or other scientific and economic evidence for summarizing the current risk information on the pest or pathogen in question. Necessary information for the PRA includes the characterization of the organism and its possible detection, information on its life cycle, dispersal potential, survival and adaptability, as well as its geographical distribution and host plant range. In addition, the potential to become established is evaluated as well as the mode of transport, possible mitigation measures, and the likely economic and environmental impact of the introduction (EPPO 2002, Cave et al. 2008).

Several biological factors affect the risk of introduction and establishment of *Phytophthora* spp., including their often large host ranges, indistinct and variable symptoms on different hosts, factors affecting latency and dormancy, long survival time of oospores and their resistance to droughts, cold temperatures and microbial decomposition as well as the rapid build-up of secondary inoculum from near undetectable levels of primary inoculum.

1.3 Detecting and identifying *Phytophthora*

Preventing the spread of *Phytophthora* requires detection techniques that are robust, specific, sensitive and rapid. The classical methods to detect and isolate *Phytophthora* spp. are based on the isolation and culture of the microbe to examine its morphology, temperature-growth relations and pathogenicity. The process is time consuming and most importantly requires high expertise. Waterhouse (1963) characterised six groups of *Phytophthora* based on apical thickening and width of the sporangia exit pore, the caducity and pedicel length of sporangia, amphigynous or paragynous antheridia and hetero- or homothallism. This morphological classification system has served as the basis for taxonomic keys (Waterhouse 1963, Stamps et al. 1990). Difficulties in identification arise from the fact that morphological characters can be indistinct, continuous between species or highly variable within a species. Modern phylogenetic studies have led to the emergence of a different and more natural grouping of *Phytophthora* species (Cooke et al. 2000, Kroon et al. 2004, Villa et al. 2006, Blair et al. 2008). The identification of species has been aided by molecular techniques such as sequencing of specific genomic areas or fingerprinting, such as Single Strand Conformation Polymorphism (SSCP) and Restriction Fragment Length Polymorphism (RFLP) (Kong et al. 2003a, Drenth et al. 2006)

For easier detection of *Phytophthora* spp. from infected plants, immunoassay-based test kits are commercially available, e.g., enzyme-linked immunosorbent assay (ELISA) and lateral flow immunochromatographic assay (LFD) (Lane et al. 2007). These tests are not species specific but are intended for the initial screening of infected material. DNA-detection tests based on PCR directly from plant material or environmental samples by species-specific primers have also been developed for several *Phytophthora* species (reviewed in O'Brien et al. 2009). Also, some level of multiplexing (e.g., detection of multiple species at the same time) can be achieved by utilizing species-specific probes in PCR-ELISA analysis (Bailey et al. 2002).

In 2008, Schena and colleagues published a set of species-specific primers for detecting 15 *Phytophthora* species in a single-round amplification from infected leaves or in a nested PCR of soil or water samples. Some cross reactions were observed in the study, i.e., primers designed for *P. cactorum* (Lebeert & Cohn) J. Schröt and *P. ilicis* Buddenh. & Young cross reacted with *P. idaei* Kennedy & Duncan and *P. nemorosa*, respectively. Multiplex-realtime-PCR has been successful in detecting two (Ippolito et al. 2004) and four (Schena et al. 2006) *Phytophthora* species in a single reaction. Multiplexing in real-time PCR is restricted by the number of fluorescent probes, which is currently limited. Furthermore, the greatest challenge in using both conventional and real-time PCR for multiplexing is optimizing the conditions for a reaction containing several primers and probes.

Micro- and macroarray analyses potentially provide a larger basis for multiplexing. Zhang et al. (2008) described a macroarray analysis, which included the detection of four *Phytophthora* species in addition to several other pathogen species at the same time. Andersson and colleagues (2006) investigated the use of microarrays for *Phytophthora* species separation, and successfully differentiated eight species using multiple probes for each of them. In 2009, van Doorn and others introduced a novel detection and identification system for plant pathogens in which fragmented ITS-PCR products are subjected to a “padlock-probe” ligation detection (LD) assay using universal cZipCode hybridization arrays. The method shows great promise for multiplex detection of several specific plant pathogens.

The ITS region of ribosomal DNA consists of rRNA subunit genes (18S, 3.8S and 28S) separated by two internal spacer regions ITS1 and ITS2. This region is the most widely used genomic region for the molecular detection of *Phytophthora* species because it is fast evolving, contains relatively low intraspecific and high interspecific variation and is present in multiple copies. These facts make ITS highly useful for molecular discrimination, especially ITS1, because the sequence is already available for almost all *Phytophthora* species. However, some closely related species can share identical sequences (Schubert et al. 1999, Brasier et al. 2004a, Hughes et al. 2006, Jung & Burgess 2009, Bezuidenhout et al. 2010, Jung et al. 2011). Other multicopy targets are mitochondrial genes, of which the cytochrome oxidase I (*cox*) gene has been used for detection of *P. ramorum* in addition to some other species (Martin et al. 2004, Tooley et al. 2006). The *cox* gene also contains variable regions for designing primers. The disadvantage is that mitochondrial genes are uniparentally inherited, which hinders the detection of hybrid isolates originating from sexual hybridisation. Schena and colleagues (2008) used the variable intron sequences of the *Ypt1* gene to develop primers for 15 *Phytophthora* species. Also *Lpv* storage protein (Kong et al. 2003b), *GPA1*, *TRP*, *RAS*-like (Ioos et al. 2006), β -tubulin (Bilodeau et al. 2007) and elicitor genes (Bilodeau et al. 2007, Lacourt & Duncan 1997) have been used to design primers for species-specific detection. Generally, detection of single-copy genes is less sensitive than multicopy genes.

1.4 *Phytophthora* spp. in Finland

Potato late blight caused by the heterothallic *Phytophthora infestans* (Mont.) de Bary is a devastating disease of potato worldwide. Prior to the late 1970s, only the A1 mating type was found outside of Mexico. By the early 1980s, the A2 mating type of *P. infestans* was introduced from Mexico to Europe and the old asexual population was replaced by a sexual one (Fry et al. 1993, Hohl & Iselin 1984). Monitoring the A2 mating type in Finland began in 1992, at which time the strain was rare in the population. Unfortunately, there were no older isolates of the pathogen available and the exact time of migration of A2 remains uncertain (Hermansen et al. 2000). Cold winters experienced at high northern latitudes limit the survival of the asexual

stage in infected tubers, and potato late blight epidemics derived from asexual sources of primary inoculum were thus delayed until the end of August (Mäkelä 1966, Seppänen 1987). However, oospores formed in a sexually reproducing population provide a new and serious overwintering source of primary inoculum in Finland (Hannukkala et al. 2007).

Phytophthora cactorum was initially isolated in Finland in the early 1990s from strawberries suffering from leather rot (Parikka 1990). Since then, the pathogen has caused problems in strawberry production as an agent of crown rot as well as causing stem lesions on silver birch in forest nurseries (Lilja et al. 1998, Parikka 1990, Hantula et al. 2000). Aliette (fosetyl-aluminium) is the registered pesticide for control. Genetic variation of *P. cactorum* has been studied by Random Amplified Microsatellite (RAMS) and Random Amplified Polymorphic DNA (RAPD) analyses. Cooke et al. (1996) were able to discriminate between the strawberry and apple pathovars using RAPD. Finnish isolates from birch have been shown to contain considerable genetic variation in contrast to the strawberry crown rot isolates that are largely clonal in Europe according to fingerprinting analyses (Hantula et al. 1997, Lilja et al. 1998, Eikemo et al. 2004, Hantula et al. 2000,). Besides being genetically separable in fingerprinting analyses, the strains causing crown rot in strawberry and stem lesions on birch have also shown to be pathogenically separate in Finland (Hantula et al. 2000).

Phytophthora ramorum is a regulated quarantine pathogen in the EU. In Finland, the Finnish Food Safety Authority EVIRA conducts plant health inspections, in the course of which symptomatic plants are screened by an EU standard species-specific PCR. In the spring of 2004, *P. ramorum* was detected for the first time on *Rhododendron* cultivars imported to Finland from Germany. During further inspections by EVIRA in the same year, one nursery having domestic production of *Rhododendron* was found to harbor *P. ramorum* based on PCR (Lilja et al. 2007).

2 AIMS

The objective of the study was to gather information on the occurrence of *Phytophthora* spp. in Finnish nurseries. Furthermore, the study sought to produce information for risk assessments of these *Phytophthora* and to establish means for their detection.

More specifically, the aims of the individual studies were:

1. To screen a forest nursery where stem lesions occur on *Betula pendula* Roth. for *Phytophthora* spp. by isolations and baiting of irrigation water (**I**)
2. To isolate *Phytophthora* spp. from *Rhododendron* plants from a nursery found to harbor *P. ramorum* based on PCR detections (**III**)
3. To develop a detection tool based on *Phytophthora*-specific primers and PCR–DGGE of infected plant material (**II**)
4. To screen for possible escape from the nursery and to determine the efficacy of sanitation protocols by annual sampling (**I, III**)
5. To screen the susceptibility of Finnish plant species to introduced *Phytophthora* spp. (**III**)
6. To gather information on the survival ability of *Phytophthora* spp. in Finnish conditions (**I, III, IV**)
7. To generate new and pertinent information for risk assessment by examining the infection biology of *P. plurivora* on *Picea abies* L. (**IV**)

3 MATERIALS AND METHODS

All of the Finnish *Phytophthora* isolates were collected from nurseries. Some isolates originated from silver birch seedlings in a forest nursery, in addition to a natural pond nearby the nursery (**I**). Isolates were also obtained from *Rhododendron* plants collected between 2005-2010 from a domestic ornamental nursery (**III**). Isolations were also made from *Syringa vulgaris* in another nursery in Central Finland (**IV**). *Phytophthora* isolates used in the development of a PCR-DGGE based molecular detection method (**II**) were mostly obtained from Centraalbureau voor Schimmelcultures (CBS) microbe collection in Utrecht, Netherlands. The isolates are listed in Table 1.

The Finnish *Phytophthora* isolates were obtained by direct isolation and plating from plant material (**I, III**), or by baiting from water samples (**I, III, IV**). Identification was achieved by comparing characteristic morphological features and morphometric data with descriptions in literature, in addition to PCR amplification and sequencing of internal transcribed spacer 1 (ITS 1) region of SSU rDNA (**I, III**) and beta tubulin gene (**I, III, IV**). Four deciduous and two coniferous tree species, in addition to seven plant species common in Finnish plant production and forest ecosystems were used in screening the susceptibility to the new *Phytophthora* spp. (**III**, Table 1). Isolate survival of cold temperature was tested in -5 °C in organic material (apples) (**III**) and in malt agar plates (**IV**). Growth responses to temperature was determined by measurements at 6 different temperatures (**IV**). Histological examination of *Picea abies* tissue colonisation by *P. plurivora* was studied by making thin layer cuttings after embedding with Kulzer Histo-Technique ISO 7100 (**IV**). Materials and methods are listed in Tables 1, 2 and 3.

Table 1. Plant materials and *Phytophthora* isolates used in the individual studies.

Cultured isolates from nursery samples		Date of isolation and host	Studies
<i>P. cactorum</i>	Ph414 (Suonenjoki, Finland)	2004, <i>w</i>	I
	Ph415 (Suonenjoki, Finland)	2004, <i>B</i>	I
	Ph416 (Suonenjoki, Finland)	2004, <i>B</i>	I
	Ph417 (Suonenjoki, Finland)	2004, <i>B</i>	I
	Ph419 (Suonenjoki, Finland)	2005, <i>w</i>	I
	Ph420 (Suonenjoki, Finland)	2006, <i>B</i>	I
	Ph421 (Suonenjoki, Finland)	2006, <i>w</i>	I, II
	Ph440 (Finland)	2007, <i>R</i>	III
	Ph445 (Finland)	2007, <i>R</i>	III
<i>P. ramorum</i>	Ph401 (Finland)	2005, <i>R</i>	III
	Ph402 (Finland)	2005, <i>R</i>	III
	Ph405 (Finland)	2005, <i>R</i>	III
	Ph426 (Finland)	2006, <i>R</i>	II, III
	Ph455 (Finland)	2009, <i>R</i>	III
	Ph457 (Finland)	2010, <i>R</i>	III
<i>P. plurivora</i>	Ph411 (Finland)	2005, <i>R</i>	III, IV
	Ph412 (Finland)	2005, <i>R</i>	III, IV
	Ph481 (Finland)	2005, <i>R</i>	III, IV
	Ph515 (Finland)	2005, <i>R</i>	III, IV
	Ph441 (Finland)	2007, <i>R</i>	III, IV
	Ph442 (Finland)	2007, <i>R</i>	III, IV
	Ph444 (Finland)	2007, <i>R</i>	II, III, IV
	Ph450 (Finland)	2008, <i>S</i>	III, IV
<i>P. pini</i>	Ph443 (Finland)	2007, <i>R</i>	III, IV
Isolates for DNA samples			
<i>P. cambivora</i>	CBS 248.60		II
<i>P. citricola</i> s.l.	CBS 368.79		II
<i>P. citrophthora</i>	CBS 111726		II
<i>P. europaea</i>	CBS 109053		II
<i>P. lateralis</i>	CBS 102608		II
<i>P. porri</i>	CBS 567.86		II
<i>P. quercina</i>	CBS 782.95		II
<i>P. pseudotsugae</i>	CBS 446.84		II
<i>P. psychrophila</i>	CBS 803.95		II
<i>P. syringae</i>	CBS 110161		II
<i>P. uliginosa</i>	CBS 109055		II
<i>Elongisporium undulatum</i> (syn. <i>P. undulata</i>)	Ph12 (Lilja et al. 1994. Eur J For Path 24: 181–192.)		II

w = from pond water, *B* = from *Betula pendula*, *R* = from *Rhododendron* sp., *S* = from *Syringa vulgaris*

Table 2. Plant materials used in the individual studies.

Plant species used in inoculation trials	Studies
<i>Alnus incana</i> , Grey alder	III
<i>Alnus glutinosa</i> , Common alder	III
<i>Betula pendula</i> , silver birch	I, III
<i>Fragaria x ananassa</i> , strawberry	II, III
<i>Malus domestica</i> Golden Delicious, apple	II
<i>Picea abies</i> , Norway spruce	II, III, IV
<i>Pinus sylvestris</i> , Scots pine	III
<i>Rhododendron</i> 'Elvira' Tigerstedt	II, III
<i>Solanum lycopersicum</i> , tomato	II
<i>Vaccinium angustifolium</i> , lowbush blueberry	III
<i>Vaccinium myrtillus</i> , blueberry	II, III
<i>Vaccinium uliginosum</i> , bog bilberry	III
<i>Viburnum lantana</i> , wayfaring tree	II, III
<i>Viburnum lentago</i> , sheepberry	III
<i>Quercus robur</i> , pedunculate oak	III
Plant materials used as baits	
<i>Betula pendula</i> , silver birch (seedling tips)	I
<i>Malus domestica</i> Golden Delicious, apple (fruits)	III
<i>Rhododendron catawbiense</i> (leaves)	III
<i>Rhododendron smirnowii</i> 'Pohjolan tytär' (leaves)	I
<i>Rhododendron tomentosum</i> (syn. <i>Ledum palustre</i>), Marsh Labrador tea (leaves)	I
Plant materials used in survival test	
<i>Malus domestica</i> Golden Delicious, apple	III

Table 3. Methods used in the individual studies.

Sampling and isolation	Studies
Isolation from plant material by plating	I, III
Baiting from water samples	I, III, IV
Plant inoculations	
Fruit inoculation	II, III
Mycelial inoculation via wound	I, II, III, IV
Inoculation by zoospore suspension	IV
Molecular techniques	
DNA extraction	I, II, III
PCR amplification and sequencing of internal transcribed spacer 1 (ITS 1) region of SSU rDNA	I, III
PCR amplification and sequencing of beta tubulin gene	I, III
Multilocus DNA fingerprinting with SSR (RAMS) primers	I, III
Multilocus genotyping of <i>P. ramorum</i> isolates using 6 microsatellite loci	III
Temperature tests	
Test of isolate survival in organic material (apples) at -5 °C	III
Test of isolate survival in malt extract agar plates at -5 °C	IV
Test for isolate growth responses to temperature	IV
Microscopical examination of infection process	
Histological examination of <i>Picea abies</i> tissue colonisation by <i>P. plurivora</i> by making thin layer cuttings after embedding with Kulzer Histo-Technique ISO 7100	IV
Statistical testing	
Kruskall-Wallis one-way analysis of variance	I
One-way ANOVA	I
Tukey's comparisons	I
Likelihood ratio test for a multinomial response/chi squared independence test	III, IV

4 RESULTS AND DISCUSSION

4.1 Occurrence and identity of *Phytophthora* spp. in Finland

Phytophthora cactorum

P. cactorum has been established in Finnish nurseries for over 15 years. In the study nursery at Suonenjoki (62°39'N 27°04' E), *P. cactorum* was isolated from stem lesions of *Betula pendula* seedlings as well as by baiting of a natural pond that supplies irrigation water to the nursery (I). The *P. cactorum* isolates from the pond and stem lesions were genetically identical according to Random Amplified Microsatellite (RAMS) analysis with two primer pairs. Both isolates also induced symptoms on *B. pendula* seedlings in an inoculation test; thus identifying the irrigation water one potential source of inoculum. In addition, isolates had considerable variation in their morphological characteristics and growth rates (I). Variation in the study population might reflect the long history of *P. cactorum* in the nursery, during which time phenotypic and physiological differences have emerged. Alternatively, *P. cactorum* might have been introduced via trading of nursery stock on multiple occasions. From a methodological point of view, RAMS analysis did not detect variation inside a population infecting birch in the study nursery, thus it remains a practical method for separating different *P. cactorum* lineages reflecting different host species (Hantula et al. 2000).

In this study, *P. cactorum* was also detected from a lake close to another nursery in Central Finland (Päijänne, 61°20'N 24°16'E), but twigs and leaves sampled from symptomatic *Syringa vulgaris* L. from the nursery yielded only *P. plurivora* (IV). Furthermore, *P. cactorum* was also isolated from *Rhododendron* that were produced domestically in Southern Finland (III).

Phytophthora ramorum

Only one of the ca. 120 domestic nurseries inspected yearly by EVIRA between 2004 and 2010 was found to harbor *P. ramorum*. In this nursery, PCR screening of symptomatic *Rhododendron* with *P. ramorum* specific primers gave a positive result every year. Also, mycelial cultures of *P. ramorum* were isolated from *Rhododendron* samples every year except 2007 (III).

RAMS fingerprinting was used to identify lineages (EU1, NA1 or NA2) present among the Finnish isolates. This method has been used earlier with *P. cactorum* and other pathogens (I, Hantula et al. 1997, Hantula et al. 2000, Rytkönen et al. 2011). However, RAMS fingerprinting could not detect any intraspecific variation between our isolates and the European and North American reference isolates of *P. ramorum*. The result indicates that *P. ramorum* in Europe and North America is rather uniform, at least in the case of these particular microsatellites. In the case of *P. cactorum*, variability is detected relatively easily with RAMS analysis (Hantula et al. 2000). This could be due to an early establishment of *P. cactorum* in the northern hemisphere long ago, and which might also have native populations in Europe contributing to genetic variability (Jung et al. 1996, Jung et al. 2000).

Because RAMS was deemed to be rather inefficient to detect variation in *P. ramorum*, the microsatellite multilocus genotype of each isolate was determined with 7 existing microsatellite markers, resulting in five of six of *P. ramorum* isolates being identified as the EU1MG1 genotype (III). Vercauteren and colleagues (2010) showed that the Belgian population of *P. ramorum* was dominated (68%) by the same genotype (EU1MG1). This was suggested to be the original genotype introduced to Belgium whereas other less common genotypes were probably derived from it (Vercauteren et al. 2010). Strain EU1MG1 might

be considered the most widespread genotype in Europe, and probably representative of the original founder population.

Phytophthora plurivora and *P. pini*

Other *Phytophthora* species isolated in the same nursery included *P. plurivora* and *P. pini* (III). Initially, these isolates were identified as *P. inflata* (Lilja et al. 2007). However, ITS sequences and the recent taxonomic revision of the *P. citricola* species complex (Jung & Burgess 2009, Hong et al. 2011) allowed their more precise identification as *P. plurivora* and *P. pini*. *Phytophthora plurivora* occurs abundantly in forests, semi-natural ecosystems and nurseries in Western, Central, Eastern and Southern Europe where it causes bark necroses, fine root losses and dieback in at least 41 woody host species (Jung & Burgess 2009) including *Quercus robur* L. (Jung & Blaschke 1996, Jung & Nechwatal 2008) and *Alnus glutinosa* L. (Jung et al. 2003, Jung & Blaschke 2004). It is also present in the US on *F. sylvatica*, *Acer saccharum* Marsh. and *Alnus* spp. (Jung & Burgess 2009, Weiland et al. 2010). *Phytophthora plurivora* has also been recently found to cause dieback and mortality of several deciduous tree species in Denmark, Norway and Poland (Talgø et al. 2009, Thinggaard 2009, Orlikowski et al. 2011). *Phytophthora pini* is widespread in the eastern US where it damages and kills the introduced species *Syringa vulgaris* and *Fagus sylvatica* L. (Jung et al. 2005, Weiland et al. 2010, Hong et al. 2011). In contrast, in Europe it has only been found in nurseries, indicating a very recent introduction (Jung & Burgess 2009). It is a new species to Finland and other Nordic countries.

4.2 Susceptibility of Nordic plant species to the introduced *Phytophthora* spp.

Risk analyses assume that the consequences of pest introduction are positively correlated with the host range of the pest (Cave et al. 2008). Thus, common Finnish trees and shrubs such as *Betula pendula*, *Alnus incana* L. (Moench), *A. glutinosa*, *Picea abies*, *Pinus sylvestris* L., *Quercus robur*, *Vaccinium uliginosum* L. and *V. myrtillus* L. and, in addition, the predominantly ornamental plants *Rhododendron* spp., *Vaccinium angustifolium* Aiton and *Fragaria x ananassa* Duchesne were tested for their susceptibility to isolates of the introduced *P. cactorum*, *P. ramorum*, *P. plurivora* and *P. pini*. (III)

In the pathogenicity trials, all four species of *Phytophthora* were able to infect shoots of *Rhododendron* while the stem tissues of *P. sylvestris* and *Q. robur* were resistant to all of them. The results of Jung et al. (1996) showed that *P. plurivora* (under its previous name *P. citricola*) was aggressive to the bark of 5-year-old *Q. robur* seedlings and mature *Q. robur* stems causing bark lesions within 3 months and 5 weeks, respectively. The duration of the assessments in the pathogenicity test here was four weeks, which might have not been enough to induce symptoms on the 6-year-old *Q. robur* seedlings; which could also be the case with *P. sylvestris*. The incidence of disease after inoculation with *P. ramorum* was highest in *Vaccinium* spp. but it also infected *Viburnum lantana* L. *Phytophthora ramorum* also caused lesions on *B. pendula* and *A. glutinosa*, but conifers were resistant (III). All these plant species are native and abundant in Finland. Consequently, *P. ramorum* would find suitable hosts if it escapes from a nursery. Alarming occurrences have been reported from Scotland and Norway where *P. ramorum* has been found on *Vaccinium myrtillus* in the heathlands and in an arboretum, respectively (Central Science Laboratory 2009, Harwood et al. 2009, Herrero et al. 2011). The stems of the native and widespread conifers *P. abies* and *P. sylvestris* seemed to be resistant to *P. ramorum*. According to previous studies (Denman et al. 2005, Moralejo et al. 2009b), it is

likely that *P. sylvestris* is not a suitable host for *P. ramorum*, and although individual needles of *P. abies* might be colonised (Denman et al. 2005), the formation of stem cankers is unlikely. However, when considering the consequences of the possible introduction of *P. ramorum* to Finnish natural ecosystems, it should be taken into account that the climate matching model (CLIMEX) predicts that the risk is low for establishment and epidemic spread in Northern Europe under current climate (RAPRA 2009).

Among other diseases, *P. cactorum* causes crown rot in Finnish strawberries (Hantula et al. 1997, 2000). However, the pathogenicity of different *P. cactorum* isolates varied on strawberry plants (III). According to previous investigations, Finnish isolates of *P. cactorum* from strawberry and birch are morphologically distinguishable in terms of the relative size of oogonia, oospores and sporangia. DNA fingerprinting have placed isolates from birch, strawberry and apple into separate clusters (Hantula et al. 1997, 2000, Lilja et al. 1998, Cooke et al. 1996). Furthermore, it was shown that isolates from birch are not pathogenic to strawberry (Hantula et al. 1997, 2000, Lilja et al. 1998). Here, all *P. cactorum* isolates were pathogenic to *Rhododendron* but differed in their pathogenicity towards strawberry. More isolates and tests would reveal the underlying patterns of host specificity. Our isolates probably represent at least two separate introductions of different *P. cactorum* genotypes, although this was not tested by molecular means. According to a Swedish study (Molin et al. 1960), *P. cactorum* was found to cause damping off in young Scots pine seedlings. In our trials, Scots pine was resistant. Older seedling age characterised by suberised vs. non-suberised stem tissue, in addition to genotype of the host and the pathogen might have affected the success of infection here.

In our pathogenicity trials, *P. plurivora* was able to infect most plants tested. It also had more hosts than *P. ramorum*. Weiland et al. (2010) compared the pathogenicity of *P. cactorum*, *P. plurivora* and *P. pini* to *F. sylvatica* and *S. vulgaris*. Isolates of *P. cactorum* were the least pathogenic and caused less necrosis than isolates of *P. pini* and *P. plurivora* (Weiland et al. 2010). Also, our results indicate that *P. pini* and *P. plurivora* cause more severe symptoms than *P. cactorum* on *B. pendula*, *V. lantana*, *F. x ananassa*, *Rhododendron* and *V. myrtillus*. It was also evident that *P. pini* and *P. plurivora* were the most aggressive species to *P. abies*. Although these species have yet to be found on *P. abies* in natural circumstances, our results suggest that at least juvenile trees and seedlings are vulnerable to these pathogens.

However it has to be noted that there can be a significant difference between the susceptibility of fine roots and stem/bark tissues. Several tree declines are driven by Phytophthora-caused fine root losses, and therefore soil infestation trials for testing the susceptibility of fine roots of Nordic tree species to *P. cactorum*, *P. plurivora*, *P. pini* and *P. ramorum* will be an important subject for future research.

4.3 Symptom development and histology of *Picea abies* tissue after inoculation

Picea abies seedlings were inoculated with *P. plurivora* and *P. pini* in order to observe symptom development. Both species were able to infect *P. abies* seedlings via mycelial inoculation of wounded stem tissue (III), but were also able to invade undamaged shoots (IV). Results indicate that these species are capable of infecting the most important tree species in Finnish nurseries, and they pose a clear threat should their prevalence increase, as has happened with *P. cactorum* (Hantula et al. 2000, Lilja et al. 2011). Some isolates of *P. plurivora* as well as the single isolate of *P. pini* caused disease in all seedlings inoculated via application of mycelia to wounded tissue or zoospore suspension. With live mycelia, the first discoloration and wilting symptoms were observed 2 days post inoculation (dpi). In the case of zoospore inoculum, symptoms developed ca. 4 dpi. In addition to adding to the epidemiological information

of this species, the four-day time lag between infection and appearance of symptoms has importance in the practical handling of seedlings. However, another major aspect to be regarded as possible invisible inoculum is the infestation of soil and fine root infections, and the persistence of resting structures in dead fine roots and root debris.

Histological observations of *P. plurivora* in *Picea abies* tissue were made in order to confirm the presence of hyphae in the diseased plant, to examine the progression of the infection and to detect possible propagules. Knowledge on latency is of great importance to prevent the spread of *Phytophthora* in infected but otherwise healthy-looking plants. It was difficult to determine which tissue is preferred for growth, in that almost all tissues were infected including the vascular system and the pith parenchyma. *P. plurivora* grew rapidly in the *P. abies* tissues after infection and was able to reach the pith in less than 4 days. Vascular vessels seemed to be most suitable tissues for longitudinal growth.

In the *P. abies* shoot tissue, *P. plurivora* seemed to grow both inter- and intracellularly. Most intracellular growth was observed in the vascular tissues. The longest stretches of hyphae were observed in the vascular vessels, which probably allow for fast growth inside the plant. No hyphae were found in the samples from nonsymptomatic tissues, even though plasmolysis was sometimes observed. It indicates that *P. plurivora* infects *P. abies* as a necrotroph, growing perhaps mostly in the apoplast and killing cells quickly. Also Portz et al. (2011) reported severe plasmolysis in *Fagus sylvatica* roots infected by *P. plurivora* (under previous name *P. citricola*), even without symptoms 5 dpi. Secretion of extracellular enzymes such as citricolin (Fleischmann et al. 2005) are likely to also be involved in *P. plurivora* infection, but it was not examined in this study.

P. plurivora is homothallic, and abundantly produces oogonia in growth media (Jung et al. 1996, Jung & Burgess 2009). However, only two structures resembling oogonia were found in tissues of *P. abies*. This can be due to the high content of resin and polyphenolic compounds in *P. abies* tissue that might inhibit the production of oogonia. Both of the observed oogonia were less than 20 µm in diameter (15 and 6 µm), which is below the range reported in the description (15–37.5 µm; isolate means 27.5–29.9 µm) (Jung & Burgess 2009). These observations suggest that these oogonia are immature. Alternatively, it is also known that due to the lower nutrient contents compared to culture media, propagules formed in plant tissue are smaller than reported in culture (Cother & Griffin 1973). More investigations are needed to confirm the production of oospores by *P. plurivora* in *P. abies* tissue, although results of this study indicate that the production of propagules in stem tissue of *P. abies* is at least very low, and does not occur less than 8 dpi. (IV)

In the asymptomatic tissue samples, no hyphae were observed. However, aggregations of small membrane vesicles and plasmolysis in the cortex parenchyma of the fresh shoot were noted. Thus, according to the present study, *P. plurivora* does not grow in shoot tissue of symptom-free *P. abies*, and therefore it is unlikely that it would produce oospores there. In addition to practical importance, the results of these histological observations should be useful in epidemiological studies and risk assessments. However, for complete picture of the disease cycle, the significance of possible soilborne inoculum and fine root infections should be further investigated.

4.4 Survival of *Phytophthora* spp. and success of sanitation practices

P. ramorum is believed to be resistant to the effects of cold temperature compared to other species, and the threshold for its 7-day survival in *Rhododendron* tissue is between -10 and -20 °C (Tooley et al. 2008). For the Finnish isolates of *P. ramorum*, the threshold temperature

for two-week survival in apple tissue was between +2 and -5 °C (III). The higher threshold temperature for *P. ramorum* in apple tissue of this study compared to *Rhododendron* tissue in the study of Tooley et al. (2008) might be due to the lower water contents of plant tissue compared to apple fruit. On MEA, all *P. ramorum* isolates were able to remain viable 2 weeks at -5 °C (IV). None of the isolates of *P. plurivora* and *P. pini* were able to grow after 3 days in apple tissues at -5 °C (III). However, the situation was different with isolates on MEA plates, where 50% of them remained viable (IV).

The difference in results from MEA and apple tissue could be attributed to differences in incubation times before transfer into -5 °C (1–4 days incubation in apples vs. 5–7 days in MEA). *P. plurivora* and *P. pini* isolates may have had enough time on MEA to produce vesicles or initiate production of oogonia before the transfer. The difference in incubation time might result also in different stages of maturation in *P. ramorum* chlamydospores. *P. cactorum* chlamydospores have been shown to germinate well following a 24 hours treatment of -23 °C (Darmono & Parke 1990). Here, *P. cactorum* remained viable in apple tissue as well as on MEA for two weeks at -5 °C (III, IV). It was also found to persist in a natural pond over consecutive years (I). Also the survival of *P. ramorum* during the Finnish winter is indicated by the recovery of isolates from the study nursery on consecutive years (III).

Finally, soil temperature under snowcover in southern and central Finland rarely drops below -1.5 °C (Hänninen et al. 2005). Thus, after surviving 2 weeks in -5 °C, soil survival of *P. ramorum*, *P. cactorum*, *P. plurivora* and *P. pini* in Finland is likely under current climatic conditions. If the winter climate ameliorates in Finland due to global warming, warmer temperatures may permit survival at an even higher frequency.

4.5 Detection and identification of *Phytophthora* spp. with PCR-DGGE

In the PCR-DGGE tool developed in this study, the PCR multiplied ITS1 regions of the different *Phytophthora* species are separated in an electrophoresis with a chemical gradient to denature the sample. The method was able to discriminate most of the 16 species of *Phytophthora* tested. Also DNA extraction from the tested plant materials resulted in a template of sufficient quality for direct PCR amplification of the target region (II). The plant species involved in the sensitivity trial were *B. pendula*, *F. x ananassa*, *M. domestica* Golden Delicious, *P. abies*, *Rhododendron*, *Solanum lycopersicum* L. and *V. lantana*. However, the method has not yet been tested with infected, suberised, polyphenol-containing bark samples from mature trees. The major advantage of this method, as with all direct molecular detection methods, is the possibility to bypass the time-consuming classical identification process, which requires high expertise. Thus, PCR-based methods to some extent can replace longterm experience in routine diagnostics of known *Phytophthora* species from tissue samples. Because PCR primers anneal at a region that is conserved in all members of the genus, the PCR-DGGE method can be used to detect and differentiate both known and previously undetected species in a single step, which is an important feature given the rapid increase of *Phytophthora* species described in recent years (Cooke et al. 2007).

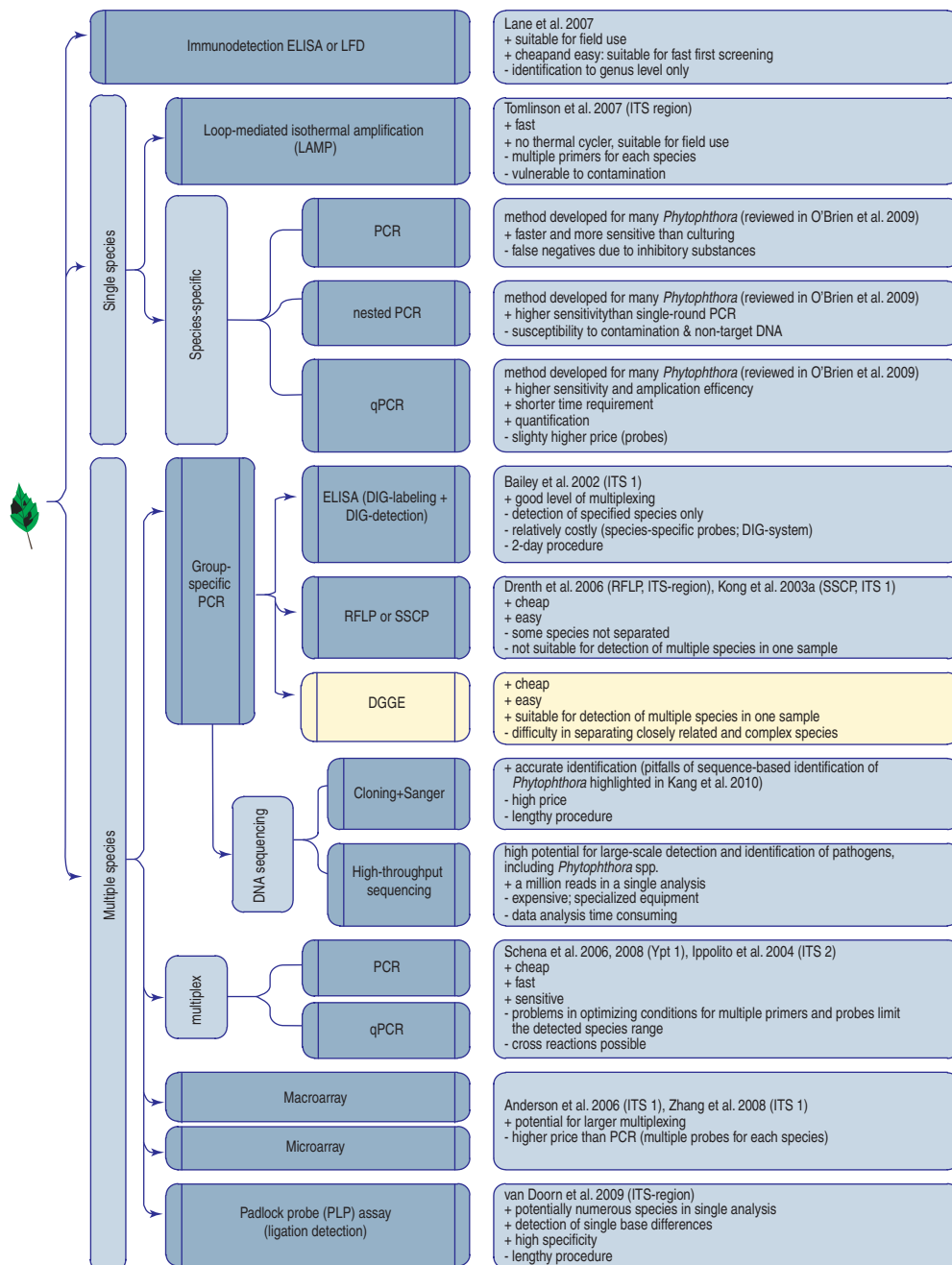
The PCR-DGGE method is able to simultaneously detect and identify at least two *Phytophthora* species from a single sample. The ability to detect multiple species in the same sample is surpassed only by high-throughput sequencing, e.g., 454 pyrosequencing. Ovaskainen et al. (2010) compared PCR–DGGE with 454 pyrosequencing as methods for fungal community description and found that although pyrosequencing gave higher estimates for fungal diversity, DGGE analysis revealed the dominant wood-inhabiting species. Thus, although it can neglect the lowest minority microbes in a sample, PCR–DGGE can detect the

common species for a much lower cost than high-throughput sequencing. One limitation of the PCR-DGGE analysis, as of other molecular detection methods in general, is posed by the fact that the primary inoculum levels of soilborne *Phytophthora* in soils are usually very low. Therefore, they might often not be detectable by molecular methods.

The ITS1 region of ribosomal DNA is the most widely used genomic region for the molecular detection of *Phytophthora* species and highly useful for molecular diagnostics. However, an important disadvantage of using the PCR–DGGE method developed here relates to the sequence diversity of the region. Difficulties in separating *Phytophthora* species arise when dealing with closely related species which can share identical or almost identical ITS sequence (II) (Schubert et al. 1999, Hansen et al. 2003, Brasier et al. 2004a, Martin & Tooley 2003, Jung & Burgess 2009, Bezuidenhout 2010, Jung et al. 2011). In such cases, species can be identified by sequence analyses of single bands, provided that databases including all alleles are available. It should also be noted that some of these restrictions can be circumvented by changing the PCR-DGGE primers to take advantage of the variation in different genomic areas.

The PCR–DGGE technique developed here (II) reliably detects *Phytophthora* in plant tissues, can separate most of the tested species from each other and indicate multi-species infections. Thus, it is a useful diagnostic tool either applied alone or in concert with traditional isolation culture techniques. The strength of PCR-based methods is in large-scale routine surveys for the presence of known *Phytophthora* species in symptomatic tissue samples. Generally, their weaknesses are the detection level of *Phytophthora* species from soil samples and the detection of new unknown species or hybrid isolates. The choice of the appropriate method depends on the individual case and often a combination of both molecular and classical detection and identification methods might be useful. Molecular and immunological methods used for detection and identification of *Phytophthora* species from plant material, including the method developed in this study, are depicted in Figure 2.

Figure 2. Molecular and immunological methods used for detection and identification of *Phytophthora* species from plant material.



5 CONCLUSIONS AND FUTURE PROSPECTS

Phytophthora cactorum has been introduced to Finland over 15 years ago, most likely via imported plant material. It is an example of an accidental introduction that has since spread widely via commercial strawberry production and nursery culture of birch seedlings. This thesis reports the establishment of this pathogen in natural waterbodies, where it can survive Finnish winter conditions and act as a source of infection when the infested water is used for irrigation. It is also reported here that new introductions of this pathogen re-occur through international trade of ornamental *Rhododendron*.

This thesis reports three new introductions of *Phytophthora* species in Finnish nurseries, namely *P. ramorum*, *P. plurivora* and *P. pini*. The only species with quarantine status, *P. ramorum*, is the most suited for growth in cooler temperatures and resistant to annual sanitation practices in Finnish nurseries. However, *P. plurivora* and the closely related *P. pini* had more hosts among Finnish tree seedlings and other plants abundant in Finnish nurseries and forest ecosystems. Also, their infectivity was high compared to *P. ramorum* and *P. cactorum*, but their higher optimum temperature for growth and poorer frost survival suggests a preference for a warmer climate. However, the survival ability of oospores was not conclusively determined in this study and thus the survival of *P. plurivora* and *P. pini* might be underestimated. This study demonstrates that all of these species have properties that contribute to a high risk of establishment and spread in Finland. *Phytophthora plurivora* and *P. pini*, which were highly infectious in pathogenicity trials, pose a real threat to commercial production of *P. abies* seedlings. Given their host range, Finnish forests might be under a serious threat should they escape from infected nurseries, which recently occurred for *P. plurivora* in other Nordic countries. These species are also easily transmitted via human activity, i.e. hiking, forest recreation, planting of infested nursery stock, transport of infested soil with forest machinery and road-building machines as well as the use of infested gravel material for building of forest roads. In addition, future climate models suggest the Finnish climate to become warmer and wetter, which will promote the spread and establishment of *Phytophthora* species in natural ecosystems, as shown e.g. by the modelling of the future distribution and establishment of *P. cinnamomi* in Europe (Brasier 1996) and by the current *Phytophthora* epidemic in Central European beech forests (Jung 2009).

The *Phytophthora* species in this study were detected by isolation, except the quarantine pathogen *P. ramorum*, which was initially detected by species-specific PCR. It is likely that by using a multiplex (i.e., capable of detecting multiple species in one analysis) molecular detection method, such as the PCR-DGGE developed here, the diversity of *Phytophthora* spp. found in Finnish nurseries would have been even higher. An additional limitation is that most of the *Rhododendron* samples examined in this study were obtained from the plant inspection authorities. In these cases, only plant lots initially found positive for *P. ramorum* by species-specific PCR were obtained and included in the isolations. The next step would be a systematic survey of nurseries including detection and identification of all *Phytophthora* spp. and testing of tissue and soil samples from both diseased and non-symptomatic plants, and by combining molecular detection with traditional isolation methods.

As increased human movement enables the effective translocation of *Phytophthora* spp. and expected climate change will facilitate the natural spread and establishment of *Phytophthora* species in Nordic countries, it is likely that other pathogenic members of this genus will become established in Finland and adjacent states unless effective phytosanitary measures are uniformly accepted and implemented by the international horticultural industry. At the moment, complete prevention of pathogen introductions is impossible, but favoring

plant species of native origin as well as regenerating of forests by natural regeneration or seeding would reduce the risk.

In the future, it is likely that research in forest pathology will necessarily focus on introductions of alien pathogens and new disease outbreaks. Research concerning future threats is currently conducted all over the world. For example, EU-funded projects such as FORTHREATS (The European network on emerging diseases and invasive species threats to European forest ecosystems) and ISEFOR (Increasing Sustainability of European Forests: Modelling for security against invasive pests and pathogens under climate change) aim to synthesize information on exotic organisms, especially forest pathogens. In addition to the before mentioned EU COST Action FP0801 which focuses specifically on alien *Phytophthora*, a new EU COST Action FP1002 “Pathway Evaluation and Pest Risk Management in Transport”(PERMIT) was started in 2010, which is focussing on reducing threats from exotic pests through promoting enhanced pathway management. The next step is to raise awareness and find the tools and political will to address these problems and reduce the threats in practice.

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