The genus *Phytophthora*; phylogeny, speciation and host specificity

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General introduction

Plant diseases are a major threat to global food security. The most devastating plant pathogens are the Fungi and the Oomycetes. The latter resemble fungi in appearance and behaviour, but during evolution they have evolved independently. In the tree of life the Oomycetes are classified in the Stramenopile lineage of the supergroup Chromalveolates, while the Fungi belong to the supergroup Unikonts (Keeling et al., 2005). Like Fungi, Oomycetes have a global distribution and thrive in quite diverse environments. They can live as (hemi-) biotrophic or necrotrophic pathogens in association with plants, animals, or other microbes, but also as saprophytes feeding on dead or decaying matter (Lamour and Kamoun, 2009). The best studied Oomycetes are the plant pathogenic ones, in particular species belonging to the genera *Phytophthora* and *Pythium,* and the downy mildews (*Peronosporaceae*). By far the most notorious *Phytophthora* species is *Phytophthora infestans*, the causal agent of potato late blight and responsible for a turning point in history. The Irish potato famine in the mid nineteenth century was due to enormous yield losses in potato caused by late blight. This lead to massive starvation and emigration of the Irish people as well as political and social upheaval that influenced the fate of Ireland, England and the USA. The first appearance of potato late blight in Europe was also an incentive for botanists to investigate the causes of other plant diseases (Agrios, 2005). The emerging concept that microorganisms were infectious agents gave rise to the birth of plant pathology as a new scientific discipline.

Nowadays, *P. infestans* is still a major threat for potato and tomato production. Together with the fungal pathogens *Puccinia graminis* (wheat stem rust), *Mycosphaerella fijiensis* (black sigatoka of banana), *Phakopsora pachyrhizi* (Asian soybean rust) and *Magnaporthe oryzae* (rice blast), *P. infestans* is in the top ten list of plant pathogens that threaten food security (Pennisi, 2010). Current annual losses of potato harvest due to *P. infestans* infection amount to 6.7 billion dollar, and that is a conservative estimate (Haas et al., 2009). In the European Union alone, the damage amounts to one billion dollar, one sixth of the total value of the potato crop in the EU (Haverkort et al., 2008).

The central themes in this thesis are all related to the diversity and dynamics in the genus *Phytophthora.* How many species are there, what is defined as a species and how do species emerge? In this introductory chapter I will first describe the basic biology of this type of organisms by briefly discussing the various stages of the life and disease cycle of the type species of the genus, i.e. *Phytophthora infestans.* This is followed by short descriptions of the history and impact of diseases caused by four prominent *Phytophthora* species. Subsequently, I will elaborate on the emergence of new species and end with the scope of this thesis.

Disease cycle of *Phytophthora infestans*

Phytophthora species can propagate through a sexual and an asexual cycle. The asexual propagules are sporangia and zoospores and the sexual propagules that result from mating are called oospores. Some *Phytophthora* species are homothallic (i.e. mating occurs in one thallus), whereas others are heterothallic with two known mating types designated A1 and A2.

P. infestans, the disease cycle of which is shown in Figure 1, is a hemi-biotrophic pathogen that propagates on the above ground parts of potato and tomato plants, mainly on leaves. Tubers and stolons of potato can become infected too, especially at the end of the growing season. In the asexual disease cycle, hyphae emerge from the infected plant tissue. These differentiate into sporangiophores on which sporangia are formed. These vegetative spores are lemon-shaped structures that are dispersed by wind or water. When they land on a suitable host, the sporangia can either germinate directly by forming a germ tube, or differentiate into zoosporangia in which the cytoplasmic content differentiates into seven or eight uninucleate zoospores which are equipped with flagella. Under wet conditions the zoospores are released from the semipapillate zoosporangia and dispersed on the host. Their release is facilitated by the papilla, a bud at the tip of a zoosporangium. Papillae consist of a plug of hydrated material that easily dissolves prior to the release of zoospores. *Phytophthora* species either have nonpapillate, semipapillate or papillate zoosporangia, a trait used by Waterhouse (1963) to classify *Phytophthora* species (Figure 2a-2h).

As a result of interaction with its host, a zoospore responds by retracting or discarding its flagella and forms a cell wall. After this process, also known as encystment, the cyst germinates. At the end of a germ tube tip, prior to penetration, an appressorium is formed (Figure 3). A penetration hypha emerging from the appressorium invades the epidermal plant cell and from there hyphae grow intercellularly in the mesophyll. Haustoria are formed, specialized feeding structures that invade the mesophyll cells to retrieve nutrients to support the growing mycelium (Judelson and Blanco, 2005). Shortly after infection the first symptoms appear, often in the form of water-soaked lesions with a necrotic centre that spread rapidly. This leads to deterioration of leaves and stems, and eventually of the whole plant. After three to five days, new sporangiophores are formed at the margins of the lesions, leading to numerous sporangia that spread and initiate new infection cycles. Under favourable conditions, zoospores can run down from the infected leaves in droplets of water and, in the case of potato, infect the tubers. Infected tubers are often responsible for initiating a new

epidemic in the next season; immediately after emergence of the new shoots the pathogen starts to sporulate, thereby creating a source of early inoculum.

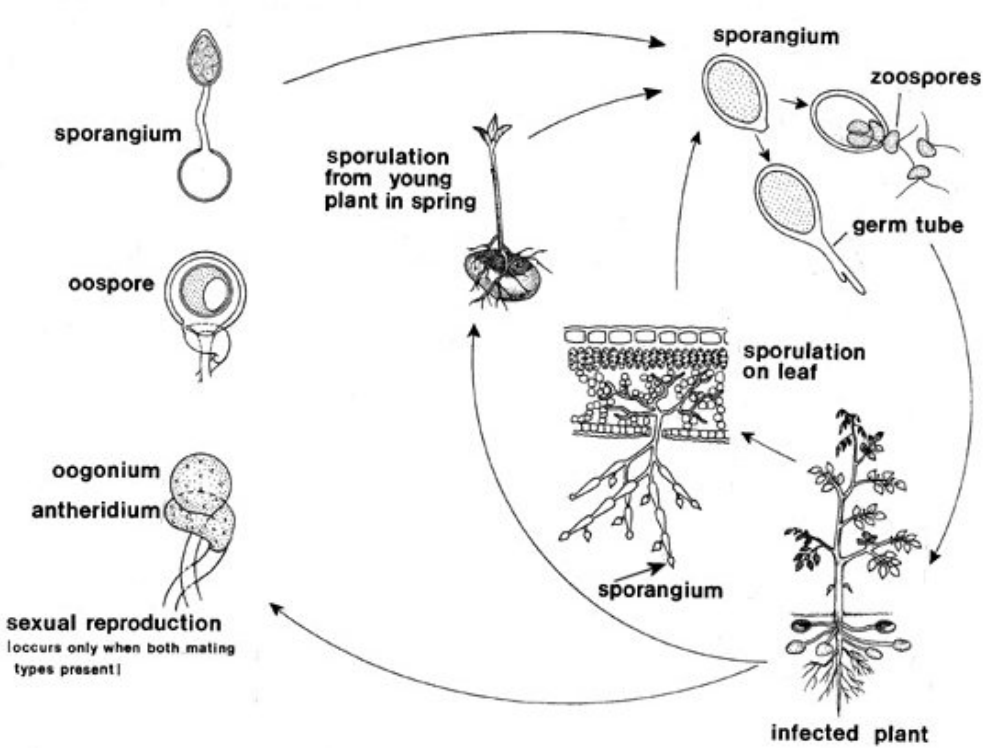

Figure 1. The disease cycle of *P. infestans*.

See: http://www.apsnet.org/education/lessonsplantpath/LateBlight/discycle.htm

P. infestans is heterothallic and thus requires two strains of opposite mating type to initiate the sexual cycle. When an A1 strain senses an A2 and vice versa, male antheridia and female oogonia are formed on each thallus. The antheridia and oogonia are delimited from the mycelium by septa. An antheridium attaches to the oogonial incept of the opposite mating type and the oogonial incept grows through the antheridium. This process is called amphigynous attachment (the male is surrounding the female, Figure 2i), as opposed to paragynous attachment (the male is besides the female), where the antheridium adheres to the side of an oogonium, close to the attachment site of the oogonium to the mycelium. Antheridium attachment is another Waterhouse criterion used to classify *Phytophthora* species (Waterhouse, 1963).

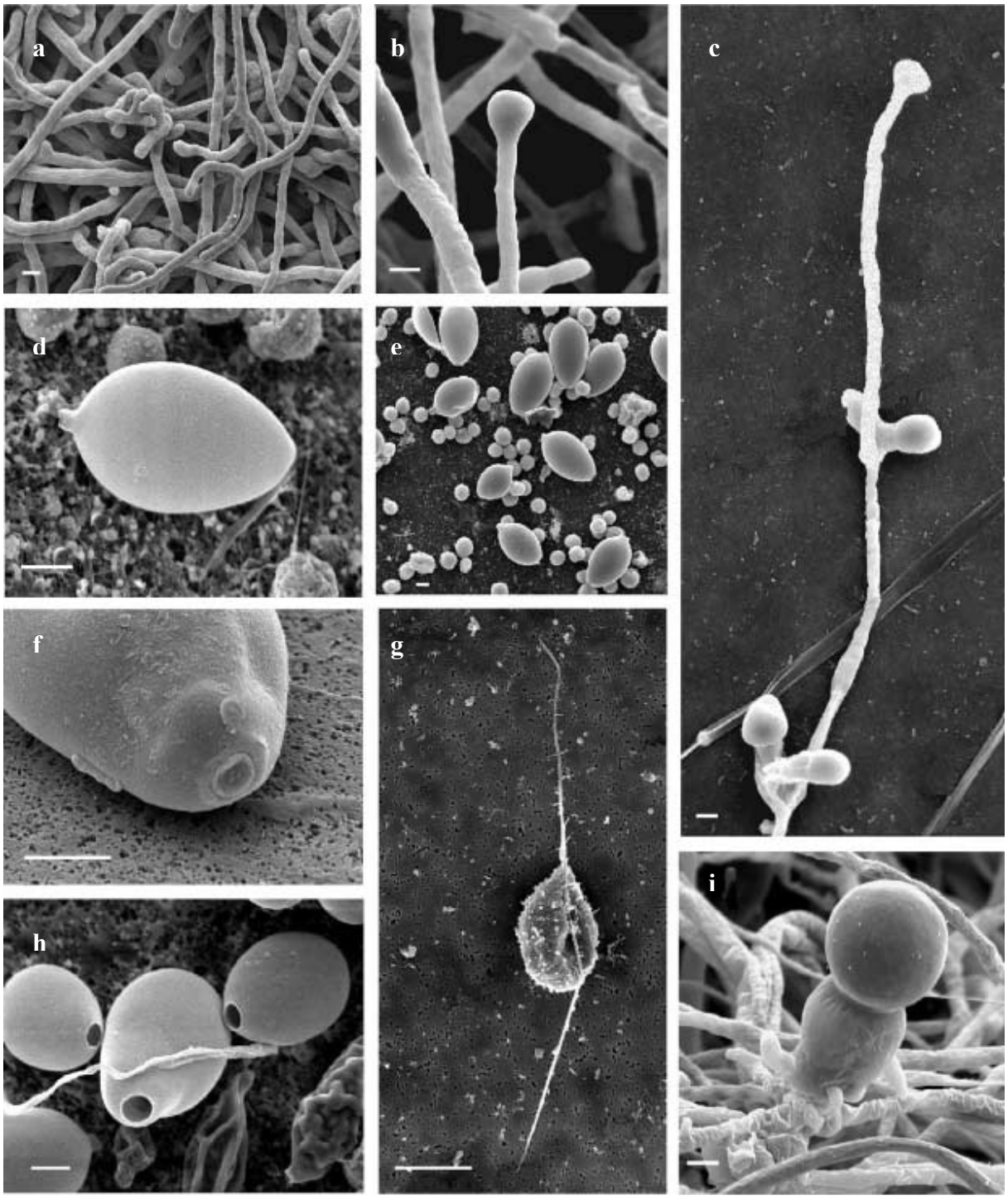

Figure 2. Stages of the development of different types of spore of *Phytophthora infestans*. The bar in each panel represents 5 μm.
a Vegetative, non-sporulating hyphae. **b** The swollen tip of an asexual sporangiophore, typical of a sporangium initial. **c** A sporangiophore containing four maturing asexual sporangia on lateral branches and a terminal sporangiophore initial. **d** An ungerminated sporangium. **e** A mixture of sporangia and zoospores. **f** The apical tip of a sporangium, showing the opening through which zoospores are released (operculum), which is now filled with an apical plug. **g** A zoospore with its two flagella attached to a central groove; decorations or mastigonemes can be seen on the upper flagellum. **h** Sporangia after having released zoospores, displaying open opercula. **i** An oospore formed from A1 and A2 mating type hyphae. [Reproduced from Judelson and Blanco (2005) with permission from the authors].

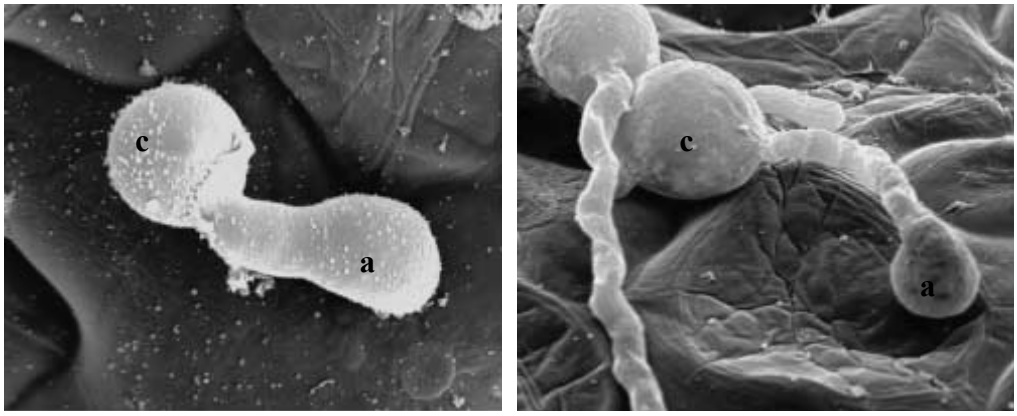

Figure 3. Cyst of *P. infestans* (c) forming an appressorium (a). [Reproduced from Judelson and Blanco (2005) with permission from the authors].

In the antheridia and oogonia, a meiotic or reductional division of the diploid genome takes place and haploid nuclei are formed. *Phytophthora* species do not form uninucleate gametes, a trait which clearly separates the Oomycetes from the true Fungi. One antheridial nucleus is transferred to the oogonium by a fertilization tube and fuses with one oogonial nucleus. The resulting oospore is a thick-walled structure that is formed in infected plant tissue.

When the plant dies and the plant tissue becomes decayed, oospores are released into the soil where they can survive for many years. When the soil is saturated with water, oospores can germinate, albeit at a very low frequency. The germinating oospores form sporangia or zoosporangia that release zoospores, depending on environmental conditions like moisture and temperature. When a host plant is present in the vicinity of germinating oospores, the zoospores respond to chemical stimuli present in exudates from the roots of the host plant and swim towards the root tips. They subsequently infect host plants and initiate new infection cycles.

Phytophthora diseases

The genus *Phytophthora* consists of over 100 described species. Some species have a narrow host range and infect one or a few plant species while others have a broad host range and attack numerous plant species. Thousands of plant species are susceptible to one or more *Phytophthora* species (Erwin and Ribeiro, 1996). *Phytophthora* pathogens are an increasing threat to natural ecosystems, forestry and agriculture. Here I describe the history and impact of four of the most damaging species in more detail, both from a socio-economic and ecologic perspective.

Phytophthora infestans

The potato late blight pathogen *P. infestans* is very destructive in agriculture. It was first described to occur on a large scale in the 1840s in Europe and the US, and its devastation of potato crops has caused widespread famine and emigration at that time. In Ireland the situation was especially grave, with the loss of one million lives and the emigration of one and a half million people during the Irish potato famine (Abad and Abad, 1997). The pathogen has remained a problem for potato production ever since, although sequential applications of crop protection products (fungicides) could control the pathogen effectively for some time. In the late 1970s, early 80s, however, farmers in Europe and the US experienced that late blight was more difficult to control. Until the late 1970s only the A1 mating type was present in most tomato and potato growing areas. Since *P. infestans* is heterothallic, sexual reproduction can only occur when both these mating types are present. The globally occurring *P. infestans* population was clonally propagated: variation in *P. infestans* strains could only arise by mutation and hence, adaptability of the *P. infestans* population was relatively low. This changed with the introduction of strains with both A1 and A2 mating type, most likely originating from a shipment of potatoes from Mexico to Europe. Due to sexual reproduction and meiotic recombination, the *P. infestans* population could now better adapt to new environmental conditions and more easily breach genetic resistance introduced in potato cultivars widely grown at the time.

Because of its long history and impact on agriculture, *P. infestans* has been the subject of study of many researchers in many different laboratories worldwide. As a consequence, most of the tools that are now used for oomycete research were first developed for *P. infestans.* It is also the oomycete species that is currently best represented in the various

genome and EST databases and as such, it can be considered as a model species for the Oomycetes (Bouwmeester et al., 2009). The latest accomplishment was the generation of the DNA sequence of the 240 megabase genome that was recently published by an international research consortium (Haas et al., 2009).

Phytophthora sojae

Phytophthora sojae, the soybean root rot pathogen, is responsible for an annual loss of up to 2 billion US dollars in soybean production (Tyler, 2007). The pathogen causes root and stem rot in soybean and damping off in seedlings. *P. sojae* is a soilborne pathogen and mainly limited to one host, *Glycine max* (soybean). It is a homothallic species with nonpapillate sporangia. Especially under wet conditions, in saturated soils, soybean roots are prone to infection by zoospores. The pathogen easily forms oospores in infected root tissue and these oospores can survive for many years in the soil causing infection in subsequent seasons (Dorrance and Grünwald, 2009).

Because of its economic impact, *P. sojae* has been intensively studied and, as *P. infestans,* has the status of a model species for the Oomycetes. It was a major effort to unravel the genetic makeup of this pathogen. The genome of *P. sojae* was the first oomycete genome to be sequenced (Tyler et al., 2006) and the analyses of the genome sequence and the functional analyses of a number of genes provided new insights in pathogenicity and virulence factors of oomycete pathogens. It was found that gene families associated with plant infections show a rapid expansion and diversification. Among these is a superfamily of 350 genes with similarly to oomycete avirulence genes that interact in a 'gene-for-gene' relationship with resistance genes. Crop resistance to *P. sojae* is mainly managed through development of cultivars which have single dominant resistance genes. Many of these genes have lost their effectiveness as isolates of *P. sojae* have adapted to these genes. The resistance genes in *G. max* have been mapped to six loci on the soybean genome (Dorrance and Grünwald, 2009).

Phytophthora cinnamomi

In Australia, the accidental introduction of *Phytophthora cinnamomi* has caused extensive damage in nature; whole ecosystems have withered due to this pathogen. The pathogen was first isolated from cinnamon trees in Indonesia in 1922 and its centre of origin is believed to be near Papua New Guinea (Hardham, 2005). The introduction of a

contaminated nursery stock in Perth in the 1920s allowed *P. cinnamomi* to "escape" into the jarrah (*Eucalyptus marginata*) forests. The undergrowth in these forests proved to be even more susceptible to *P. cinnamomi* than Eucalyptus itself, causing the disease to spread at an alarming rate. The area of native vegetation in temperate and tropical Australia affected by *P. cinnamomi* exceeds many hundreds of thousands of hectares, and continues to increase. In Western Australia alone, more than 6000 km^2 are now infested and over 40% of the approximately 6000 plant species in the South West Botanical Province are susceptible. The diseases caused by *P. cinnamomi* are a 'key threatening process to Australia's biodiversity' (Anonymous, 2008) and the impressive host range ranks *P. cinnamomi* at the top of the genus *Phytophthora* in terms of number of plant species it can infect.

Long term studies of diseased areas in Southern Australia have provided us with a better understanding of the epidemiology of diseases caused by *P. cinnamomi*. In soil, it can grow saprophytically for prolonged periods and when the opportunity arises to infect new hosts, the pathogen sporulates and produces vast amounts of zoospores. These zoospores encyst and the encysted zoospores can form a germ tube that penetrates the host plant. The disease develops swiftly after heavy rains, when the soil is waterlogged, as the pathogen needs water for the formation of zoosporangia and the release of zoospores. Under moist conditions, *P. cinnamomi* can survive at least 6 years in the form of chlamydospores, or less often, as oospores. Although the pathogen is heterothallic, most genetic variation originates by mutation in asexual populations, even when both mating types are present and sexual recombination can occur. This lack of sexual recombination resulted in the presence of three clonal lineages of *P. cinnamomi* in most parts of the world. These lineages hardly interact, but still the pathogen possesses the ability to adapt rapidly to new hosts and conditions.

Phytophthora ramorum

Although its impact on food production is low, the emergence of *P. ramorum*, or Sudden Oak Death, in the mid 1990s in California has boosted the public awareness of the impact of *Phytopthora* diseases and plant pathogens in general. Whole stands of tanoak (*Lithocarpus densiflorus*) and coast live oak (*Quercus agrifolia*) perished in California and Oregon due to this newly emerged pathogen (Grünwald and Goss, 2009), rousing public concern, mainly from prosperous land owners. In addition to oak, an ever increasing number of host plants fall victim to this disease, with 109 plant species recognized as susceptible to *P. ramorum* thus far. The disease also causes problems in nurseries and parks in Europe,

where *Rhododendron* spp. and *Viburnum* spp. were found to be affected by the same pathogen. *P. ramorum* is a heterothallic species and has semipapillate spores. Hardly any oospores are found, as the two mating types do not often coincide, and chlamydospores ensure its survival in soil when hosts are absent.

Because of the alarming damage caused by *P. ramorum* and the lack of adequate control methods, *P. ramorum* was the first *Phytophthora* species to be selected by funding agencies for whole genome sequencing. Since the value of sequencing of an unknown species that had no history of research was questioned, *P. sojae* was taken along in the genome sequencing (Govers and Gijzen, 2006). This allowed comparative genome analysis that resulted in a wealth of information on pathogenicity genes and revealed extensive synteny between species and even more interesting, synteny breakpoints that harbour the host specific virulence and avirulence genes (Govers and Gijzen, 2006; Tyler et al., 2006).

Emergence of new *Phytophthora* diseases

With the globalisation of food production and increased trade, pathogens have been brought into new areas and have adapted to new host species and environments. An additional threat is the possibility of hybridization; two distinct *Phytophthora* species can interact in soil, in host tissue or in water, and form interspecific progeny. This hybrid progeny can combine traits of both parental species and could therefore cause new disease types. Under normal circumstances, gene flow between two strains or isolates of different species is only possible when viable F1 hybrids are being produced following interspecific hybridization on a common host. This is a contradiction to the species concept, where a species is defined as a group of individuals that can only intercross within that species. For *Phytophthora* species, the boundaries between species are sometimes breached by interspecific hybridization, either via sexual or parasexual processes (Ersek and Nagy, 2008).

Sexual interspecific hybridization

Interspecific hybridization can occur through the normal sexual cycle of *Phytophthora*. Two isolates of opposite mating type can induce the sexual stage in each others mycelia. When antheridia and oogonia are formed, these can interact, and interspecific sexual progeny is formed. There are several examples of interspecific hybrids generated in the laboratory. Goodwin and Fry (1994) attempted to generate species hybrids between *P. infestans* and

P. mirabilis. These hybrids were impaired in their ability to infect the host plants of either parental species, but isozyme and DNA fingerprint analyses indicated that the isolates that were formed were indeed of hybrid constitution. The process of sexual hybridization is also possible in homothallic species, as was demonstrated by May et al. (2003) who generated species hybrids between *P. sojae* and *P. vignae*. These hybrids could infect both parental hosts, but were impaired in aggressiveness. Donahoo and Lamour (2008) showed that *P. capsici* and *P. tropicalis* could form interspecific sexual hybrids in the laboratory, whereby either parent could act as the maternal partner and contribute its mitochondrial DNA to the offspring. Not all combinations of parental isolates were successful; in a number of combinations only apomictic progeny was formed.

Several examples of naturally occurring hybridization events have been described in literature. On loquat trees in Peru and Taiwan (Hurtado-Gonzales et al., 2009) and in hydroponics in the Netherlands (Bonants et al., 2000; Man in 't Veld et al., 1998), hybrid offspring between *P. nicotianae* and *P. cactorum* was found to infect a number of new hosts. Analysis of nuclear and mitochondrial sequences and of AFLP fingerprints proved that the new pathogens were in fact species hybrids. In 2007, another species hybrid was described in the Netherlands, this time on rhododendron, involving a hybrid between *P. cactorum* and *P. hedraiandra* (Man in ' t Veld et al., 2007).

Somatic hybridization

In aquatic environments, the zoosporangia that are present on the mycelia can release zoospores, single nucleate swimming spores. These zoospores have a very fragile outer membrane. When different *Phytophthora* species are present in a common habitat, their zoospores can interact. Certain chemicals, or an electrical pulse, can partially disrupt the membranes of the zoospores. Two interacting zoospores can then fuse so that both nuclei are enveloped by one membrane. Ersek et al. (1995) described new species hybrids generated in this way between *P. nicotianae* and *P. capsici.* Hybrid progeny shared the host range of both parental species.

A second method for somatic hybridization has been described to occur by heterokaryon formation whereby hyphae of two species can merge and a species hybrid is formed. It remains unclear whether these processes play a role in interspecific hybridization between *Phytophthora* species in nature. The newly described hybrid species *P. alni* is thought to be an allopolyploid hybrid, consisting of three subspecies. Several hybridization

events and somatic or gametangial fusions form the basis for this complex species hybrid (Bakonyi et al., 2006; Bakonyi et al., 2007; Brasier et al., 1999; Brasier et al., 2004; Ioos et al., 2006).

Scope of this thesis

The aim of the research described in this thesis was to gain further insight in the impact of speciation and interspecific hybridization on populations of plant pathogenic *Phytophthora* species.

Section 2 deals with the *Phytophthora* phylogeny and starts with an introduction on *Phytophthora* species identification, including recent surveys to monitor the occurrence of *Phytophthora* species and a short overview on phylogenetic inference **(chapter 2.1)**. In **chapter 2.2**, nuclear and mitochondrial DNA sequences are used to determine the position of *P. infestans*, *P. mirabilis*, *P. ipomoeae*, and *P. andina* in the phylogenetic tree of the *Phytophthora* genus. An overview of all known *Phytophthora* species categorized by clade is presented in **chapter 2.3**. The overview not only includes officially recognized species, - i.e. those for which a valid Latin species description is present in literature- but also putative new species, which will most likely receive official status in the near future. For each species, several characteristics are provided, including their most important host species, the type of sporangia and their mating system.

The phylogenetic analyses of the genus *Phytophthora* revealed some incongruence between phylogenetic trees based on nuclear and mitochondrial DNA. Species that show this incongruence may have been involved in interspecific hybridization. **Section 3** focusses on new species and hybrids in clade 1c of the *Phytophthora* genus. In **chapter 3.1** the possibilities for interspecific hybridization are further explored using two closely related species. *P. infestans* and *P. mirabilis* originate from the same geographic location. They are indistinguishable based on ITS sequences region and show slight sequence variation in nuclear and mitochondrial genes (chapter 2.2). To investigate if they can exchange genetic material, interspecific hybrids were generated and the resulting F1 isolates were intercrossed and backcrossed to the parental species. The genetic makeup of the hybrids was verified by DNA fingerprinting and their pathogenicity and host specificity were tested in detached leaflet assays on *Mirabilis jalapa*, a host of *P. mirabilis*, and *Solanum tuberosum* and *S. lycopersicum*, hosts of *P. infestans*.

The Andes in South America, and especially Ecuador, is either a centre of origin or a centre of diversity for *P. infestans* and as such of key importance for the evolution of this species. To gain insight into the diversity of *Phytophthora* species occurring on the various host plants classified in the genus *Solanum*, isolates were collected and their genetic and pathogenic properties were characterized **(chapter 3.2)**. Many of these isolates that phenotypically resembled *P. infestans* appeared to be quite distinct at the molecular level. They could be classified into three clonal lineages **(chapter 3.3)** and these lineages were grouped into a new species that was named *Phytophthora andina*.

Section 4 deals with a spin-off of the phylogenetic analysis described in chapter 2.2: the development of an assay to distinguish between *P. ramorum* isolates that were found in the USA on oak trees, and in Europe on rhododendrons. Both groups of isolates are propagated clonally, as one mating type of *P. ramorum* is solely found in the USA and the other mating type is restricted to Europe. With the troubles of *P. infestans* in mind, where the global population became increasingly more adaptable and virulent after the introduction of the second mating type, it is essential that both populations are kept in isolation. The diagnostic assay described in **chapter 4.1** was designed based on a sequence polymorphism discovered in the phylogenetic analysis and was used by Plant Protection Agencies to screen nurseries and plant materials that are shipped between countries.

In the general discussion (**section 5)** the implications of the research described in this thesis are placed in a broader perspective with special emphasis on hybridization events in *Phytophthora,* the centre of origin of *P. infestans* and some recent developments in *Phytophthora* genomics. It is foreseen that in the near future next generation sequencing technologies will be exploited to gain more insight in the diversity, speciation and host specificity in the *Phytophthora* genus.

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2

The *Phytophthora* phylogeny

2.1

Introduction

Phytophthora is a genus on the move. In the 120 years separating the studies of Heinrich Anton de Bary (1876) and the reference monograph '*Phytophthora* diseases worldwide' by Erwin and Ribeiro (1996), 58 species of *Phytophthora* were identified and described in literature (chapter 2.3). In the last decade, the number of *Phytophthora* species has doubled and new species are added almost on a monthly basis. This enormous increase in new species is due to, one the one hand, the availability of more sophisticated tools for species determination and on the other hand, large scale surveys by several research groups for the presence of novel *Phytophthora* species in natural and agricultural settings.

Waterhouse groups

For a long time identification and classification of species within the genus *Phytophthora* were based on the key developed by Waterhouse (1963). The mycologists in the pre-molecular era used host range, spore morphology, presence or absence of chlamydospores, optimal growth temperature, colony morphology, surface structure of oospores and other "Waterhouse" criteria to define species boundaries and to position a new species in one of the six Waterhouse groups. The allocation of an isolate to a particular species was arduous work and required trained experts with a good eye and attention to detail.

The description of a new species was even more challenging, requiring the researcher to be a skilled mycologist able to distinguish the potential new species from all other species, an artist to draw spore structures by hand and a classicist to phrase the findings in Latin. Researchers often found discrepancies within a species, for example groups of isolates with much higher optimal growth temperature or aberrant oospore ornamentation. Since the Waterhouse key could often not handle these discrepancies, footnotes were made to justify why the deviating isolates were kept within the species.

Species identification based on DNA sequences

The method of species identification and classification began to change when DNA fingerprinting methods became more readily available. Like phenotypic traits, DNA fingerprinting can be used to group isolates within a particular species (Drenth et al., 1993; Drenth et al., 1994). Another frequently used method to distinguish *Phytophthora* isolates at the species level was isozyme analysis, utilizing polyacrylamide gel-electrophoresis to detect

different alleles for a number of enzymes (Elliott and Maxwell, 1984; Oudemans and Coffey, 1991). These techniques provided invaluable new insights in both population structure within species and variation between species. In the 1990s AFLP analysis became the state of the art technology for DNA fingerprinting (Vos et al., 1995). Van der Lee et al. (1997) constructed the first molecular-genetic linkage map of *P. infestans* based on AFLP markers whereas others used AFLP markers as high resolution markers to analyse the population structure of *P. infestans* (Flier et al., 2003; Flier et al., 2007; Knapova and Gisi, 2002; Purvis et al., 2001).

The exploitation of DNA sequencing provided the biggest leap in knowledge on *Phytophthora* species so far. If the sequences for particular genes or DNA regions are identical or nearly identical, the isolates supposedly belong to the same species. If DNA sequences of the same region are available for dozens of species, a phylogeny can be made. Species can then be grouped in clades, consisting of a single common ancestor and all its descendants. A clade is, simply expressed, a branch (Greek: *klados*) of the evolutionary tree which is separated from the rest of the tree by a single cut. Any branch, however large or small, that is cut off in this way is *monophyletic*, or of a single origin (Wilkins, 2007).

Large scale surveys

In the last decades several plant pathogenic *Phytophthora* species have caused huge damage to natural vegetations and ecosystems, for instance *P. cinnamomi* in Australia, *P. alni* in European forests and *P. ramorum* in California and Oregon. The impact of *Phytophthora* diseases on plant species diversity in the affected ecosystems, or economic damage on wood stands, triggered several large-scale surveys for presence and diversity of *Phytophthora* isolates (Balci et al., 2007; Balci et al., 2008; Belbahri et al., 2006; Brasier et al., 2005; Burgess et al., 2009; Jung, 2009; Jung and Burgess, 2009; Nechwatal and Mendgen, 2006; Streito et al., 2002). These surveys not only increased the knowledge on host range of *Phytophthora* species or the habitats in which these species thrived, but also resulted in the discovery of a fair number of new *Phytophthora* species. For most of these species, a connection could be made between the presence of the species and disease symptoms of a nearby host. However, several new species were discovered which were distinct from all other prevailing *Phytophthora* species based on morphology and DNA sequence, but for which no apparent host plant was found (Balci et al., 2008; Jung et al., 2002; Nechwatal and Mendgen, 2006).

Phytophthora phylogenies based on DNA sequences

One of the first DNA regions to be used in phylogenetic analysis was the 5.8S ribosomal RNA gene and the flanking internal transcribed spacers 1 and 2 (ITS1 and ITS2) (Lee and Taylor, 1992). This region contains stretches of high homology that were used to design primers for Polymerase Chain Reaction (PCR) amplification. For almost all *Phytophthora* species, the same primers can be used. The first extensive phylogenetic study of the *Phytophthora* genus based on ITS1 and ITS2 sequences was described by Cooke et al. (2000). This study, which included 234 isolates from 50 distinct *Phytophthora* species, provided the basis for the clade nomenclature currently used to group *Phytophthora* species and replaced the Waterhouse classification which was found wanting.

The advantage of the ITS approach is that the sequence of ITS1, 5.8S, and ITS2 can be readily obtained and as a result the ITS sequences of a large number of *Phytophthora* species are currently available in GenBank. A disadvantage, however, is the strict selection pressure on this genomic region, which is present in many copies within one genome. The observation that variability in ITS sequences between closely related species is low because of this selection pressure raised doubts about the applicability of ITS regions for phylogenetic inference (Alvarez and Wendel, 2003; Bailey et al., 2003). This led to a new approach for generating appropriate sequences for phylogenetic analysis of *Phytophthora* species. This approach involves the sequencing of "housekeeping" genes, i.e. genes, either mitochondrial or nuclear, that encode proteins with known functions in the metabolism or catabolism of the organism. These genes also possess highly conserved regions that are suited for universal primer design, but the nucleotide variation within the genes is higher. This approach results in better resolved phylogenies, with a reduced influence of homoplasious traits when compared to ITS-based analyses (Alvarez and Wendel, 2003). Many *Phytophthora* genes lack introns, regions of DNA within a gene that are not translated into protein. Introns often have a higher level of polymorphism when compared to exons, the DNA regions within a gene that do encode proteins. The higher levels of polymorphism make intron sequences more useful for phylogenetic studies. Because of the lack of introns, larger stretches of exon sequences are needed to create a well-resolved phylogenetic tree.

The first significant example of a study using *Phytophthora* "housekeeping" genes was published by Martin and Tooley (2003) who sequenced two mitochondrial genes,

Cytochrome Oxidase I and II (*cox*1 and *cox*2) from 51 isolates representing 27 *Phytophthora* species. However, this study had two drawbacks. Firstly, it covered only a subset of the known *Phytophthora* species with clades that were less resolved when compared to the study by Cooke et al. (2000) and, secondly, the study used only mitochondrial genes, which are uniparentally inherited. As a result, the novel clade nomenclature that Martin and Tooley (2003) introduced in their study for the genus *Phytophthora* did not substantiate.

In chapter 2.2 of this thesis we present a phylogenetic analysis based on the sequences of both nuclear and mitochondrial genes and covering 113 isolates from 48 *Phytophthora* species (Kroon et al., 2004a). Until recently, it was the most comprehensive study of the *Phytophthora* genus that expanded on the clade nomenclature introduced by Cooke et al. (2000). Separate analyses were carried out for both nuclear and mitochondrial regions, and for all regions combined. This revealed discrepancies in the phylogenetic position of a number of *Phytophthora* isolates, hinting at interspecific mating and somatic hybridization events. Phenotypic traits from the Waterhouse key were interpolated on the phylogenetic tree, in order to speculate on the ancestry of traits like mating type, sporangium morphology and attachment of the antheridia to the oogonia.

An expansion of our phylogenetic analysis in chapter 2.2 was published by Blair et al. (2008). They sequenced seven genes covering 8700 nucleotides from seven loci in 234 isolates that represented 82 species, including many new *Phytophthora* species from recent surveys. This is therefore the most detailed and complete *Phytophthora* phylogeny published to date. The data from Blair et al. (2008) combined with data on new *Phytophthora* species that have been reported in literature since the monograph of Erwin and Ribeiro (1996) are presented in chapter 2.3.

2.2

Phylogenetic Analysis of *Phytophthora* Species Based on Mitochondrial and Nuclear DNA Sequences

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Abstract

A molecular phylogenetic analysis of the genus *Phytophthora* was performed, 113 isolates from 48 *Phytophthora* species were included in this analysis. Phylogenetic analyses were performed on regions of mitochondrial (cytochrome c oxidase subunit 1; NADH dehydrogenase subunit 1) and nuclear gene sequences (translation elongation factor 1α; β-tubulin) and comparisons made to test for incongruence between the mitochondrial and nuclear data sets. The genus *Phytophthora* was confirmed to be monophyletic. In addition, results confirm that the classical taxonomic grouping as described by Waterhouse (1963) does not reflect true phylogenetic relations. *Phytophthora* species were redistributed into 8 clades, providing a more accurate representation of phylogenetic relationships within the genus *Phytophthora*. The evolution and transition of morphological, pathogenic and reproductive traits was inferred from the cladogram generated in this study. Mating system was inferred to be a homoplasious trait, with at least eight independent transitions from homothallism to heterothallism observed.

Introduction

The genus *Phytophthora* consists of more than 70 species and is classified within the diploid, algae-like Oomycetes in the Stramenopile clade of the Chromista (Cavalier-Smith, 1986; Dick, 1995; Yoon et al., 2002). The genus harbors devastating plant pathogens that have a large impact on agriculture (e.g. *P. infestans*, Potato Late Blight), arbiculture (e.g. *P. ramorum*, Sudden Oak Death), and whole ecosystems (e.g. *P. cinnamomi* in Australia).

Recently, Riethmüller et al. (2002) clarified the phylogenetic relationships of the Peronosporomycetidae on the basis of analysis of nuclear large subunit ribosomal DNA (nLSU rDNA) sequences. The Peronosporomycetidae represent one of the three subclasses within the Oomycetes, in which the genus *Phytophthora* is classified. Their results challenged the longstanding opinion that *Phytophthora* and *Pythium* are sister genera. Riethmüller et al. (2002) concluded that the genus *Phytophthora* is nested within one of the (pseudo)*Peronospora* clades, as are the genera *Peronophythora*, *Bremia*, and *Plasmopara*. Sister group to this assemblage is a clade comprising the genera *Pythium* and *Lagenidium*.

Recent molecular analysis (Cooke et al., 2000; Martin and Tooley, 2003) has substantially increased our understanding of the phylogenetic relationships between

Phytophthora species. Both studies, however, were based on either sequence information of single DNA-regions (rDNA internal transcribed spacers [ITS]) or genes (cytochrome oxidase II [*Cox*II]), or covered only a limited subset of *Phytophthora* species (for *Cox*I). Our phylogenetic study is based on 5 sets of sequence data from the nuclear and mitochondrial genome for a wide range of *Phytophthora* species with an emphasis on the phylogenetic position of *P. infestans* and related species. The value of conduction phylogenetic analysis on multiple genes has been shown recently in *Fusarium* (O'Donnel et al., 1998) and yeast phylogenies (Rokas et al., 2003) and is now a well-established practice in, for instance, the angiosperms (Davis and Chase, 2004).

Timing of proliferation of *P. infestans* and related species, such as *P. mirabilis*, *P. ipomoeae*, and *P. phaseoli*, can be important from a phytopathological point of view. DNA sequence divergence rates may serve as a measurement for the level of isolation between these species and may be indicative for the possibility of gene flow between *P. infestans* and closely related species (Goodwin et al., 1999). Such gene flow can have a major impact on pathogenicity as interspecies crosses have resulted in progeny with host ranges broader than either parental species (Man in 't Veld et al., 1998).

The principal aim of our study was to examine, in detail, the phylogenetic relationships within the genus *Phytophthora*. Because of their different mechanisms of inheritance, a comparison of phylogenies based on nuclear and mitochondrial gene sequences was fundamental to this study. Nuclear genes are inherited from both the maternal and paternal line, but mitochondrial genes are exclusively transmitted through the maternal line (Whittaker et al., 1994). The hypothesis will be tested that sequence information originating from both nuclear and mitochondrial DNA can be pooled for phylogenetic analysis. The second objective is to test the validity of *Phytophthora* sub-clades proposed in earlier studies (Cooke et al., 2000) and to establish the position of *P. infestans* and closely related species within the *Phytophthora* clade. The third objective is to explore patterns of evolution of sexual traits (homothallic vs. heterothallic mating system, antheridial attachment), morphological characteristics (presence or absence of papillae) and ecological niche preference (soil or foliage).

Materials and methods

Selection of isolates

Isolates used in this study were selected from 45 *Phytophthora* species (Table 1), with an emphasis on *P. infestans* and the related taxa *P. mirabilis* and *P. ipomoeae* (Flier et al., 2002). All isolates were classified according to Erwin and Ribeiro (1996). A *Phytophthora* isolate, of which the taxonomic identity has not yet been determined, isolated by the Dutch Plant Protection Service from *Spathiphyllum* spp. (unpublished data), was also included in this study, as was an isolate of putative hybrid origin, isolated from alder trees in the Netherlands (Brasier et al., 1999). These isolates will be referred to as *P.* sp. Spathiphyllum and *P.* hybrid-Dutch variant, respectively. In addition, a group of isolates from the Andean Highlands in Ecuador was included. This group will be referred to as *P. andina*, a putative new species described by Adler et al. (2004).

Outgroups were selected on the basis of a phylogenetic study by Riethmüller et al. (2002). *Pythium aphanidermatum* and the following Stramenopile representatives were used as outgroups in our analysis: *Fragilaria striatula* (Genbank **AB020224**), *Ophiocytium majus* (**AB000210**), *Pylaiella littoralis* (**Z72500**), *Pythium ultimum* (**AF218256**), and *Achlya klebsiana* (**J05597)**, each covering a single gene sequence in the data set.

Isolates of *P. infestans* were chosen from a wide range of host plant species and geographic locations. For *P. infestans*, *P. mirabilis* and *P. ipomoeae*, peptidase (Pep) and glucose-6-phosphate isomerase (Gpi) allozyme patterns (Goodwin et al., 1995) were used as an additional selection criterion in order maximize the likely diversity amongst the selected isolates. For *P. infestans* (37 isolates), *P. mirabilis* (15), and *P. ipomoeae* (4), isolates were pooled into groups with close to 100% sequence identity and from each of these groups, one representative isolate was selected for subsequent phylogenetic analysis. This resulted in four groups of *P. infestans* isolates, corresponding to the four *P. infestans* haplotypes (Ia, Ib, IIa, and IIb) (Griffith and Shaw, 1998), five groups of *P. mirabilis* isolates (which we will refer to as type I to V), and one group of *P. ipomoeae* isolates.

To facilitate comparison between the data presented in this paper and the analysis of the ITS-region (ITS 1 and 2 flanking the 5.8S rDNA subunit) by Cooke et al. (2000), we have used the same isolates when available (Table 1) and adopted the ITS clade nomenclature whenever applicable.

Table 1. Isolates of *Phytophthora* used in this study, their designations, origins and year of collection

Phytophthora species	Isolate numbers		Origins		
	International	Local	Host	Country	Year
P. infestans haplotype Ia	...	Pic99186	*Solanum stoliniferum*	Mexico	1999
P. infestans haplotype Ib	...	West Virginia 4	*Solanum tuberosum*	USA	...
P. infestans haplotype IIa	...	Dr98004	*Solanum tuberosum*	The Netherlands	1998
P. infestans haplotype IIb	...	Can4	...	Canada	...
P. mirabilis type I	...	Pic99129	*Mirabilis jalapa*	Mexico	1999
P. mirabilis type II	...	P3001	*Mirabilis jalapa*	Mexico	1984
P. mirabilis type III	...	Pic99145	*Mirabilis jalapa*	Mexico	1999
P. mirabilis type IV	...	G4-4	*Mirabilis jalapa*	Mexico	1998
P. mirabilis type V	...	G15-4	*Mirabilis jalapa*	Mexico	1998
P. ipomoeae	...	Pic99165	*Ipomoea longipedunculata*	Mexico	1999
P. phaseoli [a]	ATCC60171	CBS556.88	*Phaseolus lunatus*	...	...
P. andina	...	EC3421	*Solanum muricatum*	Ecuador	2001
P. arecae	...	CBS148.88	*Chamaedorea seifrizzi* x *erumpens*	USA	...
P. boehmeriae	...	CBS291.29	*Boehmeria nivea*	Japan	...
P. botryosa	IMI136916	CBS533.92	*Hevea brasiliensis*	Thailand	...
P. cactorum	...	P6183	*Rubus idaeus*	USA	...
P. hybrid-Dutch variant	...	PD92/1471	*Alnus cordata*	The Netherlands	1992
P. tropicalis	AN97/86	PD97/11132	*Rosa* spp.	The Netherlands	1997
P. cinnamomi	RADICI B	10A6	*Persea americana*	...	...
P. citricola	...	P1817	*Medicago sativa*	South Africa	...
P. citrophthora	CBS274.33	PD94/353	*Citrus limonium*	Cyprus	...
P. clandestina [a]	IMI287317	R193	*Trifolium subterranea*	Australia	1985
P. colocasiae [a]	IMI368918	...	*Colocasia esculenta*	Malaysia	1995
P. cryptogea	...	HR1/ss/pp/99	*Solanum lycopersicum*	UK	...
P. drechsleri [a]	...	CBS292.35	*Beta vulgaris var. altissima*	USA	1935
P. erythroseptica	ATCC46725	CBS951.87	*Solanum tuberosum*	Australia	...
P. fragariae var. *fragariae* I	...	A2	*Fragaria* x *ananassa*	...	...
P. fragariae var. *fragariae* II	...	NS4	*Fragaria* x *ananassa*	...	...
P. fragariae var. *rubi* I	...	FVR67	*Rubus idaeus*	...	...
P. fragariae var. *rubi* II	...	FVR30	*Rubus idaeus*	Scotland	...
P. gonapodyides [a]	...	P245	*Salix matsudana*	UK	1972
P. heveae [a]	IMI180616	CBS296.29	*Hevea brasiliensis*	Malaysia	1929
P. hibernalis	ATCC64708	CBS522.77	*Aquilegia vulgaris*	New Zealand	...
P. humicola [a]	IMI302303	...	citrus orchard soil *via* citrus bait	Taiwan	1981
P. idaei	IMI313727	R66	…	UK	...
P. ilicis	...	PD91/595	*Ilex aquifolium*	The Netherlands	1991
P. inflata [a]	IMI342898	...	*Syringa*	UK	1990
P. insolita [a]	IMI288805	...	soil	Taiwan	...
P. iranica [a]	IMI158964	CBS374.72	*Solanum melongena*	Iran	1969
P. katsurae	IMI325914	CBS587.05	soil	Taiwan	1979
P. lateralis [a]	IMI040503	CBS168.42	*Chamaecyparis lawsoniana*	USA	...
P. meadii [a]	IMI129185	CBS219.88	*Hevea brasiliensis*	India	1968
P. megakarya	IMI337098	...	*Theobroma cacao*	Equatorial Guinea	...
P. megasperma [a]	IMI133317	MEG23	*Malus sylvestris*	Australia	1968
P. multivesiculata [a]	CBS545.96	PD95/8679	*Cymbidium*	The Netherlands	1995
P. nicotianae	...	P582	*Nicotiana tabacum*	USA	...
P. palmivora	...	CBS236.30	*Cocos nucifera*	India	...
P. brassicae	...	CBS179.87	*Brassica oleracea*	The Netherlands	1987
P. pseudotsugae [a]	IMI331662	PSE1	*Pseudotsuga menziesii*	USA	...
P. quininea	...	CBS407.48	*Cinchona officinalis*	Peru	...
P. richardiae	...	CBS240.30	*Zantedeschia aethiopica*	USA	...
P. sinensis	...	P1475	-	...	...
P. sojae	...	P6497	*Glycine max*	...	...
P. syringae	IMI045169	CBS364.52	*Prunus armeniaca*	New Zealand	...
P. tentaculata [a]	...	CBS552.96	*Chrysanthemum leucanthemum*	Germany	...
P. vignae	...	CBS241.73	*Vigna sinensis*	Australia	1992
P. sp. Spathiphyllum	...	...	*Spathiphyllum* spp.	...	...
P. ramorum US-type	...	USA 0.13	*Quercus agrifolia*	USA	...
P. ramorum European-type	...	PD93/51	*Rhododendron catawbiense*	The Netherlands	1993

[a] These isolates were used in the ITS-based analysis by Cooke et al. (2000).

DNA extraction

Isolates were grown for 10-14 days at 20 °C in pea broth. This medium was prepared by autoclaving 120 g of frozen peas in one liter of tap water (20 min at 121 °C), filtering through cheesecloth and re-autoclaving (Flier et al., 2003). The mycelium was harvested, lyophilized and total DNA extracted using the Puregene kit (Gentra/Biozyme, Landgraaf, The Netherlands) according to the manufacturer's instructions. DNA pellets were dissolved in 100 µl of TE [10 mM Tris-HCl (pH 8.0), 1 mM EDTA] and stored at -20 °C.

Primer design

Primers were developed using published DNA sequences for the genus *Phytophthora*. The last nucleotide of the primers (at the 3' position, where amplification starts) was chosen to be located at the last or first base of a triplet, and was either a G or C. Forward and reverse primers were constructed to amplify a product of 900-1100 basepairs (bp), in coding regions. For each DNA region, 2-4 primers were developed and tested in pairs. Primer pairs that amplified the target sequences best in all species were selected and used for sequencing (Table 2). Primer regions were not included in the sequence alignments.

For the translation elongation factor 1 alpha gene (*EF*-1α), the *P. infestans* mRNA described by Van 't Klooster et al. (2000) was used as a basis for primer selection (accession **AJ249839**). Primers ELONGF1 and ELONGR1 amplified a 972-bp central fragment of the gene. No introns were present in this region. For ß-tubulin (ß-*tub*) primer selection, the *P. cinnamomi* mRNA accession **U22050** was used (Weerakoon et al., 1998). Amplification with TUBUF2 and TUBUR1 yielded a fragment of 989 bp, with no introns present.

For all mitochondrial regions analyzed in this study, the complete *P. infestans* mitochondrial DNA-sequence **U17009** (Paquin et al., 1997) was used as a template for primer design. For the Cytochrome *c* oxidase subunit 1 gene (*cox*1), primers COXF4N and COXR4N amplified a region of 972 bp. From a subset of isolates (*P. infestans*, *P. mirabilis*, *P. ipomoeae*, *P. phaseoli*, *P. andina*, and *P. tropicalis*) *Cox*1 was also amplified with primers used in *P. infestans* haplotyping (Griffith and Shaw, 1998). Primers F4 and R4 amplified a region of 964 bp (referred to as P4), with a 719-bp overlap with the COXF4N/COXR4N fragment. This resulted in an extension of 191 bp of sequence information for this subset of isolates, of which a region of 148 bp consists of non-coding spacer DNA. The NADH dehydrogenase subunit 1 gene (*nadh*1) is present in reverse orientation, and the primers used to amplify it were NADHF1 and NADHR1, which yielded a fragment of 897 bp of coding

sequence. For part of the ATP synthase F1 subunit α (*atp*1) gene, the glutamic acid tRNA (trnE), and part of the NADH dehydrogenase subunit 4 (*nad*4) gene (in total referred to as P2), primers F2 and R2 amplified a region of 1070 bp. Only a subset of isolates (*P. infestans*, *P. mirabilis*, *P. ipomoeae*, *P. phaseoli*, *P. andina*, *P. tropicalis*, and *P. sojae*) was used for amplification with these primers, designed for haplotyping *P. infestans* isolates (Griffith and Shaw, 1998). Two non-coding spacers (bp14102-14181 and bp14254-14282) separated the three coding regions in this fragment.

Table 2. Primers used in this study

Target DNA	Primer	Primer sequence	reference[a]	Primer orientation[b]	T_m[c]	GC[d]	Size[e]
Translation elongation factor 1α	ELONGF1	5' TCACGATCGACATTGCCCTG 3'	AJ249839	180-199	69.5	55.0	972
	ELONGR1	5' ACGGCTCGAGGATGACCATG 3'		1132-1151	69.7	60.0	
β-tubulin	TUBUF2	5' CGGTAACAACTGGGCCAAGG 3'	U22050	570-589	68.5	60.0	989
	TUBUR1	5' CCTGGTACTGCTGGTACTCAG 3'		1538-1558	61.3	57.1	
Cytochrome c oxidase subunit 1	COXF4N	5' GTATTTCTTCTTTATTAGGTGC 3'	U17009	9126-9147	53.2	31.8	972
	COXR4N	5' CGTGAACTAATGTTACATATAC 3'		10,076-10,097	50.6	31.8	
P4	F4	5' TGGTCATCCAGAGGTTTATGTT 3'	U17009	9379-9400	62.1	40.9	964
	R4	5' CCGATACCGATACCAGCACCAA 3'		10,321-10,342	69.6	54.5	
NADH dehydrogenase subunit 1	NADHF1	5' CTGTGGCTTATTTTACTTTAG 3'	U17009	27,278-27,298	52.7	33.3	897
	NADHR1	5' CAGCAGTATACAAAAACCAAC 3'		26,402-26,422	56.3	38.1	
P2	F2	5' TTCCCTTTGTCCTCTACCGAT 3'	U17009	13,613-13,633	63.5	47.6	1070
	R2	5' TTACGGCGGTTTAGCACATACA 3'		14,661-14,682	66.0	45.5	

[a] Reference to the GenBank accession containing the DNA sequence on which the primers is based.

[b] Reference to the location of the primer within the original DNA sequence.

[c] Melting temperature of the primer.

[d] GC-content of the primer.

[e] Average amplicon length.

DNA amplification and sequencing

Amplifications were performed in a PTC200 thermocycler (MJ Research, Waltham, Massachusetts, USA). The thermocycle sequence was as follows: an initial denaturation at 94 °C for 2 min; 35 cycles consisting of denaturation at 94 °C for 30 s (60 s for P2 and P4), annealing for 30 s, and extension at 72 °C for 60 s; a final extension at 72 °C for 10 min. Annealing temperatures were 60 °C for *EF*-1α and β-*tub*, 52 °C for *cox*1, 53 °C for *nadh*1, and 62 °C for P2 and P4. The reaction mix consisted of 10 to 20 ng of template DNA, 200 µM of dNTPs, 1 U of *Taq* DNA polymerase (Roche, Indianapolis, USA), 1.5 mM of $MgCl_2$, and 25 ng of each primer in a reaction volume of 25 µl. For mtDNA gene amplification, the $MgCl_2$ concentration was raised to 3.5 mM. Successful amplification was confirmed by gel electrophoresis.

PCR products were purified on Sephadex plates (Multiscreen HV, Millipore, Bedford, USA) to remove excess primers and nucleotides and sequenced with the corresponding primers using the BigDye sequencing kit (Applied Biosystems, Foster City, USA) on an ABI3700 DNA Analyzer (Applied Biosystems). The trace files were transferred to the SeqMan 5.0 module of DNASTAR (DNASTAR Inc., Madison, WI, USA). As the sequence results originated from PCR products, heterozygous sites were observed and labeled according to the IUPAC coding system. Forward and reverse sequences were linked in Editseq 5.0 (DNASTAR) and aligned using the ClustalW algorithm provided in the MegAlign module (DNASTAR). Due to length variation in the spacer regions of the P2 and P4 amplicons, some manual adjustment of the alignment of gaps was necessary.

Phylogenetic analysis

Sequence data for five coding mtDNA regions (*cox*1, *nadh*1, *atp*1, trnE, *nad*4) and two nuclear encoded genes (β-*tub* and *EF*-1α) were compared for 62 accessions. In this study an accession was defined as an isolate or group of isolates covered by a single entry in our data matrix (Table 1). For *P. infestans* (4 accessions, representing 44 sequenced isolates), *P. mirabilis* (5 accessions, representing 19 sequenced isolates), *P. fragariae* (4 accessions, representing 4 sequenced isolates), and *P. ramorum* (2 accessions, representing 5 sequenced isolates), species are represented by more than one type. For each accession, sequence entries from a single representative isolate were used. Not all regions were sequenced for all

accessions; data coverage is listed in Table 3. Phylogenetic analysis was performed using PAUP* version 4.0b10 (Swofford, 2002) running on a G4 Power Macintosh computer.

We conducted separate phylogenetic analyses for individual genes, the combined mtDNA, combined nDNA, and combined mt + nDNA data sets. Jackknife analysis was carried out in order to assess data structure and to identify significantly supported clades. In addition, between-data set congruence was tested for by comparing jackknife topologies, which was done by visual inspection. Jackknife analysis (10,000 replicates) was carried out using PAUP* with settings so as to emulate the Parsimony Jackknifer (Farris et al., 1996), i.e., percentage of characters deleted in each replicate = 37, 'fast' stepwise addition and "Jac" resampling method used. Subsequent parsimony search was performed using a heuristic search, which involved TBR branch swapping, MULTREES 'on', and 'collapse branches when maximum length is zero'. Starting trees were either generated by 500 cycles of random addition sequence (RAS) holding 3 trees at each step, or by swapping on sub-optimal trees generated from 100 RAS with no swapping, MULTREES 'off' and hence keeping one tree from each replicate, even if not optimal over all replicates.

Association between functional traits and phylogeny

The *Phytophthora* species included in this study were analyzed for selected traits. The following 'characters' were traced using MacClade 4 (Maddison and Maddison, 2000): mating system (two states; homothallic or heterothallic); presence and shape of papillae (three states; non-papillate, semi-papillate, or papillate); and type of antheridia (two states; amphigynous or paragynous). Character states were obtained from literature reports and are listed in Table 4 (Adler et al., 2004; Aragaki and Uchida, 2001; Erwin and Ribeiro, 1996; Flier et al., 2002; Ilieva et al., 1998; Man in 't Veld et al., 2002; Werres et al., 2001b).

Table 3. Data coverage for accessions included in the table

	nDNA genes		mtDNA genes		
Phytophthora species	ß-*tubulin*	*EF*-1α	*cox*1	*nadh*1	P2
P. infestans haplotype Ia	**AY564035**	**AY564093**	**AY564150**	**AY563977**	**AY564209**
P. infestans haplotype Ib	ND[a]	ND	**U17009**	**U17009**	**U17009**
P. infestans haplotype IIa	**AY564036**	**AY564094**	**AY564151**	**AY563978**	**AY564210**
P. infestans haplotype IIb	**AY564037**	ND	**AY564152**	**AY563979**	**AY564211**
P. mirabilis type I	**AY564038**	**AY564095**	**AY564153**	**AY563980**	**AY564212**
P. mirabilis type II	**AY564039**	**AY564096**	**AY564154**	**AY563981**	**AY564213**
P. mirabilis type III	**AY564040**	**AY564097**	**AY564155**	**AY563982**	**AY564214**
P. mirabilis type IV	**AY564041**	**AY564098**	**AY564156**	**AY563983**	**AY564215**
P. mirabilis type V	**AY564042**	**AY564099**	**AY564157**	**AY563984**	**AY564216**
P. ipomoeae	**AY564043**	**AY564100**	**AY564158**	**AY563985**	**AY564217**
P. phaseoli	**AY564044**	**AY564101**	**AY564159**	**AY563986**	**AY564218**
P. andina	**AY564045**	**AY564102**	**AY564160**	**AY563987**	**AY564219**
P. arecae	**AY564049**	**AY564105**	**AY564164**	**AY563991**	ND
P. boehmeriae	**AY564050**	**AY564106**	**AY564165**	**AY563992**	ND
P. botryosa	**AY564051**	**AY564107**	**AY564166**	**AY563993**	ND
P. cactorum	**AY564052**	**AY564108**	**AY564167**	**AY563994**	ND
P. hybrid-Dutch variant	**AY564053**	**AY564109**	**AY564168**	**AY563995**	ND
P. tropicalis	**AY564046**	**AY564103**	**AY564161**	**AY563988**	**AY564220**
P. cinnamomi	**AY564054**	**AY564110**	**AY564169**	**AY563996**	ND
P. citricola	**AY564055**	**AY564111**	**AY564170**	**AY563997**	ND
P. citrophthora	**AY564056**	**AY564112**	**AY564171**	**AY563998**	ND
P. clandestina	**AY564057**	**AY564113**	**AY564172**	**AY563999**	ND
P. colocasiae	**AY564058**	**AY564114**	**AY564173**	**AY564000**	ND
P. cryptogea	**AY564059**	**AY564115**	**AY564174**	**AY564001**	ND
P. drechsleri	**AY564060**	**AY564116**	**AY564175**	**AY564002**	ND
P. erythroseptica	**AY564061**	**AY564117**	**AY564176**	**AY564003**	ND
P. fragariae var. *fragariae* I	**AY564062**	**AY564118**	**AY564177**	**AY564004**	ND
P. fragariae var. *fragariae* II	**AY564063**	**AY564119**	**AY564178**	**AY564005**	ND
P. fragariae var. *rubi* I	**AY564064**	**AY564120**	**AY564179**	**AY564006**	ND
P. fragariae var. *rubi* II	**AY564065**	**AY564121**	**AY564180**	**AY564007**	ND
P. gonapodyides	**AY564066**	**AY564122**	**AY564181**	**AY564008**	ND
P. heveae	**AY564067**	**AY564123**	**AY564182**	**AY564009**	ND
P. hibernalis	**AY564068**	**AY564124**	**AY564183**	**AY564010**	ND
P. humicola	**AY564069**	**AY564125**	**AY564184**	**AY564011**	ND
P. idaei	**AY564070**	**AY564126**	**AY564185**	**AY564012**	ND
P. ilicis	**AY564071**	**AY564127**	**AY564186**	**AY564013**	ND
P. inflata	**AY564072**	**AY564128**	**AY564187**	**AY564014**	ND
P. insolita	**AY564073**	**AY564129**	**AY564188**	**AY564015**	ND
P. iranica	**AY564074**	**AY564130**	**AY564189**	**AY564016**	ND
P. katsurae	**AY564075**	**AY564131**	**AY564190**	**AY564017**	ND
P. lateralis	**AY564076**	**AY564132**	**AY564191**	**AY564018**	ND
P. meadii	**AY564077**	**AY564133**	**AY564192**	**AY564019**	ND
P. megakarya	**AY564078**	**AY564134**	**AY564193**	**AY564020**	ND
P. megasperma	**AY564079**	**AY564135**	**AY564194**	**AY564021**	ND
P. multivesiculata	**AY564080**	**AY564136**	**AY564195**	**AY564022**	ND
P. nicotianae	**AY564081**	**AY564137**	**AY564196**	**AY564023**	ND
P. palmivora	**AY564082**	**AY564138**	**AY564197**	**AY564024**	ND
P. brassicae	**AY564083**	**AY564139**	**AY564198**	**AY564025**	ND
P. pseudotsugae	**AY564084**	**AY564140**	**AY564199**	**AY564026**	ND
P. quininea	**AY564085**	**AY564141**	**AY564200**	**AY564027**	ND
P. richardiae	**AY564086**	**AY564142**	**AY564201**	**AY564028**	ND
P. sinensis	**AY564087**	**AY564143**	**AY564202**	**AY564029**	ND
P. sojae	**AY564047**	**AY564104**	**AY564162**	**AY563989**	**AY564221**
P. syringae	**AY564088**	**AY564144**	**AY564203**	**AY564030**	ND
P. tentaculata	**AY564089**	**AY564145**	**AY564204**	**AY564031**	ND
P. vignae	**AY564090**	**AY564146**	**AY564205**	**AY564032**	ND
P. sp. Spathiphyllum	**AY564091**	**AY564147**	**AY564206**	**AY564033**	ND
P. ramorum US-type	ND	**AY564148**	**AY564207**	ND	ND
P. ramorum European-type	**AY564092**	**AY564149**	**AY564208**	**AY564034**	ND
Pythium aphanidermatum	**AY564048**	ND	**AY564163**	**AY563990**	ND

[a] ND, not determined.

Table 4. Properties of taxa within *Phytophthora* clades 1-8 as identified in this study [and correlating as much as possible with the clade numbering by Cooke et al. (2000)]

Clade	Species	Group[a]	Antheridia[b]	Papillae[c]	Mating[d]	Niche[e]
1a	*P. cactorum*	I	P	P	Ho	fol/soil
1a	*P. idaei*	I	P	P	Ho	soil
1a	*P. pseudotsugae*	I	P	P	Ho	soil
1b	*P. clandestina*	I	AP	P	Ho	soil
1b	*P. iranica*	I	P	P	Ho	soil
1c	*P. infestans*	IV	A	S	He	foliage
1c	*P. andina*	…	A	S	He	foliage
1c	*P. ipomoeae*	…	A	S	Ho	foliage
1c	*P. mirabilis*	IV	A	S	He	foliage
1c	*P. phaseoli*	IV	A	S	Ho	foliage
1d	*P. nicotianae*	II	A	P	He	fol/soil
2a	*P. citrophthora*	II	A	P	He	soil
2a	*P. colocasiae*	IV	A	S	He	foliage
2a	*P. meadii*	II	A	P	He/Ho	foliage
2a	*P. tropicalis*	…	A	P	He	foliage
2a	*P. citricola*	III	P	S	Ho	soil
2a	*P. inflata*	III	P	S	Ho	soil
2b	*P. multivesiculata*	…	A	S	Ho	foliage
2b	*P. tentaculata*	I	P	P	Ho	foliage
3	*P. ilicis*	IV	A	S	Ho	foliage
4	*P. botryosa*	II	A	P	He	foliage
4	*P. palmivora*	II	A	P	He	foliage
4	*P. arecae*	II	A	P	He	foliage
4	*P. megakarya*	II	A	P	He	foliage
5	*P. heveae*	II	A	P	Ho	foliage
5	*P. katsurae*	VI	A	P	Ho	soil
6	*P. humicola*	V	P	N	Ho	soil
6	*P. gonapodyides*	VI	A	N	He	soil
6	*P. megasperma*	V	P	N	Ho	soil
7a	*P. fragariae* var. *fragariae*	V	A	N	Ho	soil
7a	*P. fragariae* var. *rubi*	V	A	N	Ho	soil
7a	*P.* hybrid-Dutch variant	…	AP	N	Ho	soil
7b	*P. sojae*	V	P	N	Ho	soil
7b	*P. sp*. Spathiphyllum	…	ND[f]	ND	ND	ND
7b	*P. sinensis*	V	A	N	Ho	foliage
7b	*P. vignae*	VI	A	N	Ho	soil
7c	*P. cinnamomi*	VI	A	N	He	soil
8a	*P. cryptogea*	VI	A	N	He	soil
8a	*P. erythroseptica*	VI	A	N	Ho	soil
8a	*P. drechsleri*	VI	A	N	He	soil
8b	*P. syringae*	III	P	S	Ho	soil
8b	*P. brassicae*	…	A	S	Ho	soil
8c	*P. hibernalis*	IV	A	S	Ho	fol/soil
8d	*P. lateralis*	V	P	N	Ho	soil
8d	*P. ramorum* European-type	…	A	S	He	foliage
8d	*P. ramorum* US-type	…	A	S	He	foliage
8e	*P. quininea*	V	P	N	Ho	soil
8e	*P. richardiae*	VI	A	N	Ho	soil
8f	*P. boehmeriae*	II	A	P	Ho	fol/soil
8f	*P. insolita*	V	ND	N	Ho	soil

[a] According to Waterhouse (1963), [b] P = paragynous attachment of the antheridium to the oogonium; A = amphigynous attachment, [c] P = papillate sporangia; S = semi-papillate sporangia; N = non-papillate sporangia, [d] Ho = homothallic species; He = heterothallic species, [e] niche = niche in which the pathogen is commonly found, [f] ND = Not Determined.

Results

Phylogenetic analysis of nDNA and mtDNA data sets

The combined nuclear DNA data set comprised 1874 characters for 61 accessions, which included 58 *Phytophthora* accessions, two *Pythium* accessions, and *Achlya klebsiana* as an outgroup. For *EF*-1α 57 accessions were represented, for β-*tub* 60, and for the two genes combined 61. The combined nuclear DNA data set contained 411 potentially phylogenetically informative characters.

The combined mitochondrial DNA data set comprised 2952 characters for 63 accessions, including 59 *Phytophthora* accessions, *Pythium aphanidermatum*, *Fragilaria striatula* (Fragilariophyceae), *Ophiocytium majus* (Xantophyceae), and *Pylaiella littoralis* (Phaeophyceae); the latter three were used as outgroup. For the P2 region, 14 accessions were represented, for *cox*1 63, for *nadh*1 59, and for all mtDNA regions combined 63. The combined mtDNA data set contained 511 characters that were potentially phylogenetically informative.

Jackknife analyses of the separate mitochondrial and separate nuclear gene data sets resulted in largely congruent topologies (data not shown). Only *P. meadii*, *P. heveae*, and *P. citricola* were located on unexpected branches based on *cox*1, *P. katsurae* and *P. hibernalis* based on *nadh*1, and *P. insolita* in the analysis of the *EF*-1α region. However, as omitting these six taxa from the combined nuclear and mitochondrial data set did not alter the resulting overall tree topology (data not shown), we opted to combine the mitochondrial genes and the nuclear genes into two data sets and to include the six taxa *P. meadii*, *P. heveae*, *P. citricola*, *P. katsurae*, *P. hibernalis*, and *P. insolita* in our analysis.

Analyses of these combined mitochondrial and combined nuclear data sets were carried out in order to assess congruence between the resultant phylogenetic trees. Jackknife consensus trees from the combined nDNA and combined mtDNA analyses are shown in Figure 1A and 1B. The nDNA based jackknife topology contains at least four main (sub)clades (1, 2a, 7a, and 7b) that also occur in the mtDNA based topology. Incongruence between the phylogenetic placement in the mitochondrial and nuclear sequence based trees was evident in six species (underlined in Figure 1) at the 63% jackknife level. A poor resolution is evident at the basal nodes of both the nuclear and mitochondrial trees. The heuristic search for the nDNA data set yielded 27 most parsimonious trees (MPT) of 1957 steps long which were distributed on a single tree island (CI = 0.38, RI = 0.70).

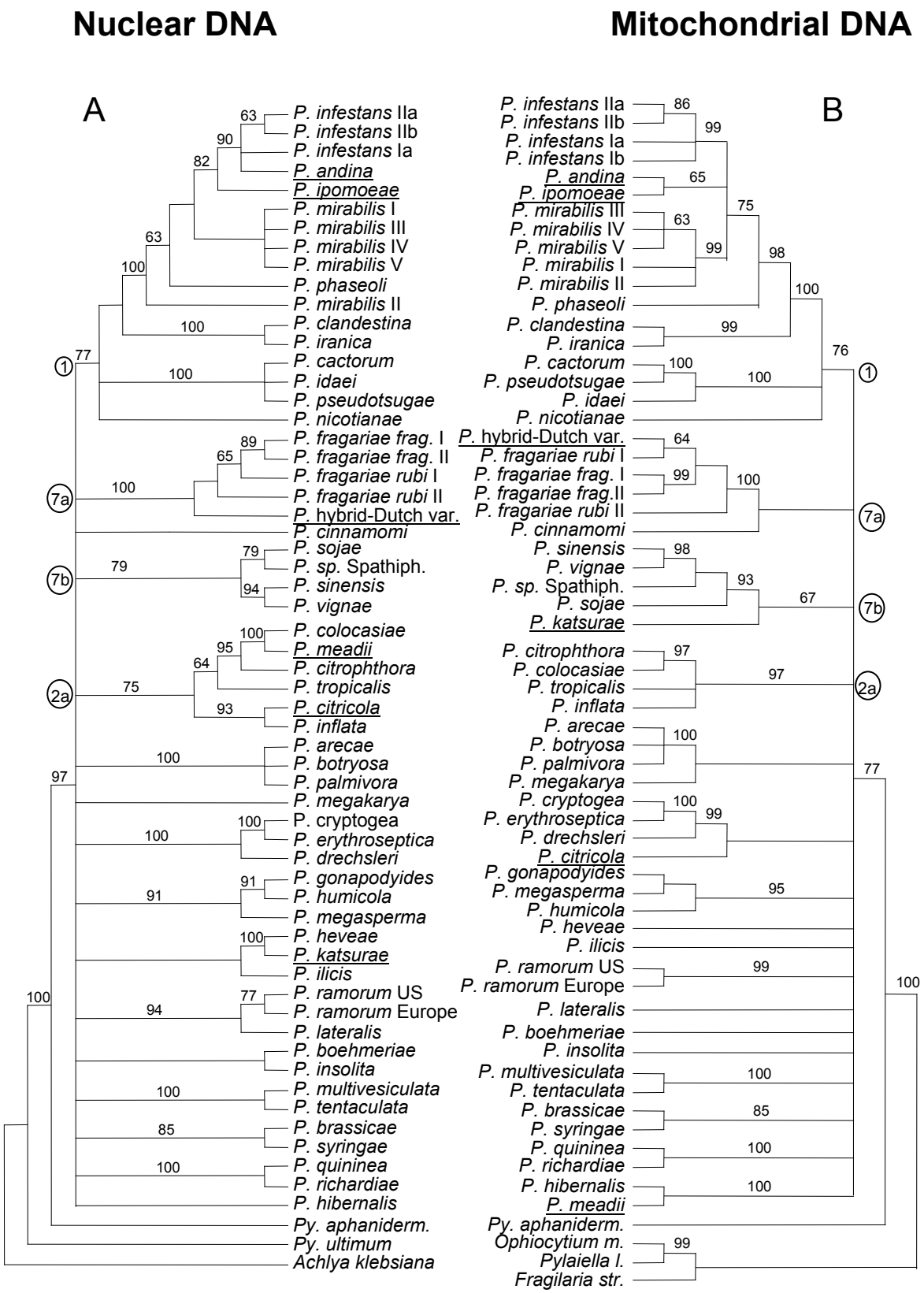

Figure 1. Nuclear and mitochondrial DNA sequence data compared: jackknife consensus trees (10,000 replicates) of combined nuclear encoded genes (ß-*tubulin* + *EF*-1α; A) and combined mtDNA encoded genes and spacer sequences (B). Numbers on branches indicate jackknife frequencies, underlined names indicate incongruently placed taxa at >63% jackknife support level. Outgroup taxa *Pythium ultimum* and *Achlya klebsiana* were not available for the mtDNA data set, whereas *Ophiocytium*, *Pylaiella* and *Fragilaria* were not included in the nuclear data set. Numbers indicate the largest supported (sub)clades.

The strict consensus tree topology of these 27 MPTs contains 32 nodes with >63% jackknife support (Figure 2A).

The mtDNA based jackknife topology contains less clades with >63% jackknife support as compared to the nuclear DNA based jackknife topology (12 vs. 14 supported (sub)clades). Heuristic search of the combined mtDNA data set yielded 22 most parsimonious trees of 2572 steps long, distributed on a single tree island (CI = 0.44, RI = 0.63). The strict consensus tree topology of these 22 MPTs contains 32 nodes with >63% jackknife support (Figure 2B).

Combined data sets

The pooled nDNA and mtDNA sequences were analyzed using the same jackknife and heuristic search settings as described above. The combined nuclear + mtDNA data set comprised 4826 characters for 63 accessions, including 59 *Phytophthora* accessions, *Pythium aphanidermatum*, *Fragilaria striatula, Ophiocytium majus*, and *Pylaiella littoralis*. The latter three were used as outgroup. *Achlya klebsiana* and *Pythium ultimum* were excluded to avoid potential long branch-attraction artifacts.

The spine of the combined tree again lacked well-supported nodes (Figure 3A), but the overall number of supported nodes (35) was greater than for the trees based on nDNA (32 nodes) and mtDNA (32 nodes) alone. The heuristic search yielded 6 MPTs of 4459 steps long, again, all situated on one single island (CI = 0.41, RI = 0.66). The differences between these six MPTs were minimal, involving only minor shifts in the placement of *P. nicotianae* and *P. infestans* type Ib. In the strict consensus tree of the 6 MPTs based on the combined data (Figure 3A) the six taxa demonstrating incongruence between the nuclear and mtDNA data (Figure 1 and 2) were grouped broadly in line with the nuclear data. Finally, branch lengths were estimated for the MPT with the highest likelihood (Figure 3B), on the basis of the 9-parameter model (GTR+I+Γ) in PAUP*. Prior to branch length optimization, outgroups were pruned in order to avoid error in branch lengths due to long branch-attraction artifacts.

Main clades

Based upon the strict consensus tree shown in Figure 3A, and in comparison with the clade nomenclature proposed by Cooke et al. (2000), 8 main clades were identified in the genus *Phytophthora* (Table 4). Clade 1, 2, 7, and 8 were (arbitrarily) divided into sub-clades,

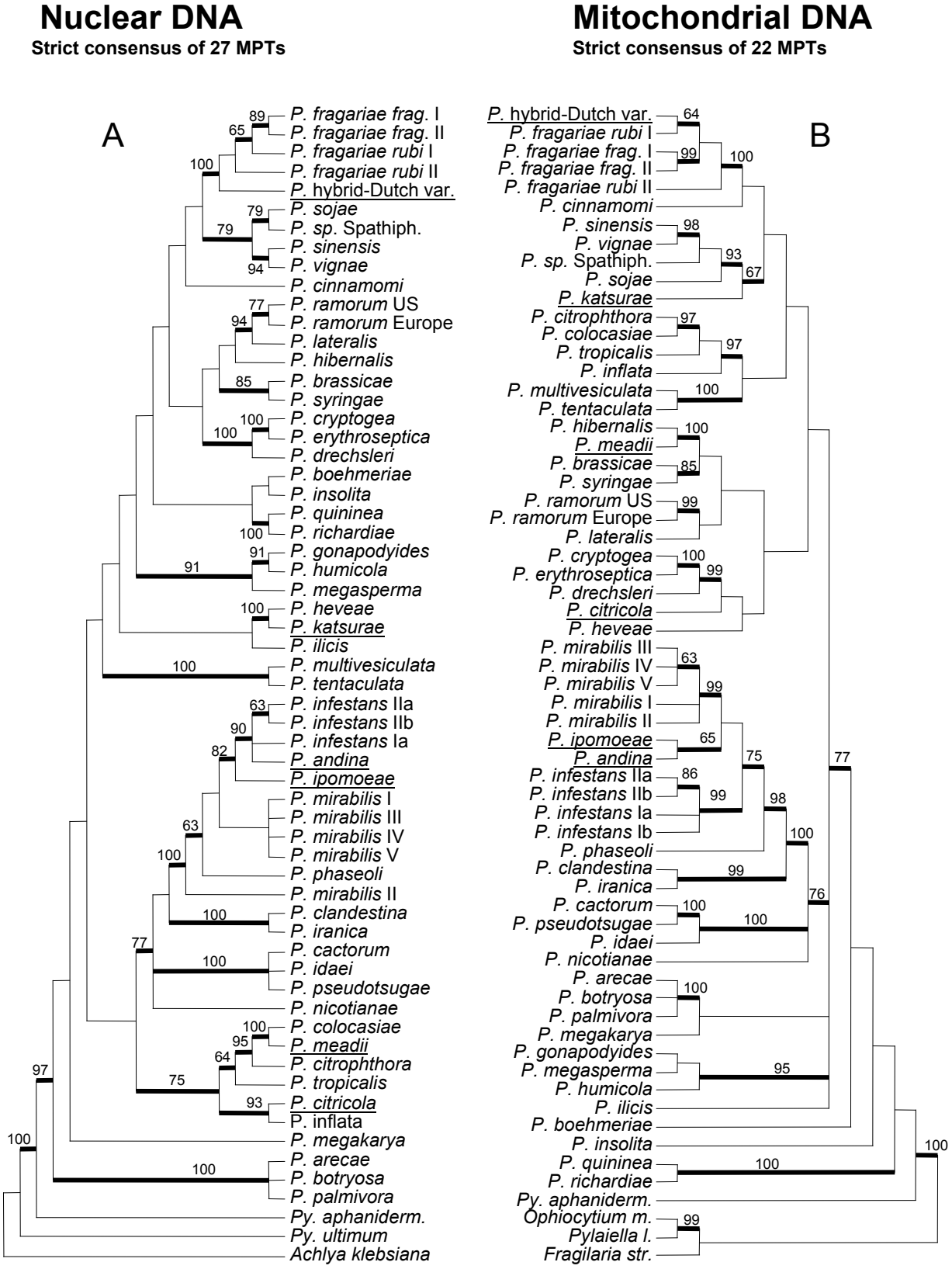

Figure 2. Nuclear and mitochondrial DNA sequence data compared: strict consensus trees of 27 most parsimonious trees calculated from the combined nuclear DNA data set (A) and of 22 MPTs calculated from the combined mtDNA data set (B). Numbers on branches indicate jackknife frequencies, thick lines indicate significantly supported branches (>63% jackknife) and underlined names indicate incongruently placed taxa at >63% jackknife support level.

Nuclear and mitochondrial genes (and spacers) combined

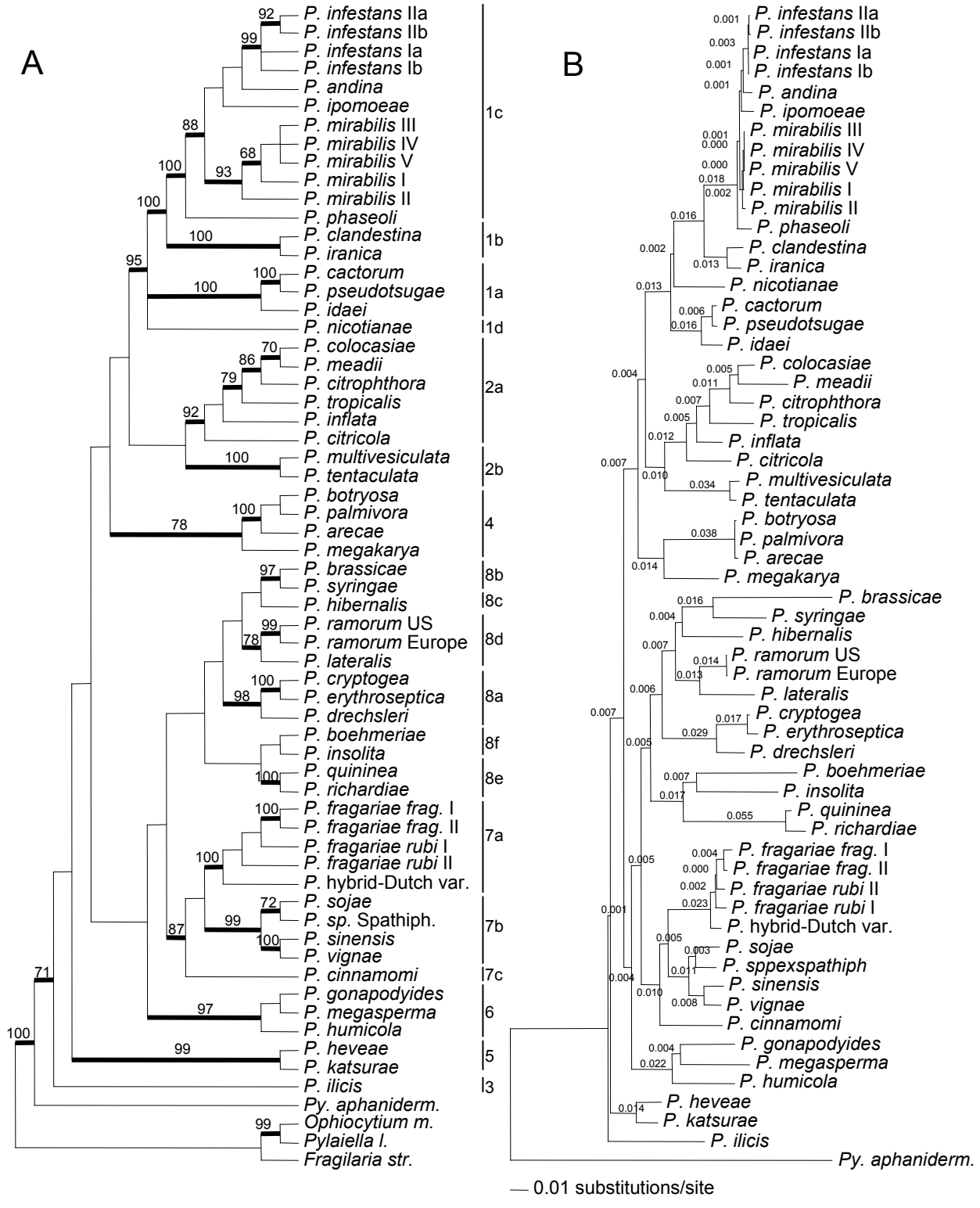

Figure 3. Nuclear and mitochondrial DNA sequence data combined: strict consensus tree (A) of 6 most parsimonious trees with numbers on branches indicating jackknife frequencies; thick lines indicate significantly supported branches (>63% jackknife). Bars indicate the (sub)clades as described in Table 2; numbers refer to clades as identified in this study [and correlating as much as possible with clade numbering by Cook et al., 2000]. (B) One of the 6 MPTs (the one with greatest overall likelihood) with non-Pythiacean outgroups pruned, and with branch lengths optimized based on a 9 parameter model (see text); numbers on branches indicate the number of substitutions per site. Note that *Pythium ultimum* and *Achlya klebsiana* were excluded in order to avoid long-branch attraction artifacts.

as within these clades significantly (jackknife) supported clusters of *Phytophthora* species could be identified.

P. nicotianae was less closely related to other taxa in clade 1, joining at a basal position, and therefore defined as sub-clade 1d. Character traits also supported this separation (Table 4). In this study, *P. tentaculata* was consistently shown to be a sister taxa to *P. multivesiculata* in sub-clade 2b, which differed from the ITS-based study (Cooke et al., 2000) in which *P. tentaculata* was shown to share a common ancestor with clade 1 taxa.

Ambiguity in the relatedness of *P. meadii* and *P. citricola* (Figure 1 and 2) to other clade 2 taxa resulted from unusually low sequence similarity in the *cox*1 gene compared to all other genes. The consensus tree, however suggests they share a common ancestor with all clade 2 species. The species *P. botryosa* was not included in clade 2 (in which it was placed in Cooke's analysis), since it showed high similarity with *P. arecae* and *P. palmivora*.

P. botryosa, P. arecae, and *P. palmivora* were closely related and alongside their nearest relative, *P. megakarya*, were included in clade 4. Despite the lack of jackknife support for including *P. megakarya* in clade 4 in the separate nDNA and mtDNA analyses it was supported by their character states (Table 4) and by the combined phylogenetic analysis (Figure 3A).

Clade 3, which consisted of *P. ilicis* and *P. quercina* in Cooke's analysis, was marked by low bootstrap support and was diverse in character states for the ITS phylogeny. As *P. quercina* was not included in this study and *P. ilicis* was included in no other clade in our combined analysis, clade 3 *sensu* Cooke et al. (2000) was retained.

There is strong support for the close relationship of *P. heveae* and *P. katsurae* in clade 5 of our analysis of nuclear gene sequences, but on the basis of mtDNA data, the sister taxa of *P. katsurae* are the non-papillate species in clade 7 (Figure 1b and 2b). The composition of clade 6 is identical in both studies, with high jackknife support for nDNA and mtDNA data.

In the well-supported clade 7, *P. cinnamomi* joins at a basal position and has thus been nominated clade 7c, because there was no support for including it in either sub-clade 7a or 7b. *P.* hybrid-Dutch variant from alder trees (Brasier et al., 1999) clusters with *P. fragariae* isolates (clade 7a) and the isolate found on *Spathiphyllum* spp. was most closely related to taxa in sub-clade 7b.

There is considerable diversity amongst the taxa in clade 8 and only some of its sub-clades have significant jackknife support. *P. cryptogea*, *P. erythroseptica*, and *P. drechsleri* form clade 8a. In clade 8b, the newly described species *P. brassicae* (Man in 't Veld et al., 2002) is clustered with *P. syringae*. The Sudden Oak Death pathogen *P. ramorum* (Werres et

al., 2001b) clusters closely with *P. lateralis* and these species are included in clade 8d. *P. quininea* and *P. richardiae* together form clade 8e, and *P. boehmeriae* and *P. insolita* form clade 8f; note that the last two sub-clades constitute a basal grade in the analysis of the mtDNA data set (Figure 2B), however without support.

P. infestans and closely related species

The intraspecific variation in the clade 1c taxa, *P. infestans* and *P. mirabilis* was in general lower than the interspecific variation. An exception, however, was the *P. mirabilis* isolate P3001 (*P. mirabilis* II in Figure 2A) in which the ß-*tubulin* gene sequence differed markedly from the other *P. mirabilis* isolates. *P. phaseoli* shares the most distant common ancestor with the rest of the clade (Figure 3A), based on both nDNA and mtDNA analyses. *P. ipomoeae* and *P. andina* are related to both *P. infestans* and *P. mirabilis*, showing closer sequence similarity to *P. infestans* in the case of nDNA and to *P. mirabilis* in the case of mtDNA (Figure 1 and 2).

Association between functional traits and phylogeny

Waterhouse (1963) assigned *Phytophthora* species to six groups, based on morphological characteristics. In order to re-assess the validity of this classification, the combined nDNA + mtDNA strict consensus tree topology was used to explore possible correlations between functional characteristics and phylogeny (Figure 4A-C). Functional characteristics (syndromes) were expressed as multi-state 'characters' and optimized on the cladogram, enabling inference of evolutionary patterns.

Discussion

Our analysis provides the first multi-gene based phylogeny of a broad range of *Phytophthora* species. We propose a revision (as presented in Table 4) of the classification of *Phytophthora* species, based on our combined nDNA and mtDNA sequence based phylogeny, as well as on morphological traits and niche preference. The traditional classification, based on morphological traits and growth characteristics, has several limitations. Growth characteristics (like colony morphology and optimal growth temperature) are not unequivocal and traits depend on the method used for measurement (e.g. the effect of growth media or host

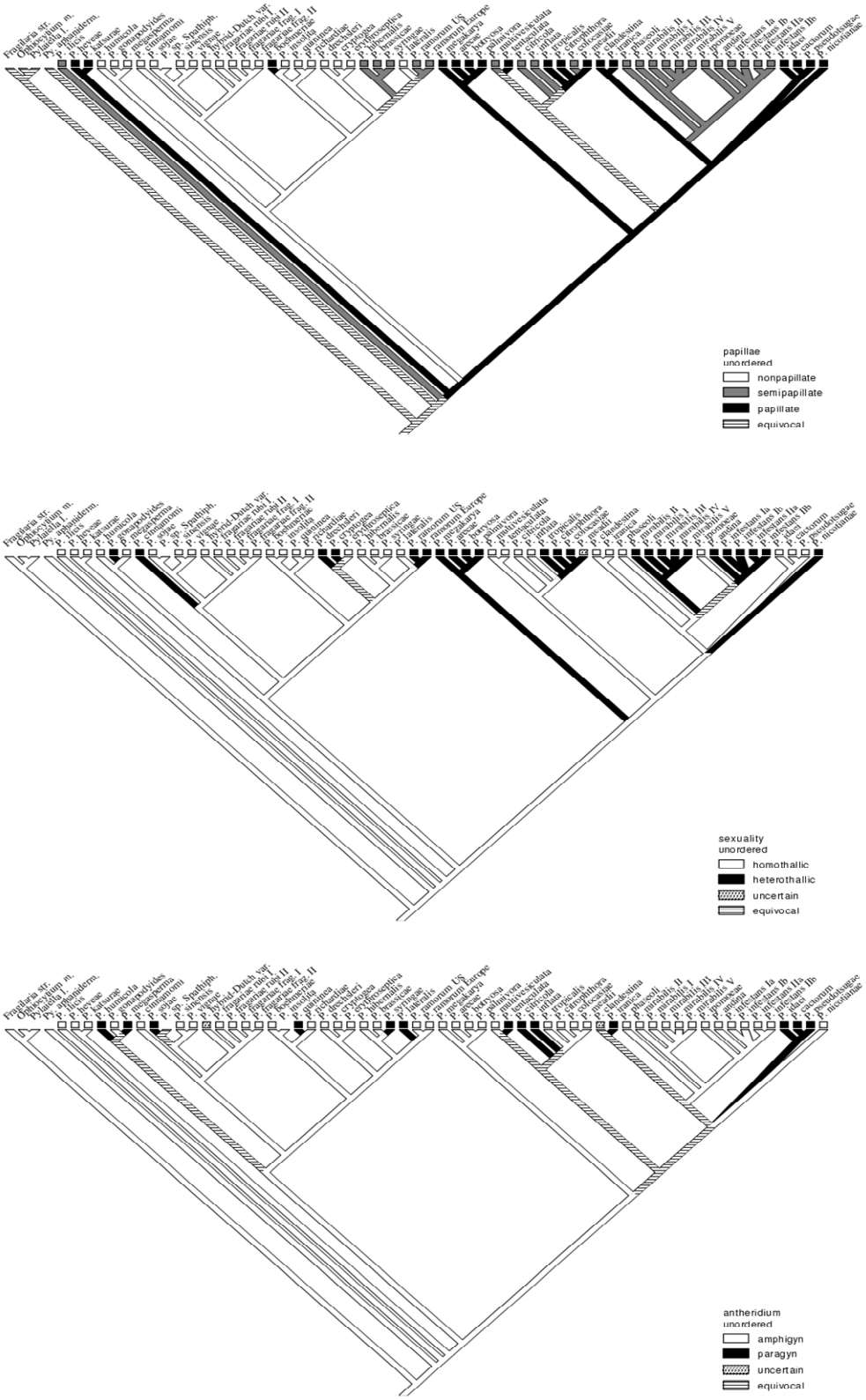

Figure 4. (A-C) Strict consensus tree of nuclear + mtDNA sequence data sets combined (the same topology as in Figure 3) and traced with character syndromes.

tissue on oospore size), or may vary because of ambiguity in trait description by observers (e.g. papillate vs. semi-papillate). The problems associated with assigning isolates to either *P. drechsleri* or *P. cryptogea* exemplify this situation (Erwin and Ribeiro, 1996; Forster et al., 2000). Additionally, growth characteristics and morphological traits are phenotypic, and groups of species sharing similar traits do not necessarily reflect evolutionary relatedness, since they may have evolved independently (convergent evolution).

In our study, we found some incongruence between phylogenies for nuclear and mitochondrial DNA. Sexual hybridization of related (Brasier et al., 1999) and unrelated (Man in 't Veld et al., 1998) species has been reported. Such hybridization events and rapid evolution within daughter species will likely confound phylogenetic analysis and a branched tree may not always be the optimal way to present such phylogenetic relationships. It is only by studying phylogenetic reconstructions based on multiple mitochondrial and nuclear genes that such relationships may be revealed. In case of recurrent hybridization events and nested species radiation, reticulate evolution may provide a more realistic explanation of these relationships (Koch et al., 2003).

The phenomena encountered in the study of *Phytophthora* hybrids in alder (Brasier et al., 1999) are exemplary of how hybridization events might influence phylogenetic inference. In these hybrids, two species are considered to have fused, resulting in tetraploid (allodiploid) offspring. The karyotype of the offspring was however not stable, as complete chromosomes were lost in subsequent meiosis, resulting in a heteroploid hybrid swarm (Delcan and Brasier, 2001). Crossing-over events between homeologous chromosomes in meiosis may result in hybrid gene fragments and either parent may contribute its mitochondrial DNA. All of these processes will affect the consistency of phylogenetic analyses over different genes or genomes for species that have been involved in hybridization events (Posada and Crandall, 2002; Sang and Zhong, 2000). These processes may be responsible for the observed incongruity in the classification of *P. katsurae*. The placement of *P. megasperma* in clade 2b instead of clade 6 for the β-*tubulin* sequence analysis might be another illustration of reticulate evolution. In both nuclear and mitochondrial cladograms, clade 2b (*P. multivesiculata* and *P. tentaculata*) had 100% jackknife support, while in ITS studies (Cooke et al., 2000), using identical isolates, these species were clearly placed in distinct clades. This may be another example of reticulate evolution.

For other sequence results, the situation is more difficult to explain by reticulation alone. For example, *P. meadii* and *P. citricola* appear to share a common ancestor in clade 2a on the basis of β-*tubulin*, *nadh*1, and *EF*-1α regions, however, the *cox*1 data groups *P. meadii*

with *P. hibernalis* (clade 8c) and *P. citricola* with clade 8a taxa. For *nadh*1, *P. hibernalis* clustered with *P. nicotianae*, and *P. katsurae* had 100% homology with the isolate from *Spathiphyllum*. Sansome et al. postulated that *P. meadii* might be a hybrid species, based on variability and instability in chromosome structure, observed in meiosis (Sansome et al., 1991). This observation could explain for the inconsistencies in the classification of *P. meadii* in our phylogenetic analysis. A group of *P. meadii* isolates was found to be tetraploid, presumably allopolyploid. Several *Phytophthora* species were mentioned by Sansome et al. as potential parental species for these hybrid isolates: *P. palmivora*, *P. capsici*, *P. botryosa*, and *P. heveae*. Our sequence results indicate that *P. colocasiae* is a more likely candidate, moreover because this species also occurs on rubber (*Hevea brasiliensis*) in south-east Asia (Ho et al., 1984). Based on homology in the *cox*1 region, *P. hibernalis* could be the other parental species involved in the formation of this hybrid species. Isozyme analysis and DNA fingerprinting experiments may give additional information on the origin of the *P. meadii* hybrids.

An explanation for the observed inconsistencies in the classification of *P. meadii* and *P. citricola* could be provided by the process of recombination in mitochondrial DNA (Hagelberg, 2003; Lössl et al., 1999; Maynard Smith and Smith, 2002; McVean, 2001; Staedler and Delph, 2002). In sexually reproducing organisms, paternal mitochondria are mostly restricted from entering the ovule. There is, however, some indication of "leakage" of paternal mitochondria, which can result in contact between maternal and paternal mtDNA and recombination events between these two mitochondrial types. For *Phytophthora* hybrids, the occurrence of parental leakage might even be more common than in other organisms; if hybrids arise from cell fusion of parental isolates (e.g. fusion of zoospores (Bakonyi et al., 2002; English et al., 1999; Ersek et al., 1995)), it is likely that there are no restrictions for contact and subsequent recombination of mtDNA. Mitochondrial DNA recombination may provide a more satisfying explanation for the observed anomalous mutation patterns as compared with homoplasy or mutation hotspots. In addition, translocated pieces of mtDNA present in the nuclear genome may be mistaken for authentic organellar mtDNA. These sequences are highly similar to the original mtDNA sequences, and may confound the phylogenetic analysis because they have a different phylogenetic history compared with those in the mtDNA (Thalmann et al., 2004).

Some species exhibit such high sequence similarity that the separation into distinct species could be questioned. In this study, data are mainly based on single isolates, so additional sequencing is needed to resolve the presence of species that can be regarded as

synonyms. For *P. arecae* and *P. palmivora*, the situation is more clear; our sequencing data confirm the proposition of Oudemans and Coffey (1991), McHau and Coffey (1994), and Martin and Tooley (2003) that *P. arecae* is not a distinct species, but should be regarded as a synonym of *P. palmivora*.

The taxonomic status of *P. botryosa* remains uncertain; this species was placed in clade 2 using ITS sequences (Cooke), but in our study it was placed in clade 4. In both studies, support for the grouping was high, but it should be noted that the isolates of *P. botryosa* used in each study differed. Additional research should clarify the taxonomic status of *P. botryosa*.

Cooke et al. (2000), based on Neighbor Joining analysis of ITS data, described two additional clades comprising *P. macrochlamydospora* and *P. richardiae* ('clade 9') and *P. insolita* ('clade 10'). These two clades were placed separately from the other eight *Phytophthora* clades in their analysis, supporting Cooke's theory of *Phytophthora* being paraphyletic. This pattern is also visible in our separate analysis of the mtDNA data set (Figure 2B). However, in our nDNA analysis (Figure 2A), as well as in the analysis of the combined data set (Figure 3A), *P. insolita* and *P. richardiae* are located within the main *Phytophthora* clade. Thus, *P. insolita* and *P. richardiae* are grouped in clade 8 in our study rather than placing them in the small separate clades 9 and 10. Since all species under study form one well-supported clade (Figure 2A and 3A), we postulate *Phytophthora* to be monophyletic.

The occurrence of two separate lines of evolution, one towards non-papillate species specializing in pathogenesis of root systems and tree trunks, the other towards (semi-) papillate species attacking the foliage of host plants, is clearly visible in the functional trait analysis (Table 3 and Figure 4A). This observation has been made previously by Brasier (1983) and Cooke et al. (2000). Clades 6, 7 and 8 form a distinct branch in the cladogram, comprising only non- and semi-papillate species, which are predominately soil-borne.

Mating system was inferred to be a homoplasious trait (Figure 4B), i.e., the observed similarity is based on at least eight independent transitions from homothallism to heterothallism. Homothally was reconstructed on our phylogeny as the ancestral condition. At least three indications of secondary homothallics (Brasier, 1983) are present in our data set: *P. ipomoeae* in clade 1c, the homothallic isolates of *P. meadii* in clade 2, and *P. erythroseptica* in clade 8a. The species *P. phaseoli* in clade 1c might be another example of secondary homothallism in the genus *Phytophthora* (Cooke et al., 2000). These results indicate that the switch from homothallism to heterothallism, and *vice versa*, has occurred

quite often in the genus *Phytophthora*. This observation brings up the question of how the underlying genetic mechanism of the switch from homothallism to heterothallism (and *vice versa*) operates.

Comparisons with well studied fungal mating systems (Casselton, 2002; Coppin et al., 1997; Metzenberg and Glass, 1990; Pöggeler, 1999; Yun et al., 1999) may be worthwhile in examining the patterns of inheritance and regulation of homo- and heterothallism in *Phytophthora* (Randall et al., 2003).

For antheridial attachment to the oogonium, it can be concluded that amphigyny is likely to be the ancestral state. The transition from amphigynous to paragynous attachment of the antheridium to the oogonium (Figure 4C) has occurred independently throughout the tree. All heterothallic species have an amphigynous attachment of the antheridium (Brasier, 1983; Cooke et al., 2000).

In this study we utilize DNA sequences from nuclear and mitochondrial origin as sources of phylogenetic markers in order to obtain a phylogenetic hypothesis for *Phytophthora* that reconstructs the evolutionary pathways as accurately as possible. Using nuclear and mitochondrial encoded sequences offers the advantage of independently evolving sets of characters in phylogenetic reconstruction. This has resulted in a robust phylogeny which is largely in congruence with the phylogenies described by Cooke et al. (2000) and Martin and Tooley (2003). As we base our phylogenetic tree on a significantly larger data set (900 bp [ITS, Cooke et al., 2000] versus 1468 bp [*Cox*II & ITS, Martin and Tooley, 2003] versus 4826 bp [our study]) we consider our phylogenetic tree as best possible estimate of *Phytophthora* phylogeny published thus far. In the light of the discussion on the applicability of ITS-regions in phylogenetic inference in angiosperms (e.g. Álvarez and Wendel, 2003; Bailey et al., 2003), we strove to conduct phylogenetic analysis without the use of ITS-regions. However, the ITS-based topologies of Cooke et al. (2000) and Martin & Tooley (2003) are in line with the results we obtained, indicating that the use of ITS-sequences in phylogenetic inference of the genus *Phytophthora* is appropriate.

For *P. infestans* and the closely related species *P. mirabilis*, *P. ipomoeae*, *P. phaseoli*, and *P. andina*, which comprise clade 1c in our analysis, ITS-sequence similarity was very high (99.9%). The phylogenetic relations between these five species could be further resolved in the analysis of the combined nDNA and mtDNA data set. The position of *P. andina*, placed intermediately between *P. infestans* and *P. mirabilis*, is surprising. This species is found in Ecuador, thousands of miles from the presumed center of origin of *P. infestans*, *P. mirabilis*, and *P. ipomoeae*, the Toluca Valley in central Mexico. The latter three species may have

undergone sympatric speciation in this valley (Flier et al., 2002; 2003; Goodwin et al., 1999) and they presumably originate from a single ancestor. The findings of this new intermediate species *P. andina* in Ecuador brings up new questions on the migration of clade 1c species throughout Central and South America during history. Ambiguous gene sequence patterns for nDNA regions in *P. andina* could indicate a hybrid origin of this species, wherein *P. infestans* is one of the parental species.

The data set resulting from this study should serve as a framework for future classifications of *Phytophthora* species. As more sequence data will become available, phylogenetic relationships will become more resolved, and intra-species variation can be measured. Molecular phylogeny will also serve as a validation tool in the process of describing a new species. A third application of this data set exploits sequence polymorphisms for designing rapid diagnostic tests. Based on a sequence difference in the *cox*1 region of *P. ramorum*, an SNP-assay was recently designed to distinguish between European and American isolates of this pathogen, assisting Plant Health authorities in preventing spread and contact of the European and American sub-populations (Kroon et al., 2004b).

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2.3

The *Phytophthora* genus anno 2010

An overview of new *Phytophthora* species and their position in the *Phytophthora* phylogeny

Chapters 2.1 and 2.3 will be submitted for publication

This chapter gives an overview of the ten clades that are currently distinguished within the genus *Phytophthora*. It includes the species presented by Erwin and Ribeiro (1996) and a large number of new species that have been described in the scientific literature since 1996. Some new species are presented that have no official species description as yet and are included as *Phytophthora* species (e.g. *P.* sp. *alticola*, *P.* sp. Salixsoil) where the suffix describes the putative species name, or the host or environment in which the isolates were found. Per clade, the most notable characteristics for each new species are covered. Some clades are divided into subclades indicating close relationships between species within a subclade. For most species the Waterhouse groups are included, linking the molecular data to phenotypic data.

Clade 1

Clade 1 is a well-studied clade; it contains the most widely known species of *Phytophthora*: *P. infestans*, next to 12 other species. Since 1996 (Erwin and Ribeiro) three new species have been added to this clade, one of which is *P. andina* in clade 1c, a close relative of *P. infestans* and described in detail in chapter 3.3 of this thesis. Another new species that is a close associate of *P. infestans* is *P. ipomoeae* (Flier et al., 2002), which was found in the Toluca valley in central Mexico, a centre of diversity of *P. infestans* and *P. mirabilis*. The third new species is *P. hedraiandra,* a pathogen of *Viburnum* shrubs, that is provisionally positioned in clade 1a (de Cock and Levesque, 2004). *P. hedraiandra,* however, differs from the other species in this subclade in *cox*1 and ITS sequences. The origin of this discrepancy was recently resolved when species hybrids were discovered that seem to be the offspring of *P. hedraiandra* and *P. cactorum* (Man in 't Veld et al., 2007). Hybrid offspring could be mistaken for *P. hedraiandra* isolates, or gene-flow between *P. cactorum* and *P. hedraiandra* could be the source of these discrepancies in sequence homology.

Clade 1 contains *Phytophthora* species that are papillate or semi-papillate, with only one type present in each subclade. The zoosporangia of species in clades 1a and 1b are papillate, the attachment of antheridia to the oogonia is paragynous, and the pathogens mainly infect the roots of their host plants. The zoosporangia of species in clade 1c are semi-papillate, have amphigynous antheridia, and are foliar pathogens. *Phytophthora nicotianae* is singular in this clade; it could not be placed in one of the subclades of clade 1 based on sequence analysis and it has amphigynous antheridia and papillate sporangia.

Table 1. Clade 1 *Phytophthora* species

Phytophthora sp.	group								Origins	
	clade	WH[a]	study[b]	Host[c]	Infected tissue[d]	sex[e]	A/P[f]	papill.[g] [h]	author[i]	year[j]
P. nicotianae	1	II	ECKB	*Nicotiana tabacum*	roots/foliage	He	A	P M	Breda de Haan	1896
P. cactorum	1a	I	ECKB	*Rubus idaeus*	roots/foliage	Ho	P	P M	Schröeter	1886
P. hedraiandra*	1a	I	B	*Viburnum* spp.	foliage	Ho	P	P M	De Cock	2004
P. idaei	1a	I	ECKB	*Rubus idaeus*	roots	Ho	P	P S	Kennedy	1995
P. pseudotsugae	1a	I	ECKB	*Pseudotsuga menziesii*	roots	Ho	P	P S	Hamm	1983
P. clandestina	1b	I	ECKB	*Trifolium subterraneum*	roots	Ho	P	P S	Taylor	1985
P. iranica	1b	I	ECKB	*Solanum melongena*	roots	Ho	P	P S	Ershad	1971
P. tentaculata [1]	1b	I	ECKB	*Chrysanthemum* spp.	roots	Ho	P	P S	Kröber	1993
P. andina*	1c	IV	KB	*Solanum* spp.	foliage/fruits	He	A	SP M	Oliva	2010
P. infestans	1c	IV	ECKB	*Solanum tuberosum*	foliage	He	A	SP M	de Bary	1876
P. ipomoeae*	1c	IV	KB	*Ipomoea longipedunculata*	foliage	Ho	A	SP S	Flier	2002
P. mirabilis	1c	IV	ECKB	*Mirabilis jalapa*	foliage	He	A	SP S	Galindo	1985
P. phaseoli	1c	IV	ECKB	*Phaseolus lunatus*	foliage/fruits	Ho	A	SP S	Thaxter	1889

***** A 'new' species not described in the monograph "*Phytophthora* species worldwide" by Erwin and Ribeiro (1996).
[1] This species was previously included in clade 2, due to wrong data or a misidentified isolate (Kroon et al., 2004a).
[a] Group according to the key of Waterhouse (1963).
[b] Study in which the species was included: E = Erwin and Ribeiro (1996), C = Cooke et al. (2000), K = Kroon et al. (2004a) B = Blair et al. (2008).
[c] Host on which the pathogen was initially found, or most the important host.
[d] Most prominent tissue infected on the majority of host plants, or niche (rhizosphere, soil).
[e] He = heterothallic species; Ho = homothallic species.
[f] A = amphigynous, P = paragynous attachment of antheridia.
[g] P = papillate; SP = semipapillate ; NP = nonpapillate.
[h] M = pathogen is affecting multiple hosts, from a variety of families; S = pathogen is known to infect a limited number of hosts.
[i] First author to describe the species (for species described by Erwin and Ribeiro (1996); their data on authorship is used).
[j] Year of appearance of species description in literature.

Clade 2

With the addition of eight new species since 1996 clade 2 has become one of the largest clades in the *Phytophthora* phylogeny. Isolates of three new clade 2 species, *P. mengei*, *P. multivora* and *P. plurivora,* were previously considered as distinct subgroups within the *P. citricola* complex. One group of isolates was pathogenic on avocado trees, where they infected feeder roots and trunks. The pathogen responsible for this disease is now named *P. mengei*. It can be classified in clade 2, based on sequence homology with *P. capsici* and *P. tropicalis*, but its morphological traits place it just outside clade 2b (Hong et al., 2009). *P. multivora* was found in the rhizosphere of declining Eucalyptus trees in Australia (Scott et al., 2009). In Europe, large-scale surveys for soil-borne *Phytophthora* species were conducted in more than a thousand forests, nurseries and semi-natural stands. *P. citricola* was thought to be the cause of several devastating declines and diebacks of major forest tree species, but DNA analysis indicated that another new species was responsible, i.e., *P. plurivora* (Jung and Burgess, 2009).

Another new species in clade 2, *P. bisheria*, causes root rot on strawberry in the USA, roses in The Netherlands, and raspberry in Australia (Abad et al., 2008). It is closely related to another new species in this clade, *P. multivesiculata* (Ilieva et al., 1998). A very similar species is *P. frigida*, found on infected roots and collars of Eucalyptus trees in South Africa (Maseko et al., 2007).

Soil and water stream monitoring experiments revealed the presence of a homothallic species in Oregon, *P. siskiyouensis* (Reeser et al., 2007). Positioning in clade 2 was only based on ITS-sequencing; additional sequence information should resolve its exact position in this clade. Within *P. capsici*, a group of isolates was placed which were later reclassified as *P. tropicalis*, a pathogen on Macadamia trees (Aragaki and Uchida, 2001). In the early 1990s, a root rot disease of cultivated tobacco was observed in burley production areas in Brazil. The disease, called yellow stunt, is caused by *P.* sp. *glovera* (Shew et al., 1999).

Species in clade 2 all have papillate or semipapillate zoosporangia.

Table 2. Clade 2 *Phytophthora* species

Phytophthora sp.	group									Origins	
	clade	WH[a]	study[b]	Host[c]	Infected tissue[d]	sex[e]	A/P[f]	papill.[g]	[h]	author[i]	year[j]
P. bisheria*	2	III	B	*Fragaria* x *Ananassa*	roots	Ho	P	SP	S	Abad	2008
P. citricola	2	III	ECKB	*Citrus* spp.	fol./fruits/roots	Ho	P	SP	M	Sawada	1927
P. frigida*	2	II	-	*Eucalyptus smithii*	roots	He	A	P	S	Maseko	2007
P. mengeii*	2	III	-	*Persea americana*	roots	Ho	P	SP	S	Hong	2009
P. multivesiculata*	2	IV	CKB	*Cymbidium*	foliage	Ho	A	NSP	S	Ilieva	1998
P. multivora*	2	III	-	*Eucalyptus* spp.	rhizosphere	Ho	P	SP	M	Scott	2009
P. plurivora*	2	III	-	*Fagus sylvatica*	rhizosphere	Ho	P	SP	M	Jung	2009
P. siskiyouensis*	2	III	-	*Umbellularia californica*	rhizosphere	Ho	P	SP	S	Reeser	2007
P. botryosa [1]	2a	II	ECKB	*Hevea brasiliensis*	foliage	He	A	P	S	Chee	1969
P. citrophthora	2a	II	ECKB	*Citrus* spp.	fruits/roots	He[2]	A	P	M	Smith	1906
P. colocasiae	2a	IV	ECKB	*Colocasia esculenta*	foliage	He	A	SP	S	Raciborski	1900
P. inflata	2a	III	ECKB	*Ulmus americana*	foliage	Ho	P	SP	S	Caroselli	1949
P. meadii	2a	II	EKB	*Hevea brasiliensis*	fruits/foliage	Ho	A	P	S	McRae	1918
P. capsici	2b	II	ECB	*Capsicum annuum*	fol./fruits/roots	He	A	P	M	Leonian	1922
P.* sp. *glovera*	2b	III	B	*Nicotiana* spp.	roots	Ho	PA	SP	S	Shew	1999
P. mexicana	2b	II	EB	*Solanum lycopersicum*	fruits	Ho[2]	A	P	S	Hotson	1923
P. tropicalis*	2b	II	KB	*Macadamia integrifolia*	foliage	He	A	P	M	Aragaki	2001

For * and footnotes a-j see Table 1.

[1] This species was previously included in clade 4 due to wrong data or a misidentified isolate (Kroon et al., 2004a).

[2] There is some ambiguity on the mating type of these species (Erwin and Ribeiro, 1996).

Clade 3

Previously *Phytophthora ilicis* was the only species placed in clade 3 until Blair et al. (2008) included *P. nemorosa*, *P. pseudosyringae* and *P. psychrophila* in their analysis. *P. nemorosa* has been found in the same regions and ecosystems as *P. ramorum*. It was isolated from myrtlewood and Californian bay and causes lethal bole cankers on tanoak and coast live oak (Hansen et al., 2003). *P. pseudosyringae* was found in Europe in rhizosphere samples collected in oak declining soils and is associated with fine root and stem necrosis of beech (*Fagys sylvatica*) and alder (*Alnus glutinosa*) (Jung et al., 2003). *P. psychrophila* was also isolated from rhizosphere samples of oak (*Quercus robur*) although no clear host could be found that was affected by this species (Jung et al., 2002).

All species in clade 3 have semi-papillate sporangia, are homothallic, and pathogenic on trees.

Table 3. Clade 3 *Phytophthora* species

Phytophthora sp.	Group									Origins	
	clade	WH[a]	study[b]	Host[c]	Infected tissue[d]	sex[e]	A/P[f]	papill.[g]	[h]	author[i]	year[j]
P. ilicis	3	IV	ECKB	*Ilex aquifolium*	foliage	Ho	A	SP	S	Buddenhagen	1957
P. nemorosa*	3	IV	B	*Umbellularia californica*	foliage	Ho	A	SP	M	Hansen	2003
P. pseudosyringae*	3	III	B	*Quercus robur*	rhizosphere	Ho	P	SP	M	Jung	2003
P. psychrophila*	3	IV	B	*Quercus* spp.	rhizosphere	Ho	A	SP	S	Jung	2002

For * and footnotes a-j see Table 1.

Clade 4

Clade 4 is another small clade that expanded rapidly: the number of species has more than doubled as compared to 1996 (Erwin and Ribeiro). During a large survey in the USA in 2004, isolates of the species *P. quercetorum* were frequently found with an oak leaf baiting method from soil samples (Balci et al., 2008). Although the pathogen has the ability to cause infection on oak, it has never been found to be associated with oak decline. A species that is responsible for oak decline is *P. quercina*, found on *Quercus* spp. in Germany, Hungary and Italy (Jung et al., 1999). In South Africa, a new clade 4 species, *P. alticola* was found on Eucalyptus trees, causing root and collar rot (Maseko et al., 2007).

Species in clade 4 have papillate sporangia and are mainly pathogenic on roots.

Clade 5

Clade 5 is the smallest distinct clade comprising only two species, *P. heveae* and *P. katsurae.* These two species were already known in 1996 when Erwin and Ribeiro published their inventory of the *Phytophthora* genus.

The clade 5 species have papillate sporangia and are homothallic with amphigynous antheridia.

Table 4. Clade 4 and clade 5 *Phytophthora* species

Phytophthora sp.	Group									Origins	
	clade	WH[a]	study[b]	Host[c]	Infected tissue[d]	sex[e]	A/P[f]	papill.[g]	[h]	author[i]	year[j]
P. alticola*	4	II	B	*Eucalyptus dunnii*	roots	Ho	A	P	S	Maseko	2007
P. arecae	4	II	ECKB	*Areca catechu*	foliage/fruits	He	A	P	M	Pethybridge	1913
P. litchii* [1]	4	I	-	*Litchi chinensis*	fruits	Ho	P	P	S	Chen	1961
P. megakarya	4	II	ECKB	*Theobroma cacao*	roots	He	A	P	S	Brasier	1979
P. palmivora	4	II	ECKB	*Theobroma cacao*	fol./fruits/roots	He	A	P	M	Butler	1919
P. quercetorum*	4	I	B	*Quercus robur*	rhizosphere	Ho	P	P	S	Balci	2008
P. quercina* [2]	4	I	CB	*Quercus* spp.	roots	He	P	P	S	Jung	1999
P. heveae	5	II	ECKB	*Heveae brasiliensis*	fruits/roots	Ho	A	P	M	Thompson	1929
P. katsurae	5	II	ECKB	*Castanea crenata*	trunk	Ho	A	P	S	Ko	1979

For * and footnotes a-j see Table 1.

[1] This species was previously described as *Peronophytora litchii* by Ko et al. (1978), but was transferred to the genus *Phytophthora* by Göker et al. (2007).

[2] This species was included in clade 3, based on the study of Cooke et al. (2000).

Clade 6

This is another clade that has doubled in size since 1996 (Erwin and Ribeiro). One of the new species is *P. pinifolia,* first described in 2008 as the cause of a new disease on pine trees in Chile (Duran et al., 2008; Duran et al., 2009). It infects needles and shoots resulting in defoliation of the trees in winter that could even lead to plant death. This foliar pathogen is somewhat unique in clade 6, which until now only included soil-borne *Phytophthora* species.

P. rosacearum was previously classified as a subgroup of *P. megasperma*. Based on host range and ITS sequences, however, the group of isolates now forming the new species differed sufficiently to warrant a classification as a new species (Hansen et al., 2009). The same situation applies to the group of isolates now classified as *P.* sp. *asparagi* (Saude et al., 2008). The isolates of this species infect the spears and crown of asparagus plants and cause significant damage in asparagus production areas. The same pathogen has also been isolated from infected agave plants in Australia.

In the rhizosphere of reed stands (*Phragmites australis*) of Lake Constance, Germany, a previously undiscovered *Phytophthora* species was found that is closely related to *P. gonapodyides*. This species has no formal name yet and is classified as *P.* sp. *Salixsoil* (Nechwatal and Mendgen, 2006). In disease assays, it is pathogenic on leaves of *Salix alba*, but no apparent disease symptoms have been found associated with *P.* sp. *Salixsoil* so far. It is widely occurring in flooded habitats. Species of *Phytophthora* that are widely spread in ecosystems, but are not causing an apparent disease on available hosts may play a role in the breakdown of plant litter. It cannot be excluded, however, that they are pathogens of considerable significance (Brasier et al., 2003a). Another pathogen thriving in flooded ecosystems is *P. inundata*. It was found on horse chestnut (*Aesculus hippocastanum*) and willow (*Salix matsudana*) in the UK and on flooded olive trees in Spain (Brasier et al., 2003b).

A new species of *Phytophthora*, *P.* sp. *cranberry*, is pathogenic on cranberry and is closely related to *P. cinnamomi* and *P.* sp. *raspberry*. Since there is hardly any molecular data for the reference isolate of this species, it is difficult to conclude whether this is indeed a new species or a variant of a previously described species (Polashock et al., 2005).

All species in clade 6 have nonpapillate sporangia and are mostly infectious on roots or present in the rhizosphere.

Table 5. Clade 6 *Phytophthora* species

Phytophthora sp.	Group									Origins	
	clade	WH[a]	study[b]	Host[c]	Infected tissue[d]	sex[e]	A/P[f]	papill.[g]	[h]	author[i]	year[j]
P.* sp. *asparagi*	6	VI	CB	*Asparagus officinalis*	spear/crown	Ho	A	NP	S	Saude	2008
P.* sp. *cranberry*	6	VI	-	*Vaccinium macrocarpon*	roots	Ho	A	NP	S	Polashock	2005
P. gonapodyides	6	VI	ECKB	*Malus pumila*	roots/fruits	He	A	NP	S	Petersen	1910
P. humicola	6	V	ECKB	*Citrus spp.*	soil	Ho	P	NP	S	Ko	1985
P. inundata*	6	VI	B	*Aesculus hippocastanum*	roots	He	A	NP	S	Brasier	2003
P. megasperma	6	V	ECKB	*Brassica* spp.	roots	Ho	P	NP	M	Drechsler	1931
P.* sp. *personii*	6	V/VI	B	*Grevillea mccutcheonii*	soil	-	-	NP	-	Abad	-
P. pinifolia*	6	V/VI	-	*Pinus radiata*	foliage	-	-	NP	S	Duran	2008
P.* sp. *raspberry*	6	V/VI	-	*Rubus idaeus*	roots	Ho	AP	NP	S	Brasier	2003
P. rosacearum*	6	V	-	*Malus domestica*	rhizosphere	Ho	P	NP	S	Hansen	2009
P.* sp. *Salixsoil*	6	V/VI	-	*Salix alba*	soil	-	-	NP	-	Brasier	2003

For * and footnotes a-j see Table 1.

Clade 7

One of the newly described species in clade 7 appears to be a species hybrid. *P. alni* is a pathogen of alder in the UK and in northern Europe (Brasier et al., 2004). Three subspecies of *P. alni* are recognized: *P. alni* subsp. *alni*, *P. alni* subsp. *uniformis*, and *P. alni* subsp. *multiformis*. The subspecies are believed to be a group of emergent heteroploid hybrids between two phylogenetically close *Phytophthora* species. A combination of nuclear and mitochondrial DNA analyses suggests that *P. alni* subsp. *alni* may have been generated on several occasions by hybridization between *P. alni* subsp. *uniformis* and *P. alni* subsp. *multiformis*, or their respective ancestors. *P. alni* subsp. *uniformis* might have *P. cambivora* as a species ancestor, whereas P. *alni* subsp. *multiformis* seems to have been generated either by an ancient reticulation or by autopolyploidization (Ioos et al., 2006).

Two other new species found in central Europe and also placed in this clade are *P. europae* and *P. uliginosa* (Jung et al., 2002). Both were found in the rhizosphere of oak (*Quercus robur*), but only *P. uliginosa* was really pathogenic on oak.

A group of isolates previously described as *P. megasperma* was found on pistachio trees and named *P. pistaciae* (Mirabolfathy et al., 2001). The group of isolates known as the *P. megasperma* complex houses a number of putative new species that can be distinguished based on sequence analysis.

All species in clade 7 are nonpapillate and mostly pathogenic on the roots of their host.

Table 6. Clade 7 *Phytophthora* species

Phytophthora sp.	Group									Origins	
	clade	WH[a]	study[b]	Host[c]	Infected tissue[d]	sex[e]	A/P[f]	papill.[g]	[h]	author[i]	year[j]
P. alni* [1]	7a	VI	KB	*Alnus* spp.	bark	Ho	A	NP	S	Brasier	2004
P. cambivora	7a	VI	ECB	*Aesculus hippocastanum*	roots	He	A	NP	M	Buisman	1927
P. europaea*	7a	V	B	*Quercus* spp.	rhizosphere	Ho	P	NP	S	Jung	2002
P. fragariae	7a	V	ECKB	*Fragaria* x *ananassa*	roots	Ho	P	NP	S	Hickman	1940
P. rubi* [2]	7a	V	ECK	*Rubus idaeus*	roots	Ho	P	NP	S	Man in 't Veld	2007
P. uliginosa*	7a	V	B	*Quercus robur*	rhizosphere	Ho	P	NP	S	Jung	2002
P. cajani	7b	VI	ECB	*Cajanus cajan*	stem	Ho	A	NP	S	Amin	1978
P. cinnamomi	7b	VI	ECKB	*Cinnamomum burmannii*	roots	He	A	NP	M	Rands	1922
P. melonis	7b	VI	ECB	*Cucumis sativus*	roots	He	A	NP	S	Katsura	1976
P. niederhauserii*	7b	VI	B	*Banksia* spp.	-	He	A	NP	M	Abad	2010
P. pistaciae*	7b	V	B	*Pistachia vera*	trunk / roots	Ho	P	NP	S	Mirabolfathy	2001
P. sinensis	7b	VI	ECKB	*Cucumis sativus*	foliage / fruits	He	A	NP	S	Yu	1982
P. sojae	7b	V	ECKB	*Glycine max*	roots / stem	Ho	P	NP	S	Kaufmann	1958
P. vignae	7b	VI	ECKB	*Vigna unguiculata*	roots / stem	Ho	A	NP	S	Purss	1957

For * and footnotes a-j see Table 1.

[1] This species was included as *P.* hybrid-Dutch variant in the study of Kroon et al. (2004a).

[2] This species was known as *P. fragariae* var. *rubi* (Erwin and Ribeiro, 1996).

Clade 8

At present clade 8 has the same number of species as clade 2 making these two the largest clades in the *Phytophthora* phylogeny. Since 1996 (Erwin and Ribeiro) six species have been added.

Isolates that are now classified as *P. sansomeana* were previously grouped in the *P. megasperma* complex (Hansen et al., 2009). All the isolates in the *P. megasperma* complex shared morphological traits, but other characteristics, like host range and optimal growth temperature, differed significantly. *P. sansomeana* was isolated from infected soybean plots. Soybean crops with resistance or tolerance to *P. sojae* suffered great damage from this new disease and the pathogen isolated from infected plants differed from *P. sojae* based on sequence analyses.

In Patagonia, the pathogen *P. austrocedrae* causes damage on the conifer *Austrocedrus chilensis* (Greslebin et al., 2007). It is closely related to *P. syringae* based on ITS sequence-data.

The most notorious new species in clade 8 is *P. ramorum*, the causal agent of Sudden Oak Death (Werres et al., 2001b). This species was found in Europe on Rhododendron and Viburnum and in the USA on oaks (Rizzo et al., 2002). Since the late 1990s it has had a major impact on ecosystems in the USA, and nowadays it ranks in the top of well-known *Phytophthora* species. During the Sudden Oak Death survey in the USA, a new pathogen of Azalea was found that was sufficiently different to define it as a new species: *P. foliorum* (Donahoo et al., 2006).

In clade 8b, isozyme and molecular data confirmed that a group of isolates placed in the species *P. porri* were in fact a different species. This pathogen was named *P. brassicae* (Man in 't Veld et al., 2002), it infects roots and collars of cabbage and can cause massive post-harvest damage.

All species in clade 8a are nonpapillate and those in clade 8b and 8c are semipapillate. The one exception is *P. lateralis*, which was typed as nonpapillate by Tucker (Erwin and Ribeiro, 1996). However, recent examination of several isolates, including the type isolate, indicated that *P. lateralis* isolates produce both nonpapillate and semipapillate sporangia (Werres et al., 2001a; De Cock, personal communication).

Table 7. Clade 8 *Phytophthora* species

Phytophthora sp.	Group									Origins	
	clade	WH[a]	study[b]	Host[c]	Infected tissue[d]	sex[e]	A/P[f]	papill.[g]	[h]	author[i]	year[j]
P. cryptogea	8a	VI	ECKB	*Solanum lycopersicum*	roots	He	A	NP	M	Pethybridge	1919
P. drechsleri	8a	VI	ECKB	*Solanum tuberosum*	roots	He	A	NP	M	Tucker	1931
P. erythroseptica	8a	VI	ECKB	*Solanum tuberosum*	roots	Ho	A	NP	M	Pethybridge	1913
P. sp. ***kelmania****	8a	V/VI	B	*Gerbera* spp.	-	-	-	NP	S	Abad	-
P. medicaginis	8a	V	ECB	*Medicago sativa*	roots	Ho	PA	NP	S	Hansen	1991
P. richardiae[1]	8a	VI	ECKB	*Zantedeschia aethiopica*	roots	Ho	A	NP	S	Buisman	1927
P. sansomeana*	8a	V	B	*Glycine max*	rhizosphere	Ho	P	NP	S	Hansen	2009
P. trifolii	8a	V	ECB	*Trifolium* spp.	roots	Ho	P	NP	S	Hansen	1991
P. austrocedrae*	8b	IV	-	*Austrocedrus chilensis*	roots / stem	Ho	A	SP	S	Greslebin	2007
P. brassicae*	8b	IV	KB	*Brassica oleracea*	roots / stem	Ho	A	SP	S	De Cock	2002
P. porri	8b	III	ECB	*Allium porrum*	foliage	Ho	PA	SP	S	Foister	1931
P. primulae	8b	III	ECB	*Primula* spp.	roots	Ho	PA	SP	S	Tomlinson	1952
P. syringae	8b	III	ECKB	*Syringa vulgaris*	foliage	Ho	P	SP	M	Klebahn	1909
P. foliorum*	8c	III	B	*Azalea* spp	foliage	Ho	PA	SP	S	Donahoo	2006
P. hibernalis	8c	IV	EKB	*Citrus* spp.	foliage / fruits	Ho	A	SP	S	Carne	1925
P. lateralis	8c	V	ECKB	*Chamaecyparis lawsoniana*	roots	Ho	P	NP	S	Tucker	1942
P. ramorum*	8c	IV	KB	*Rhododendron spp.*	trunk / roots	He	A	SP	M	Werres	2001

For * and footnotes a-j see Table 1.
[1] This species was included in clade 10, based on the study of Cooke et al. (2000).

Clade 9

Clade 9 was one of the less-resolved clades in the phylogeny published by Kroon et al. (2004a) (chapter 2.2) but has expanded enormously since 1996 (Erwin and Ribeiro).

One of the new species is *P. parsiana*, a pathogen of pistachio, almond and fig (Mostowfizadeh-Ghalamfarsa et al., 2008). *P. parsiana* seems to be similar to *P. hydropathica* described by Hong et al. (2008b). The latter, also placed in clade 9, has been recovered from necrotic leaves and blighted shoots of Rhododendron.

Another new species in this clade is *P. irrigata*, isolated from irrigation reservoirs and natural waterways (Hong et al., 2008a). It was found to be pathogenic on Azalea.

P. polonica was isolated from the rhizosphere of infected alder trees in Poland. Belbahri et al. (2006) placed the species in clade 8c sensu Kroon (Kroon et al., 2004a), but in the phylogeny by Blair et al. (2008) that has a better resolution at the base of the phylogenetic tree, *P. polonic*a could be assigned to clade 9. Several new pathogens of Eucalyptus trees have been described in recent years. Two of these, *P. captiosa* and *P. fallax*, associated with a crown disease in Eucalyptus plantations in New Zealand were also added as new species to clade 9 (Dick et al., 2006).

Species in clade 9 are nonpapillate (except for *P. macrochlamydospora*) and mainly found in the soil.

Clade 10

As clade 3, clade 10 previously comprised only one species. Three new species were added, one of which is *P. gallica* (Jung and Nechwatal, 2008) that was responsible for a decline in stands of oak and reed in Germany and France. Another recently described species in clade 10 is *P. kernoviae*, a pathogen of beech found in Cornwall in the UK (Brasier et al., 2005). The third species is *P. morindae*, that caused a severe foliar blight and fruit rot disease of noni (*Morinda citrifolia* L. var. *citrifolia*) on the island of Hawaii in 1999 (Nelson and Abad, 2010).

Species in clade 10 are papillate and pathogenic on foliage and stem with the exception of *P. gallica*. New phylogenetic analyses on the complete set of *Phytophthora* species may shift *P. gallica* from clade 10 to clade 9.

Table 8. Clade 9 and clade 10 *Phytophthora* species

Phytophthora sp.	group								Origins	
	clade	WH[a]	study[b]	Host[c]	Infected tissue[d]	sex[e]	A/P[f]	papill.[g] [h]	author[i]	year[j]
P. captiosa*	9	VI	B	*Eucalyptus* spp.	crown	Ho	A	NP	Dick	2006
P.* sp. *cuyabensis*	9	V	B	rainforest soil	soil		P	NP		
P. fallax*	9	V	B	*Eucalyptus* spp.	crown	Ho	PA	NP	Dick	2006
P. hydropathica*	9	VI	-	*Rhododendron catawbiense*	foliage	He	A?	NP S	Hong	2008
P. irrigata*	9	VI	-	*Azalea* spp.	irrigation w.	He	A	NP S	Hong	2008
P. insolita	9	V/VI	ECKB	*Citrus* spp.	soil	Ho	-	NP S	Ann	1980
P.* sp. *lagoariana*	9		B	rainforest soil	soil					
P. macrochlamydospora[1]	9	III/IV	ECB	*Glycine max*	roots	-	-	SP S	Irwin	1991
P. parsiana*	9	VI	-	*Pistacia vera*	trunk	He	A	NP S	Mostowfizadeh	2008
P. polonica*	9	V	B	*Alnus glutinosa*	rhizosphere	Ho	P	NP S	Belbahri	2006
P. quininea	9	V	EKB	*Chinchona officinalis*	roots	Ho	P	NP S	Crandal	1947
P. boehmeriae	10	II	EKB	*Boehmeria nivea*	foliage	Ho	A	P S	Sawada	1927
P. gallica*	10	V/VI	-	*Quercus robur*	rhizosphere	-	-	NP S	Jung	2008
P. kernoviae*	10	II	B	*Fagus sylvatica*	stem	Ho	A	P S	Brasier	2005
P. morindae*	10	II	-	*Morinda citrifolia*	foliage / fruit	Ho	A	P S	Nelson	2010

For * and footnotes a-j see Table 1.
[1] This species was included in clade 10, based on the study of Cooke et al. (2000).

Lost species

In the reference work of Erwin and Ribeiro (1996), eight species are described that are not presented in the phylogenies presented by us in chapter 3.2 (Kroon et al., 2004a) nor by Blair et al. (2008). Sometimes, the isolates used to describe the new species have been lost because they could not be cultured at that time or were not properly stored for long term preservation. Since no sequence data are available for these species, they can never be

included in the current phylogenies. There is even a possibility that isolates from one of these "lost" species has been put forward as a new species, since no sequence homology could be found with other species that have been sequenced. Althought many new species have been described based on Waterhouse criteria, no thorough examination has been made to check if the new species coincides with another, previously described species. The absence of reference isolates for these "lost" species decreases the chance for positive identification. Because the Waterhouse grouping largely coincides with particular clades or subclades, and species within a (sub)clade share habitat or host type, we made an attempt to deduce the position of these "lost" species in the *Phytophthora* phylogeny.

P. cyperi and *P. cyperi-bulbosi* are semipapillate species that are pathogenic on *Cyperus* spp. and most likely belong in clade 2, 3, 8b or 8c.

P. eriugena causes necrosis on leaves and stems of Cypres (*Chamaecyparis lawsoniana*) and although it has papillate sporangia, it was placed in Waterhouse group IV. This exceptional status makes it hard to find a suitable clade for this species.

P. italica is a root pathogen of myrtle (*Myrtus communis*) in Italy. Since it has papillate sporangia and is a root pathogen, it could fall in clade 1, 2, 4, 5 or 10, which all contain papillate species. Of these only clade 1 and clade 4 contain species that are paragynous. Since *P. italica* has strictly paragynous antheridia and is very similar to *P. iranica*, it likely belongs to clade 1.

P. japonica is pathogenic on rice (*Oriza sativa*) and has nonpapillate sporangia. It could belong in either clade 6, 7, 8, or 9.

P. lepironiae is a pathogen of the reed *Lepironia mucronata* that most likely belongs to clade 2, 3, 8b or 8c.

P. undulata is a peculiar species. Some researchers describe it as being a *Pythium* species, some as a *Phytophthora*. Several sequences are available in Genbank and some indeed cluster with sequences from *Phytophthora* species and some with *Pythium* sequences. According to the phylogenetic study of Levesque and De Cock (2004), this species clearly belongs in the genus *Pythium* and should be referred to as *Pythium undulatum*. Sequences clustering with *Phytophthora* species are probably derived from misidentified isolates.

P. verrucosa was described as a pathogen causing foot rot on tomato and having nonpapillate sporangia. However, it could not be cultured. It could be a member of clade 8a, which consists of other species that are pathogenic on roots of solanaceous host, are nonpapillate, and have both paragynous and amphigynous antheridia.

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3

Phytophthora clade 1c: hybrids and new species

3.1

Inheritance of host specificity in hybrid progeny of the oomycete pathogens *Phytophthora infestans* and *Phytophthora mirabilis*

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Abstract

Toluca valley in central Mexico is a proposed centre of origin of *Phytophthora infestans*, the causal agent of potato late blight. This valley also hosts *Phytophthora mirabilis,* a close relative of *P. infestans* and a pathogen of *Mirabilis jalapa*. As the two species are not separated geographically, we were interested to investigate whether the two species could mate and generate viable offspring. We performed a series of artificial crosses between Mexican isolates of *P. infestans* and *P. mirabilis* and were able to obtain viable F1, BC1 and F2 progeny. Subsequently, we were able to prove that the progeny represents true hybrids as AFLP and mitochondrial DNA marker analyses performed on both the parents and the progeny showed clear transgression of parental markers. We could also show that a substantial fraction of the progeny was pathogenic on both tomato (*Solanum lycopersicum* cv. Moneymaker) and potato (*Solanum tuberosum,* cv. Bintje). These results indicate that interspecific crosses between *P. infestans* and *P. mirabilis* could occur in nature. Tomato, a known host of *P. infestans* that widely occurs in the Toluca valley, may not only act as a bridging host for the interspecific hybrids, but may also provide the right conditions for reproduction and selection of viable and virulent intra- and interspecific recombined individuals that are pathogenic on *Mirabilis jalapa*, tomato and potato.

Introduction

Species belonging to the oomycete genus *Phytophthora* are regarded to be among the most devastating plant pathogens in agriculture (Erwin and Ribeiro, 1996). The highlands of central Mexico harbour two closely related species: *Phytophthora infestans* and *Phytophthora mirabilis* (Grünwald and Flier, 2005). Their pathogenicity on different host plants has been the main criterion for separating these two species (Galindo and Hohl, 1985). *P. infestans* infects Solanaceous hosts of which potato (*Solanum tuberosum*) and tomato (*Solanum lycopersicum*) are the most important. So far, the only host known for *P. mirabilis* is *Mirabilis jalapa* L., belonging to the family of Nyctaginaceae. The two *Phytophthora* species show high homology at the genome level (Blair et al., 2008; Kroon et al., 2004) and have similar morphology, growth characteristics and infectious behaviour with a preference for leaves and stems of their respective host plants (Flier et al., 2002; Goodwin et al., 1999). Since the geographical distribution of *P. infestans* and *P. mirabilis* overlaps (Galindo and Hohl, 1985),

the two species may have co-evolved by a process referred to as sympatric speciation (Doebeli and Dieckmann, 2000; Gavrilets, 2003; Goodwin et al., 1999; Kondrashov and Kondrashov, 1999). Whether both *P. infestans* and *P. mirabilis* share a single common ancestor and gradually developed by divergent evolution and adaption to different host plants, or whether each species adapted to its hosts independently remains unclear.

Goodwin and Fry (1994) reported that both species are not able to infect reciprocal hosts, but experiments with a large set of *P. infestans* and *P. mirabilis* isolates collected in the Toluca Valley in 1997 and 1998 showed that some isolates could infect both *M. jalapa* plants and *S. tuberosum* cv. Alpha in detached leaf assays. Senescing leaves were particularly susceptible to both *Phytophthora* species. In addition, isolates of *P. mirabilis* were frequently found to be resistant to the oomicide metalaxyl, whereas *M. jalapa* plants were never treated with this agrochemical that is commonly used in potato production (Flier et al., 2003; Grünwald and Flier, 2005). The latter observation might suggest active gene flow between *P. infestans* and *P. mirabilis*, although Goodwin et al. (1999) reported low estimates of gene flow between the two pathogens.

Additional research on sexual compatibility between the two species reported by Goodwin et al. (1999) supports the current view that *P. infestans* and *P. mirabilis* are two separate species. Although isolates of *P. infestans* and *P. mirabilis* could generate first generation hybrid offspring (F1) when crossed, this F1 progeny was impaired in generating F2 and backcross (BC1) offspring due to hybrid inviability. Goodwin et al. (1999) postulated the existence of genetic mechanisms of reproductive isolation for *P. infestans* and *P. mirabilis*. In addition to this genetic reproductive isolation, the F1 hybrid progeny was shown to be largely non-pathogenic, as only a fraction of F1 progeny was able to infect either *S. tuberosum*, *M. jalapa,* or *S. lycopersicum* in detached leaflet assays. This is referred to as post mating reproductive isolation, as opposed to pre-mating reproductive isolation, where two species are unable to produce sexual progeny.

DNA sequence comparison between *P. infestans* and *P. mirabilis* would give more insight in the evolutionary distance between the two species. The sequences of the rDNA internal transcribed spacer regions [ITS1 and ITS2] (Cooke et al., 2000) and the genes encoding ß-tubulin, translation elongation factor1α [EF-1α], NADH dehydrogenase subunit 1 [nadh1] and Cytochrome c oxidase subunit 1 [cox1] in *P. infestans* and *P. mirabilis* are highly homologous (Kroon et al., 2004). This observation prompted us to re-investigate the possibility of gene flow between the two species. Recently, new outbreaks of *Phytophthora*-related diseases have been reported that are caused by hybrid progeny of two separate

Phytophthora species (Bonants et al., 2000; Brasier et al., 2004; Ersek and Nagy, 2008; Man in 't Veld et al., 2007). Insight in the mechanisms of interspecific hybridization could shed more light on the emergence of these new diseases, and the impact of this phenomenon on agriculture. To test whether gene flow between *P. infestans* and *P. mirabilis* is possible, a set of *P. infestans* and *P. mirabilis* isolates collected from the Toluca Valley was used for exploring the possibility of making interspecific crosses.

The objectives of this study were (i) to generate interspecific hybrids between *P. infestans* and *P. mirabilis* isolates *in vitro,* (ii) to analyze the genetics of host plant specificity in the F1 progeny of crosses between *P. infestans* and *P. mirabilis* isolates, (iii) to analyze the inheritance of mating type, mitochondrial DNA haplotype and factors determining linear growth rate in progeny of these interspecific crosses, and (iv) to test the viability and host range of progeny in subsequent generations (F2 and BC1).

Materials and methods

Isolates and cultures

Isolates of *P. infestans* and *P. mirabilis* used in this study are presented in Table 1. They were collected in the Toluca Valley in central Mexico in 1997 and 1998 from *Solanum demissum* and *Mirabilis jalapa* plants, respectively (Grünwald et al., 2001). Cultures were maintained on Rye A agar (Caten and Jinks, 1968) at 20°C in darkness and were stored in DMSO (15%) in liquid nitrogen at Plant Research International, Wageningen, The Netherlands. For infection experiments, isolates were taken from liquid nitrogen storage and transferred to fresh medium with a one-month interval. For production of zoosporangia, isolates were either grown on tomato leaves (cv. Moneymaker) or on Rye A agar plates.

Mating type assessment

To determine mating type, collected isolates were paired with *P. infestans* tester strains. For each isolate, two mycelial plugs were placed 4 cm apart on 90-mm plates containing Rye A agar amended with 0.05 g of ß-sitosterol per litre. Opposite to these plugs, two plugs of *P. infestans* tester isolates of either mating type A1 (Pic97757) or mating type A2 (Pic97750) were placed. For the mating type determination of F2 and BC1 progeny, two *P. mirabilis* tester isolates G9-1 (mating type A1) and G9-5 (mating type A2) were used.

Cultures were assessed for oospore formation following two weeks of incubation at 20 °C in the dark. A cross was considered successful when oospores were present in the centre of the plates of compatible interactions, where the hyphae of the isolates of opposite mating types met (Flier et al., 2001). In this region, extensive stimulation of submerged hyphal growth was observed at the interaction zone between the two compatible parental strains, resulting in a distinct visible band. In incompatible interactions, hyphae showed growth inhibition in the central region of the plate, resulting in an uncolonized zone between parental colonies and hyphae of isolates of the same mating type showed growth repulsion.

Table 1. Characteristics of the various *Phytophthora* isolates used in this study.

				host plant		
Isolate[a]	Origin	MT[b]	Haplotype[c]	*S. tuberosum*	*S. lycopersicum*	*M. jalapa*
P. mirabilis G4-1	*M. jalapa*, Mexico 1998	A2	Ha*Pmir*I	-[d]	-	++
P. mirabilis G4-4	*M. jalapa*, Mexico 1998	A2	Ha*Pmir*I	-	-	++
P. mirabilis G5-2	*M. jalapa*, Mexico 1998	A1	Ha*Pmir*I	-	-	+[e]
P. mirabilis G9-1	*M. jalapa*, Mexico 1998	A1	Ha*Pmir*I	-	-	+
P. mirabilis G9-5	*M. jalapa*, Mexico 1998	A2	Ha*Pmir*I	-	-	+
P. mirabilis G15-4	*M. jalapa*, Mexico 1998	A2	Ha*Pmir*I	-	-	++
P. mirabilis G15-9	*M. jalapa*, Mexico 1998	A2	Ha*Pmir*I	-	-	++
P. infestans Pic97210	*S. demissum*, Mexico 1997	A1	Ha*Pinf*Ia	++[f]	++	-
P. infestans Pic97701	*S. demissum*, Mexico 1997	A2	Ha*Pinf*Ia	++	++	-
P. infestans Pic97743	*S. demissum*, Mexico 1997	A1	ND	+	++	-
P. infestans Pic97750	*S. demissum*, Mexico 1997	A2	ND	+	+	-
P. infestans Pic97757	*S. demissum*, Mexico 1997	A1	Ha*Pinf*Ia	++	++	-
P. infestans NL80029	*S. tuberosum*, NL, 1980	A1	ND	++	++	-
LK99002	F1 hybrid	A1	Ha*Pmir*I	-	++	-
LK99004	F1 hybrid	A2	Ha*Pmir*I	-	++	-
LK99005	F1 hybrid	A1	Ha*Pinf*Ia	-	++	-
LK99006	F1 hybrid	A2	Ha*Pmir*I	-	++	-
LK99009	F1 hybrid	A1	Ha*Pmir*I	-	++	-
LK99010	F1 hybrid	A1	Ha*Pmir*I	-	++	-

[a] Isolates used in this study, either as a parent in F1, F2 or BC-crosses, or as a reference for radial growth experiments. The seven *P. mirabilis* isolates shown in this Table are a representative subset of the 22 isolates used for the radial growth experiments of which the results are presented in Table 3.

[b] MT = mating type.

[c] Mitochondrial haplotype classification according to Flier et al. (2002), ND= not determined.

[d] (-) represents an interaction between isolate and host plant predominantly scored as disease severity type - or HR.

[e] (+) represents an interaction between isolate and host plant predominantly scored as disease severity type +.

[f] (++) represents an interaction between isolate and host plant predominantly scored as disease severity type ++.

For definition of the indexes see materials and methods.

Single oospore progeny

Oospores produced in the cross between *P. mirabilis* isolate G4-4 and *P. infestans* isolate Pic97757 were extracted from the agar plates and subsequently separated from somatic mycelia after Novozym treatment followed by centrifugation (Spielman et al., 1990). Agar sectors containing oospores were excised, transferred to 50-ml centrifuge tubes (Greiner, Kremsmünster, Austria) and homogenized in 9 ml of sterile tap water using a tissue grinder (Ultra Turrax T25; IKA Labortechnic, Staufen, Germany) at 20.000 rpm for one minute. Novozym (1 ml of 10 mg ml^{-1}) was added to the homogenate prior to overnight incubation under continuous motion (Spiramix 10; Denley Instruments Ltd., Sussex, UK) at 20 °C. The Novozym lytic enzyme dissolves the membranes of zoosporangia and mycelium resulting in lysis and disintegration of all mycelial structures except for the thick-walled oospores.

Following Novozym treatment, the oospores were spun down and the supernatant was removed. Oospores were washed three times with sterile tap water to prevent the frail oospore germ tube from being lysed at later stages. Oospore concentration was determined in a Fuchs/Rosenthal haemocytometer, and approximately $1x10^3$ oospores were transferred to 90-mm Petri dishes containing 0.8% water agar (WA) amended with ampicillin (100 µg ml^{-1}). Petri dishes were incubated in a climate room at 16°C, under fluorescent light (16 hrs of light/ 8 hrs darkness).

Germinating oospores were detected using a reversed image microscope (Nikon TMS) two to three days after incubation. After a brief period of linear growth most of the germ tubes produced a zoosporangium. Germinated oospores with attached zoosporangia were collected using an ethanol-sterilized eyelash, glued to a toothpick. The thin end of the eyelash was manoeuvred under the germ tube connecting the oospore and the zoosporangium. The germinated oospore adhered to the eyelash, and was transferred to a 25-well square replidish (Greiner) with 1.5 ml of Rye A agar per well. Putative F1 progeny were transferred to Rye A medium containing ampicillin (100 µg ml^{-1}) and subsequently stored in liquid nitrogen. Individual germinating oospores could be picked up from the water agar plates during a period of up to 10 days. Germination rates of oospores were generally low, not exceeding 1 to 2.5 % in the first 10 days post incubation (results not shown).

Two crosses between interspecific F1 isolates were made in order to obtain F2 progeny. The same oospore isolation procedure as described above was followed and putative F2 progeny was picked up for the crosses F2a and F2b (Table 2). In addition, three backcrosses were made, by pairing F1 progeny with *P. infestans* and *P. mirabilis* isolates. The

parental lines of backcrosses BC1a, BC1b and BC1c are listed in Table 2. Distinctly different parental strains were used for back-crosses when compared to the F1 cross, to ensure that enough informative AFLP-fragments were available for discrimination between true progeny and inbreds.

Table 2. Overview of crosses between *Phytophthora infestans* and *P. mirabilis*, and their interspecific offspring.

Cross	A1 parent	A2 parent	# progeny	Mating type A1/A2	Haplotype Ha*Pmir*I / Ha*Pinf*Ia
F1	Pic97757	G4-4	11	8:3	10:1
F2a	LK99006	LK99009	14	8:6	- [a]
F2b	LK99004	LK99010	23	14:7	- [a]
BC1a	LK99002	G9-5	33	26:7	- [a]
BC1b	LK99002	Pic97701	11	11:0	9:2
BC1c	NL80029	LK99004	6	2:4	6:0

[a] in these crosses, both parental isolates had the Ha*Pmir*I mitochondrial haplotype.

Determination of linear growth rate

Linear growth of *P. infestans* and *P. mirabilis* isolates (Table 1) and F1, F2 and backcross progeny (Table 2) was monitored for a three-day period, on three replicate agar plates (90 mm) containing modified Plich medium (Van der Lee et al., 1997). The Plich medium contained: glucose (25 g), KH2PO4 (0.50 g), MgSO4.7H2O (0.25 g), L-Asparagine (1.0 g), Yeast extract (0.50 g), Thiamine (1 mg), ß-sitosterol (10 mg) and agar (15 g) per litre of demineralised water.

DNA extraction

Isolates of *P. infestans*, *P. mirabilis* and F1, F2 and BC progeny were taken from liquid nitrogen storage, grown on Rye A agar plates, and grown for 10-14 days at 20 °C in 20 ml of Pea Broth in 9 cm Petri dishes. Pea Broth was prepared by autoclaving 120 g of frozen peas in one litre of tap water. The peas were removed by filtering through cheesecloth and the remaining broth was autoclaved again (Flier et al., 2003). The mycelium was harvested and lyophilized, and total DNA was extracted from mycelium (10 mg) using the Puregene kit (Gentra/Biozyme, Landgraaf, The Netherlands) according to the manufacturer's instructions. In addition, an extra protein precipitation step was performed after resuspension of DNA and

RNase A treatment, by adding 100 µl of TE-buffer (pH 8.0) and 50 µl protein precipitation solution from the Puregene kit, followed by isopropanol precipitation and ethanol rinsing. The DNA pellet was dissolved in 100 µl TE (10 mM Tris/HCl, pH 8.0; 1 mM EDTA) and stored at –20°C. DNA integrity and concentration was determined by gel electrophoresis (2 hrs at 100 Volts) on 1.0 % agarose gels (MP, Roche, Mannheim, Germany) in 0.5 x TBE buffer. Gels were stained with ethidium bromide and DNA was visualized under UV light, and compared with a DNA concentration standard consisting of *Eco*RI/*Hin*dIII digested λ-DNA fragments.

AFLP analysis

AFLP DNA fingerprinting was used to investigate the hybrid nature of the F1, F2 and BC progeny. AFLP analysis was performed as described previously (Adler et al., 2004). Imagemaster software (Amersham Pharmacia) was used to analyze the AFLP fingerprints and to generate a binary data matrix for presence or absence of parental DNA fragments in the F1, F2 and BC progeny.

Mitochondrial haplotypes

The P2 and P4 regions of the mitochondrial genome were amplified using primers and methods described by Griffith and Shaw (Griffith and Shaw, 1998). Reactions were performed in a PTC200 thermocycler. PCR products, digested with the restriction enzymes *Msp*I and *Eco*RI, yielded restriction fragment patterns that could be classified either as *P. infestans* haplotype Ia or *P. mirabilis* haplotype *Pmir*I (Flier et al., 2002). In heterothallic *Phytophthora* species, each parental isolate involved in a cross is able to act both as male and female partner, contributing with either its male (antheridium) or female (oogonium) sexual organs to the formation of the oospore. In Oomycetes, the parental isolate contributing to the oogonium (female parent) will transmit its mitochondrial DNA to the offspring.

Determination of host range

Parental isolates and F1 and F2 progeny were tested for their ability to infect *Solanum tuberosum* cv. Bintje, *Mirabilis jalapa*, and *Solanum lycopersicum* cv. Moneymaker, which were grown in the greenhouse. Detached leaflets were placed in 15 cm Petri dishes with 30 ml of 1.5 % WA. The leaflets were inoculated till runoff with sporangium suspensions of each isolate, either made by rinsing tomato leaves which showed extensive sporulation, or by rinsing the surface of colonies on 9 cm Rye A agar plates with 5 ml of sterile tap water (when infection of tomato leaflets could not be achieved). Hyphae were removed by filtration over sterile gauze (1/16, Van Heek Medical, Beugen, The Netherlands). The zoosporangium concentration was measured using a Beckman Coulter counter (Beckman Coulter, Fullerton CA, USA) and adjusted to $3x10^4$ zoosporangia ml^{-1}. The inoculated leaflets were transferred to a climate room at 15 °C (light regime: initial 24 hrs of darkness, then cycles of 16 hrs of light / 8 hrs of darkness) until the leaflets were scored for symptoms. Disease symptoms were scored at 7 and 14 days post inoculation using a disease severity scale; 0 = no visible symptoms; HR = restricted hypersensitive response, no sporulating lesions; + = spreading lesions, < 50% leaf surface infected; ++ = spreading lesions, > 50% leaf surface infected. Sporangia were only observed on leaves scored as + or ++ on the disease severity scale.

Results

Segregation of AFLP markers in interspecific progeny

A total of eleven putative F1 hybrids were recovered from a cross between *P. infestans* isolate Pic97757 and *P. mirabilis* isolate G4-4. First generation interspecific progeny showed AFLP banding patterns consisting of a combination of both *P. infestans* and *P. mirabilis* markers (Figure 1). For example, F1 isolate LK99003 shared 60 out of 77 AFLP markers with *P. infestans* parental isolate Pic97757 and 55 out of 66 markers with *P. mirabilis* parental isolate G4-4. Of the shared markers, 21 were present in both Pic97757 and G4-4. These results indicate that the F1 isolates are true sexual interspecific hybrids. Markers present in a heterozygous state in the parental *P. infestans* or *P. mirabilis* isolate showed clear segregation in the F1 progeny (Figure 1).

A total of 14 putative F2 progeny was picked up from the cross F2a, 23 putative F2 progeny from cross F2b, 33 putative BC1 progeny from BC1a, 11 putative BC1 progeny from BC1b,

and 6 putative BC1 progeny from BC1c (Table 2). Based on AFLP marker segregation patterns, it is concluded that both the F2 populations and the three BC1 populations are true hybrids. Also for the F2 and BC1 progeny, sexual intercrossing is the most likely origin. There are no indications of selfing, somatic fusion, or apomixis (Kondrashov, 1985).

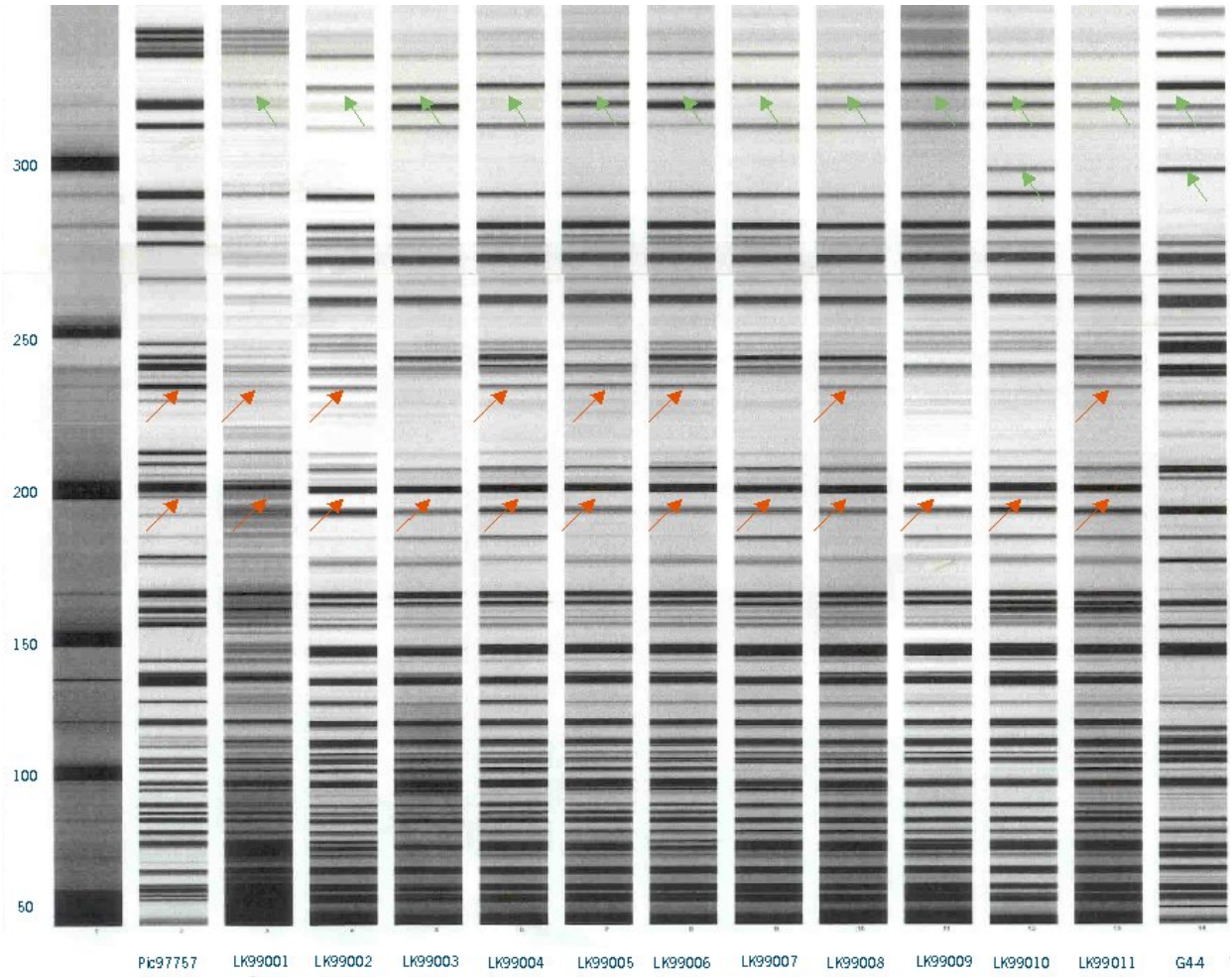

Figure 1. AFLP fingerprint of eleven F1 progeny (LK99001 to LK99011), the *Phytophthora infestans* (Pic97757) and *Phytophthora mirabilis* (G4-4) parental isolates; the left panel shows the mobility of markers each differing 50-bp in size. Indicated by the red arrows are clear examples of markers originating from *the P. infestans* parental isolate of which the lower one appears in all F1 progeny, whereas the upper one shows segregation in F1 progeny. The bands with green arrows are examples of markers originating from the *P. mirabilis* parental isolate, of which the upper one appears in all F1 progeny, and the lower shows segregation in F1 progeny.

Inheritance of mating type in interspecific progeny

Irregular distribution of mating type was observed in the F1 progeny, and in the F2 and BC1 progeny derived from the hybrid offspring (Table 2). Mating type assessment in the progeny of the cross G9-5 x LK99002 (BC1a) was difficult to perform as only few oospores were formed in test crosses with *P. mirabilis* strains G9-1 (A1) and G9-5 (A2). *P. infestans* tester strains were used to determine mating type of the BC1a progeny when no conclusive results were obtained with the *P. mirabilis* tester strains.

Increased linear growth rate in F1 progeny

Hybrid F1 progeny showed a significant increase in linear growth rate ($P < 0.001$) on minimal Plich medium as compared to *P. mirabilis* strains originating from the Toluca Valley and a diverse group of *P. infestans* strains (Table 1). The heterosis effect was lost in subsequent inter- and backcrosses, as the average linear growth rate of F2 and BC1 isolates did no longer deviate significantly from the *P. infestans* and *P. mirabilis* control groups or the parental strains (Table 3). The BC1 progeny derived from cross F80029 x LK99004 showed an increased growth rate when compared to the parental isolates, but this increase proved not statistically significant.

Mitochondrial DNA haplotype

In the F1 progeny, ten out of 11 isolates contained mitochondrial DNA originating from the *P. mirabilis* parent G4-4 (Ha*Pmir*I) whereas only one F1 isolate contained the mitochondrial DNA from the *P. infestans* parent Pic97757 (Ha*Pinf*Ia) (Table 1, Table 2). In the F2 progeny, and in the BC1a progeny, *P. mirabilis* mitochondrial DNA was present in both parental isolates and as a consequence all progeny contained haplotype Ha*Pmir*I. In the progeny from backcrosses BC1b and BC1c, the ratio Ha*Pmir*I:Ha*Pinf*Ia was 9:2 and 6:0, respectively. Overall, the number of progeny isolates inheriting the *P. mirabilis*-type mitochondrial DNA in crosses involving both Ha*Pmir*I and Ha*Pinf*Ia is significantly higher ($P<0.05$) than the number of isolates receiving the *P. infestans* type mitochondrial DNA. The expected ratio would be 1:1, assuming that both parental isolates have equal chance to act as a female partner, thus contributing its mitochondrial DNA to the progeny.

Table 3. Mean values of linear growth of isolates of *Phytophthora infestans* and *Phytophthora mirabilis*, and the interspecific offspring of crosses between these two species.

# individuals	Population	Linear mycelial growth[a]	
1	G4-4	4.9	bc[b]
1	Pic97757	5.6	abc
6	*P. infestans*	4.9	bc
22	*P. mirabilis*	5.0	bc
11	F1	7.1	a
14	F2a	4.6	c
21	F2b	5.0	bc
33	BC1a	5.5	abc
11	BC1b	6.6	ab
6	BC1c	4.9	c

[a] Mean linear growth in mm day^{-1}.

[b] isolates/populations which have no letters in common show a significant ($P<0.001$) difference in radial growth.

Host plant specificity

Pathogenicity indexes for the F1, F2 and BC1 hybrids are listed in Figure 2. F1 progeny derived from cross Pic97757 x G4-4 was highly pathogenic on tomato leaflets, whereas no symptoms were observed on potato and *M. jalapa* leaflets. One isolate, LK99001, was not pathogenic on either of the hosts tested, and its growth on agar and its sporangia production were impaired.

In the F2a progeny, ten out of 14 isolates infected tomato leaflets, whereas only one of those ten isolates infected potato leaflets. None of the F2a progeny could infect *M. jalapa* leaflets. In the progeny of cross F2b, ten out of 21 isolates infected tomato leaflets, of which seven could also infect potato leaflets. One of these isolates infected *M. jalapa*, tomato and potato (two replicates), thus combining the host range of both parental isolates (as tested in these experiments). Pathogenicity on potato was associated with pathogenicity on tomato; only isolates pathogenic on tomato leaflets also infected potato leaflets.

Discussion

The first objective of our study was to test the possibility of sexual reproduction between *P. infestans* and *P. mirabilis* isolates, and thus to examine possible occurrence of gene flow between these two species. Results from AFLP, mitochondrial DNA, and host range experiments indicate that the offspring generated in the cross between *P. infestans* isolate Pic97757 and *P. mirabilis* isolate G4-4 is the result of sexual recombination. AFLP analysis indicated that both parents transmitted one haploid nuclear genome to the F1 progeny, supported by the observation that a number of heterozygous markers from parental isolates did not show up in the F1 progeny. This confirms that genomes of the F1 progeny are the result of meiotic recombination rather than from a somatic fusion between parental isolates, as has been observed in other hybrids (Brasier et al., 2004; Ersek and Nagy, 2008). The F1 progeny shared most, but not all AFLP markers with the *P. infestans* and *P. mirabilis* parental isolates. Of the total number of markers in *P. infestans* isolate Pic97757, on average 22% did not show up in the F1 progeny. The estimated number of heterozygous markers in isolate Pic97757 would therefore amount to 44%. In the *P. mirabilis* parental isolate G4-4 the number of observed heterozygous markers is somewhat lower (estimated at 33%).

We propose a simple model that could describe the genetics of host specificity in *Phytophthora* species in clade 1c (Kroon et al., 2004) (Table 4). Pathogenicity on *S. lycopersicum* appears to be under simple genetic control. Based on our data, the locus regulating pathogenicity on tomato (designated as T for tomato) is presumably present in a homozygous dominant condition (T/T) in the *P. infestans* parental isolate Pic97757 and in a homozygous recessive condition (t/t) in the *P. mirabilis* parental isolate G4-4. Thus, the F1 progeny will be heterozygous (T/t) for pathogenicity on tomato. All the F1 progeny (except the non-fit isolate LK99001) was able to infect tomato, indicating that pathogenicity on tomato is inherited in a dominant fashion. This hypothesis holds true in the F2a progeny, where 75% of the isolates was able to infect tomato, which follows Mendelian genetics. However, in the F2b progeny, only 50% of the isolates did infect tomato leaflets, which might be due to an underestimation of infection potential. Part of the isolates is genetically able to infect tomato, but is impaired in causing infection of tomato possibly due to other (genetic) factors. An alternative explanation might come from observed lethality of certain combinations of genes from the two parental strains. Both mating type distribution and pathogenicity on tomato show a segregation ratio close to 2:1 in the F2b progeny, which might be an indication for differential lethality in part of the offspring.

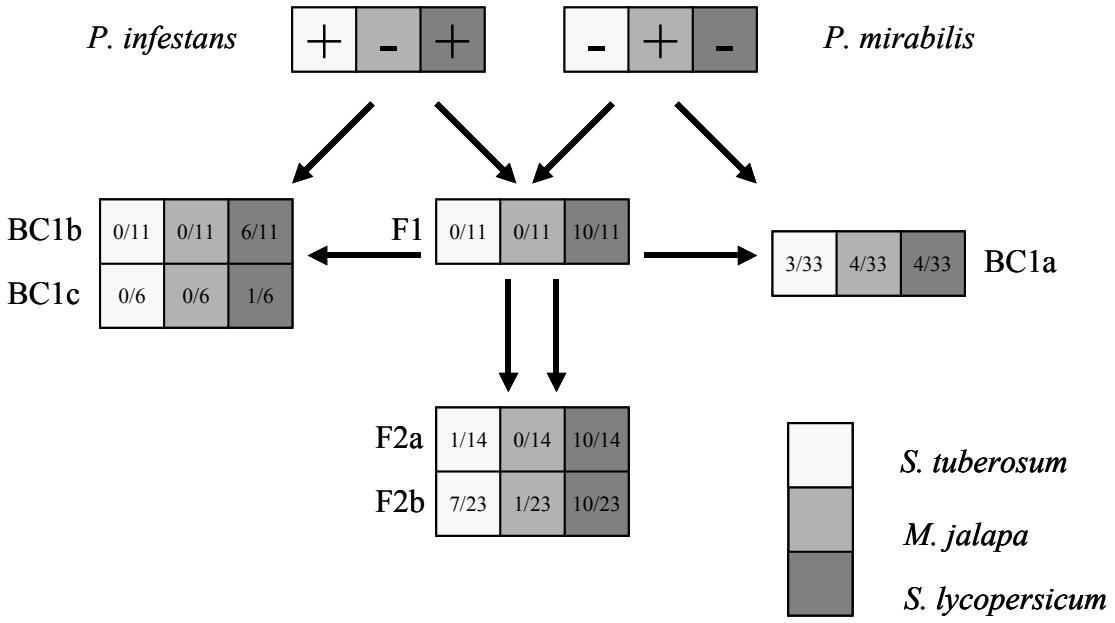

Figure 2. Mating scheme used for the generation of F1, F2, and BC1 offspring, involving *P. infestans* x *P. mirabilis* strains (Table 1). Numbers indicate the number of progeny that is pathogenic on the various hosts (*S. tuberosum*, *M. jalapa*, and *S. lycopersicum*) out of (/) the total number of progeny for that combination. + = pathogenic; - = not pathogenic.

Pathogenicity on potato and tomato could be under similar genetic control. Seven out of eight hybrid F2 progeny that did infect tomato did also infect potato. Thus, one locus controlling pathogenicity on tomato might also control pathogenicity on potato. It could be postulated that an incompatibility factor or elicitor (Jiang et al., 2008) is involved as a modulator (designated as P for potato). In most incompatible interactions, that resulted from inoculating hybrid progeny on potato leaves, a hypersensitive response (HR) was observed (Vleeshouwers et al., 2000). *P. infestans* isolate Pic97757 may be homozygous recessive (p/p = virulent) for this incompatibility factor and *P. mirabilis* isolate G4-4 homozygous dominant (P/P = avirulent). As a result the F1 progeny will be heterozygous (P/p = avirulent) and not able to infect potato; this in turn will result in an HR on potato leaflets for F1 progeny. In the F2a population, it was found that one out of 14 progeny infects both potato and tomato, nine out of 14 progeny infects tomato and causes an HR on potato, whereas four out of 14 progeny infects neither tomato nor potato, nor causes an HR on potato. The observed ratios are statistically in agreement with the expected ratios in the proposed model (Table 4).

For *M. jalapa*, the genetic regulation of pathogenicity appears to be more complex. The genetic background of the *M. jalapa* tester plants is not known, and these plants may contain race-specific resistance genes (*R*-genes). The *P. mirabilis* parental strains used in this

study did infect the *M. jalapa* genotype used, but only one F2b isolate was able to infect the same *M. jalapa* tester plant.

Skewed segregation ratios for mating type have been reported in earlier studies on crosses involving *P. infestans* isolates (Judelson et al., 1995; Van der Lee et al., 1997). The mechanism regulating the inheritance of the mating type locus appears to be complex, and has not been fully resolved. A1 isolates are considered heterozygous at the mating type locus (*A*/*a*) and A2 isolates homozygous (*a*/*a*) (Randall et al., 2003). It is postulated that deleterious or lethal loci are linked to the mating type locus, resulting in a complex locus with the characteristics of a balanced lethals system (Judelson et al., 1995). This lethality will result in the recovery of just two out of four possible meiotic rearrangements of alleles linked with mating type in the progeny. Some crosses result in a high rate of sterile progeny (Judelson, 1996). It is to be expected that these processes will often cause a deviation of the predicted Mendelian segregation ratio of 1:1 for mating type, as has been found in this study.

Mitochondrial DNA is uniparentally inherited in *Phytophthora*; the mating partner that provides the oogonium in a cross between two isolates transmits its mtDNA to the offspring. In theory, both isolates have an equal chance to be the "female" parent in a cross. Galindo and Gallegly (1960) and Goodwin and Fry (1994) already postulated the presence of "strong females" in crosses involving *P. infestans*. The latter found in their hybridization experiments with *P. infestans* and *P. mirabilis* that the *P. infestans* parental isolate predominately acted as the female partner. In our hybridization experiments, the *P. mirabilis* isolate proved to be the strongest female parent. These results indicate that strong femininity is to be regarded as an intraspecific rather than an interspecific trait in crosses between *P. infestans* and *P. mirabilis*.

Although typically a within-species phenomenon, hybrid vigour, or heterosis, can occur in sexual progeny of two distinct species (Shull, 1948; Gu and Ko, 2001). An increased growth rate is observed when parents involved in hybridization have accumulated deleterious mutations with a negative effect on growth rate (inbred lines), and are able to compensate each other's mutations in the offspring (Ingvarsson and Whitlock, 2000). This compensation will lead to enhanced vigour in the F1 progeny. The heterosis effect is only observed if both parents have a similar genetic constitution. When the differences in genetic constitution between parental strains are too high, the progeny is often impaired in growth due to genetic imbalance. The heterosis effect encountered in the F1 progeny indicates that the genetic constitution of *P. infestans* and *P. mirabilis* does not differ substantially.

Our data clearly demonstrate that *P. infestans* and *P. mirabilis* are sexually fully compatible. Viable, pathogenic, and fertile F1, F2 and BC progeny is produced in

interspecific crosses. Pathogenicity on tomato and potato appears to be under simple Mendelian genetic control in the hybrid progeny. The only restriction for interspecific hybridization and gene flow in nature between *P. infestans* and *P. mirabilis* seems to be the absence of a common host. This pre-zygotic reproductive isolation (Goodwin and Fry, 1994) proves to be a strong barrier, as only low levels of actual gene flow are observed between *P. infestans* and *P. mirabilis* populations in central Mexico

Our results indicate that tomato (*S. lycopersicum*) could act as a bridging host for *P. infestans* x *P. mirabilis* progeny. In both the F1 and F2 hybrid progeny, isolates were able to infect this host. Should sexual interaction between *P. infestans* and *P. mirabilis* take place in nature, for instance on senescent potato plants, *S. lycopersicum* could provide a suitable niche for F1 progeny. On this host the F1 hybrid progeny showed vigorous growth and extensive sporulation. This F1 progeny can then intercross, leading to F2 hybrid progeny, or F1 hybrids can mate with *P. infestans* isolates. This could even lead to progeny with an expanded host range when compared to the parental species, as has been shown in this study. Therefore the use of *S. lycopersicum* as a crop in areas where interspecific hybridization between *P. infestans* and *P. mirabilis* could occur should be avoided.

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3.2

Genetic Diversity of *Phytophthora infestans* sensu lato in Ecuador provides new insight into the origin of this important plant pathogen

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Abstract

The metapopulation structure of *Phytophthora infestans* sensu lato is genetically diverse in the highlands of Ecuador. Previous reports documented the diversity associated with four putative clonal lineages of the pathogen collected from various hosts in the genus *Solanum.* This paper simultaneously analyzes diversity of the complete collection of isolates, including a large number that had not yet been reported. This analysis confirmed the existence of three pathogen populations, which all appear to be clonal lineages, and which correspond to those previously reported as US-1, EC-1 and EC-3. No evidence was found from the analyses of recently collected isolates that would contradict earlier reports about these three lineages. In contrast, new data from a group of isolates from several similar hosts caused us to modify the previous description of the clonal lineage EC-2 and its previously proposed hosts, *Solanum brevifolium* and *Solanum tetrapetalum*. Given the uncertainty associated with the identification of these hosts, which all belong to the section *Anarrhichomenum*, we refer to them as the *Anarrhichomenum* complex, pending further taxonomic clarification. New pathogen genotypes associated with the *Anarrhichomenum* complex were isolated recently that are A1 mating type and Ia mitochondrial DNA (mtDNA) haplotype, and therefore differ from the previously described EC-2 lineage, which is A2 and Ic, respectively. Because of uncertainty on host identification, we do not know if the new genotypes are limited to one host species and therefore represent yet another host-adapted clonal lineage. For now, we refer to the new genotypes and previously described EC-2 genotypes, together, as the pathogen group attacking the *Anarrhichomenum* complex. Two A2 isolates identical to the previously described EC-2 archetype were collected from severely infected plants of pear melon (*Solanum muricatum*). Pear melon is generally attacked by US-1 and this is the first clear case we have documented where two distinct pathogen genotypes have caused severe epidemics on the same host. Based on presence of unique marker alleles (restriction fragment length polymorphism [RFLP], and mtDNA) and genetic similarity analysis using RFLP and amplified fragment length polymorphism (AFLP) data, EC-3 and isolates from the *Anarrhichomenum* complex are genetically distinct from all genotypes of *P. infestans* that have been reported previously. No current theory of historical migrations for this pathogen can adequately support a Mexican origin for EC-3 and genotypes of the *Anarrhichomenum* complex and they may, therefore, be palaeoendemic to the Andean highlands. To date, we have identified 15 hosts in the genus *Solanum*, in addition to the *Anarrhichomenum* complex and some unidentified species, of *P. infestans* sensu lato in Ecuador. Five of the *Solanum*

hosts are cultivated. One isolate was collected from *Brugmansia sanguinea*, which represents the first report from Ecuador of a host of this pathogen that is not in the genus *Solanum.* However, *P. infestans* sensu lato was only found on flower petals of *B. sanguinea.* This study provides new insights into the population structure of highly specialized genotypes of *P. infestans* sensu lato in the Andean highlands. The results are discussed in the light of previous hypotheses regarding the geographic origin of the pathogen.

Introduction

The late blight pathogen, *Phytophthora infestans* (Mont.) de Bary, which has a global distribution on potatoes and tomatoes, is believed to have originated in the highlands of central Mexico, causing mild epidemics on native wild tuber-bearing *Solanum* species (Goodwin et al., 1992; Grünwald et al., 2001; Reddick, 1939). Mexico is considered the center of origin of *P. infestans* because both mating types (A1 and A2) are present and there is a high genetic diversity for this pathogen in this region (Goodwin and Drenth, 1997; Grünwald et al., 2001; Niederhauser et al., 1954; Tooley et al., 1986). In contrast, only the A1 mating type and low genetic diversity had been found outside Mexico until the mid 1980s (Goodwin et al., 1992; Grünwald et al., 2001; Hohl and Iselin, 1984). An alternative hypothesis proposed the Andes, center of origin of the cultivated potato, as the center of origin for *P. infestans (*Abad and Abad, 1997). This hypothesis is based primarily on historical accounts of potato disease in the Andes, and appears to have little support in the scientific literature.

Late blight of potato first appeared in the US and Europe in the middle 1800s. Devastating late blight epidemics destroyed potato crops in Ireland during 1845 and 1846, contributing to poverty, starvation and emigration. Until recently, most isolates of *P. infestans* found outside North America belonged to the US-1 clonal lineage. This led to a hypothesis that US-1 had caused the original epidemics in Europe in the 1800s and then spread globally, presumably with seed trade (Fry et al., 1993; Goodwin et al., 1994). Recent analyses, however, of mitochondrial DNA of *P. infestans* in herbarium material presented evidence that a genotype different from US-1 was involved in the original epidemics in Europe (Ristaino et al., 2001).

During the 1980s, the A2 mating type of *P. infestans* was detected in Europe along with several new alleles for known markers (Drenth et al., 1993; Hohl and Iselin, 1984). A

second global migration from Mexico had taken place. The pathogen population in Europe is now highly diverse and there is evidence for sexual reproduction in several European countries (Andersson et al., 1998; Drenth, 1994; Turkensteen et al., 2000). As a variant to the hypothesis for the first and second global migrations of *P. infestans,* some authors proposed that earlier migrations also took place from Mexico to South America (Andrivon, 1996; Tooley et al., 1989) One hypothesis also advances the idea that *P. infestans* was originally introduced into Europe from South America and not directly from Mexico (Andrivon, 1996; Perez et al., 2001).

The brief description of the origins and migrations of *P. infestans* given above can be found in much greater detail in recent reviews (Andrivon, 1996; Goodwin, 1997). Nonetheless, the conclusions presented in these reviews are based primarily on studies involving isolates of *P. infestans* originating from potato, and to a lesser extent tomato. This is probably because late blight is an economically important disease on potatoes worldwide and possibly also because there appear to be few alternative hosts, apart from tomato, in the temperate zone where most of the research on the pathogen was done. In other parts of the world however, *P. infestans*, or species similar to *P. infestans*, are known to attack several other wild and cultivated hosts in the family Solanaceae. In Mexico, *P. infestans* attacks several wild tuber-bearing species of *Solanum.* Evidence from recent studies in Mexico indicates that these wild *Solanum* species are all attacked by *P. infestans* (Grünwald et al., 2001) although only limited gene flow exists between the subpopulations of the pathogen on wild *Solanum* species and cultivated potatoes (Flier et al., 2003). A new homothallic *Phytophthora* closely related to *P. infestans* was described recently as *Phytophthora ipomoeae* (Flier et al., 2002). In the Toluca valley of central Mexico, epidemics on solanaceous hosts are exclusively caused by *P. infestans* as the host range of *P. mirabilis* (Galindo and Hohl, 1985) and *P. ipomoeae* is restricted to non-solanaceous hosts.

The situation appears to be more complex in South America where a *Phytophthora* species very similar to *infestans* was found on hosts then described as *S. brevifolium* and *S. tetrapetalum* (Ordoñez et al., 2000). This pathogen group was designated the EC-2 clonal lineage of *P. infestans* sensu lato and it does not attack cultivated potatoes or tomatoes. Four host-adapted groups of *P. infestans* sensu lato have recently been described in Ecuador. Little variability was found within these groups and there appeared to be little or no gene flow among them, so all were considered to be clonal lineages. Their designations were EC-1, found on potato and tuber-bearing wild *Solanum* species (Forbes et al., 1997); EC-2, described above; US-1, found on tomato, cultivated pear melon (*Solanum muricatum*) and

Solanum caripense (Forbes et al., 1997; Oyarzun et al., 1998); and EC-3 found on cultivated tree tomato (*Solanum betaceum*) (Erselius et al., 1999). Novel RFLP bands were found in fingerprints of EC-2 and EC-3 genotypes , and the EC-2 lineage was characterized by a new mitochondrial DNA (mtDNA) haplotype, subsequently designated as Ic (Erselius et al., 1999; Oliva et al., 2002; Ordoñez et al., 2000). These results demonstrate that the Ecuadorian highlands harbor wide genetic diversity within *P. infestans* sensu lato that has not been detected in studies done elsewhere on pathogen populations attacking potato and tomato. Furthermore, preliminary assessment of recent isolates collected in Ecuador indicated that the full extent of pathogen diversity has not yet been described. In the present study, we describe genotypes of *P. infestans* sensu lato collected recently from several cultivated and wild hosts in the highlands of Ecuador. The new data cause us to modify some previous conceptions about the *P. infestans* sensu lato in Ecuador. The origin and historical migrations of this pathogen are discussed in light of our current understanding of the pathogen population structure in the Ecuadorian highlands.

Materials and Methods

Definitions

An operational definition for the taxonomic status of host-adapted populations of *P. infestans* sensu lato was applied throughout this paper. There is evidence suggesting that certain host-adapted clonal lineages present in Ecuador may belong to one or more *Phytophthora* spp. that have not yet been formally described (Ordoñez et al., 2000). The *P. infestans* sensu lato metapopulation is defined as a group of local pathogen populations with varying levels of genetic isolation based on geographic distance, temporal effects and host specialization. Nonetheless, for the purpose of the present publication, we consider these populations to be part of *P. infestans* sensu lato as no other described species accommodates them better.

Collection and isolation of P. infestans sensu lato

We collected and assessed with at least 3 markers over 450 single lesion isolates throughout the highlands of Ecuador from 1995 through 2002 (Table 1). Except for specific studies on pathogen populations attacking potato (Forbes et al., 1997) and tomato (Erselius et al., 2000; Oyarzun et al., 1998) no specific sampling plan was followed for the collection.

Collections from wild tuber-bearing species were oriented on previous trips by plant taxonomists (Spooner et al., 1992), but we also visited previously unexplored areas. Researchers in the national agricultural research institute (INIAP) gave useful information on the locations of plantations of the cultivated hosts pear melon (*S. muricatum*) and tree tomato (*S. betaceum*). In general, Ecuador is a center of diversity for the genus *Solanum* (Hijmans and Spooner, 2001) and most of the wild non tuber-bearing hosts we describe are common. Therefore, numerous trips were planned to unexplored areas based on knowledge on climatic conditions favorable for late blight development. Isolations were made by trapping with potato tuber slices, or with a selective medium (Forbes et al., 1997; Ordoñez et al., 2000; Oyarzun et al., 1998). Some isolates originating from plants of the *Anarrhichomenum* complex and from tree tomato were described previously (Erselius et al., 1999; Ordoñez et al., 2000). Some of the isolates from potato and tomato were also described using several genetic and phenotypic markers (Forbes et al., 1997; Oyarzun et al., 1998). A brief description of two isolates with A2 mating type isolated from pear melon (*S. muricatum)* was published recently

Table 1. Number of isolates of *P. infestans* sensu lato from different hosts in Ecuador evaluated for different markers

Host species	RFLP classification	Base[a]	MtDNA[b]	RFLP[c]	AFLP[d]	Metalaxyl[e]		
						R	I	S
Tuber bearing								
Solanum andreanum	EC-1, US-1	17	15	7	…	…	2	10
S. colombianum	EC-1	91	71	60	2	2	5	12
S. minutifoliolum	EC-1	11	9	11	2	…	…	4
S. paucijugum	EC-1	19	17	15	3	1	…	8
S. regularifolium	EC-1	1	1	1	1	…	1	…
S. solisii	EC-1	1	1	1	…	…	…	1
Solanum spp.		14	13	8	2	…	1	4
S. tuberosum, S. phureja (potato)	EC-1	49	43	22	5	11	8	19
S. tuquerrense	EC-1	9	8	5	…	…	…	9
Non tuber-bearing								
Brugmansia sanguinea	EC-2	1	1	1	…	…	…	1
S. betaceum (tree tomato)	EC-3	34	31	17	5	3	3	19
Anarrhichomenum complex	EC-2, EC-2.1[f]	67	66	29	13	…	1	36
S. caripense	US-1	25	22	8	1	…	10	7
S. lycopersicum (tomato)	US-1	77	49	13	4	2	21	28
S. muricatum (pear melon)	US-1, EC-2.1[f]	19	18	11	…	…	3	4
S. ochranthum	US-1	16	15	4	…	…	2	13
S. radicans	?	1	1	…	…	…	…	…
Total		452	381	213	38	…	…	…

[a] The base set of markers consists of mating type and banding patterns for *glucose-6-phosphate isomerase* and *peptidase*. [b] Mitochondrial DNA haplotype [c] Restriction fragment length polymorphism (RFLP) fingerprint [d] Amplified fragment length polymorphism (AFLP) fingerprint [e] R= resistant to metalaxyl, I = intermediate and S = sensitive. [f] The EC-2.1 sub-lineage was described previously (Ordoñez et al., 2000). The RFLP fingerprint of EC-2.1 differs from EC-2 by 3 bands.

(Adler et al., 2002). All isolates were maintained for short periods on Rye A or Rye B medium (Caten and Jinks, 1968) at 18ºC in the dark. Isolates are being stored for longer periods on Rye A agar slants at 15ºC with a 12 hour photoperiod in Quito, Ecuador (contact G. Forbes), and in liquid nitrogen at Plant Research International in Wageningen, the Netherlands (contact W. Flier).

Characterization of isolates

All isolates reported here (458) were tested for mating type, glucose-6-phosphate isomerase (*Gpi*) and peptidase (*Pep*) (Table 1). The majority of isolates from each host was also tested for mitochondrial DNA haplotype. Smaller samples from each host were fingerprinted using RFLP and/or AFLP technology. Metalaxyl resistance was also tested for a subset of isolates from most hosts. At least 2 isolates from each of the major pathogen groups were compared for sporangial dimensions and papilla shape using light microscopy and a video image system.

Metalaxyl resistance

Isolates were tested for resistance to 5 and 100 μg per ml metalaxyl in 10% unclarified V8 medium and classified as resistant, intermediate, or sensitive. Conditions of the test and criteria for classification were described previously (Forbes et al., 1997).

Isozyme electrophoresis

Isozyme electrophoresis for the enzymes *Gpi* and *Pep* was done on starch gels (Spielman, 1991) and on polyacrylamide gels. Polyacrylamide gel electrophoresis (PAGE) was done using 1 mm thick 7.5% gels with 25 mM Tris-0.19 M glycine, pH 8.8 as separating gel and electrode buffer. Bands were clearer when a 1 cm stacking gel (2.5% acrylamide 0.06 M Tris-HCl, pH 6.7) was used (Davis, 1964). PAGE gels were run with a constant current of 5 mA for 1 h and then increased to 10 mA. Voltage rose continuously throughout, from about 50 to 280 V. Electrophoresis was terminated when the bromophenol blue dye reached the bottom of the gel, at about 16 cm running length. Allozyme genotypes (inferred from banding pattern phenotypes) were scored as described by Spielman et al. (1991). Scorings represent mobility of the enzyme alleles relative to an allele designated as 100. Isolates with known alleles from the collection of W.E. Fry, Cornell University, were used for comparison.

DNA extraction

Isolates were grown for 10 to 14 days at 20 ºC in pea broth prepared by autoclaving 120 g of frozen peas in 1 l of water. The peas were removed by filtering through cheesecloth and the broth was autoclaved again. The mycelium was grown on pea broth, harvested, lyophilised and stored at –80 ºC. Lyophilised mycelium (10 to 20 mg) was ground in micro-centrifuge tubes with a pestle and sterile sand. Total DNA was extracted using the Puregene kit (Gentra/Biozym, Landgraaf, the Netherlands) according to manufacturers instructions or as previously described (Ordoñez et al., 2000). DNA was dissolved in 100 µl of TE (10 mM Tris-HCl [pH 8.0], 1mM EDTA [pH 8.0]) and stored at –20 ºC.

MtDNA haplotype

Mitochondrial DNA **(**mtDNA) haplotypes were determined by amplification of DNA of each isolate using primers designed for specific regions of the mitochondrial genome of *P. infestans* (Griffith and Shaw, 1998). PCR reactions were performed in a thermocycler (PTC100; MJ Research, Waltham, MA). Digestion of the amplified regions with *Cfo*I, *Msp*I*,* and *Eco*RI restriction enzymes yields restriction patterns by which the isolates can be classified into five haplotypes: Ia, Ib, IIa, IIb and Ic (Carter et al., 1990; Griffith and Shaw, 1998; Oliva et al., 2002).

RFLP fingerprinting

A total of 213 isolates were characterized using the moderately repetitive clone RG-57 (Goodwin et al., 1992) and the non-radioactive enhanced chemiluminescence (ECL) kit (Amersham, Eindhoven, the Netherlands) according to the manufacturer's instructions.

AFLP fingerprinting

DNA (250 ng) was digested in a 50-µl reaction volume with *Eco*RI (10 units) and *Mse*I (10 units) for 6 h at 37 ºC in restriction ligation buffer (10 mM Tris/Ac [pH 7.5], 10 mM MgAc, 50 mM KAc, 5 mM ditihiothreitol, and 50 ng μl^{-1} bovine serum albumin). Digestion was confirmed on agarose gels. Restriction fragments were ligated to *Mse*I adapters (5'GACGATGAGTCCTGAT/CTACTCAGGACTAGC 5') and *Eco*RI adaptors (5' CTCGTAGACTGCGTACC/CATCTGACGCATGGTTAA 5') using 0.1 µM *Eco*RI adapter, 1.0 µM *Mse*I adapter, 0.2 mM ATP and 2.4 units of T4 DNA ligase (Amersham Pharmacia Biotech, Uppsala, Sweden) (Baayen et al., 2000). Ligation was performed overnight at 10-

12ºC and the ligation products were diluted 10 times with filtered ultra pure water. Non-selective PCR amplification was performed using primers E00 (5' GACTGCGTACCAATTC 3') and Mse00 (5' GATGAGTCCTGAGTAA 3') for all restriction fragments. Non-selective PCR amplifications were performed in a PTC200 thermocycler (MJ Research) as described previously (Baayen et al., 2000). The amplified restriction fragment products were checked on 1.0% agarose gels. Selective PCR was performed in a 50 µl reaction volume with 5 µl of 20 X diluted amplification products with 200 µM dNTP and 5 ng of Cy5-labeled fluorescent Eco21 primer (5' CTCGTAGACTGCGTACC), and 30 ng of Mse16 (5' GATGAGTCCTGAGTAACC) primer. Products were loaded on Sequagel (Gentra/Biozym) polyacrylamide gels and run on an ALFexpress automatic sequencer (Amersham, Pharmacia Biotech). Conditions were 1500 V, 60 mA, 35 W, and 55ºC. On each gel, 36 samples were loaded together with flanking Cy5-labelled fluorescent 50 bp ladders (Amersham, Eindhoven, the Netherlands) and two reference isolates (Pic99016 and VK6C).

Data analysis

A subset of 37 recently collected isolates from a wide range of host plant species was selected for comparison of the resolution of multilocus and AFLP genotypes. Isozyme and mating type data were combined with RG57 RFLP fingerprints as described previously (Forbes et al., 1998) to create multilocus genotypes. AFLP patterns were analysed using Imagemaster ID software (Amersham, Eindhoven, the Netherlands). A total of 67 distinct and reproducible AFLP bands were identified using the primers Eco-21 and Mse-16. Bands were treated as putative single AFLP loci and a binary matrix containing the presence or absence of these reproducible bands was constructed and used for further analysis. The AFLP marker matrix is available upon request (W. Flier). Multilocus and AFLP genotypes were in separate cluster analyses using GENSTAT 6.1 (Lawes Agricultural Trust, Rothamsted Experimental Station UK). Similarity matrixes of multilocus and AFLP genotypes were constructed using the Jaccard method (Van Tongeren, 1995); and similarity between and within *P. infestans* populations was estimated using the HDISPLAY procedure in GENSTAT (Genstat 5, release 3). Trees were constructed from the distance matrixes using the unweighted pair-group method of averages (UPGMA) algorithm and visualized with TREECON version 1.3b (Van Peer and De Wachter, 1997).

Results

Collections of *P. infestans* sensu lato were made in all parts of the Ecuadorian highlands but were concentrated in the central provinces of Tungurahua and Pichincha (Figure 1). Locations for each collection event were logged and after 1999 each event was geo-referenced using a global positioning system; all data are stored in a database. Disease is ubiquitous and generally serious on potato and tomato during each of the two rain periods in the highlands, but especially in the most intense rainy season occurring between February and May. The situation was quite different for other hosts. Disease may be severe on the other cultivated hosts but it is generally sporadic. Disease is sporadic to rare on wild hosts, although at times severe epidemics were encountered. Our sample size was too small to make observations on the epidemiological dynamics of the diverse pathosystems we studied.

Figure 1. Collection sites in Ecuador and hosts of the collected isolates of *Phytophthora infestans* sensu lato obtained between 1995 and 2002. Each number symbolizes a specific host: 1 = potato (*Solanum tuberosum* or *S. phureja)*, 2 = tomato (*S. lycopersicum)*, 3 = *S. andreanum*, 4 = *S. colombianum*, 5 = *S. minutifoliolum*, 6 = *S. paucijugum*, 7 = *S. regularifolium*, 8 = *S. solisii*, 9 = *Solanum* spp., 10 = *S. tuquerrense*, 11 = *Brugmansia sanguinea*, 12 = tree tomato (*S. betaceum*), 13 = *Anarrhichomenum* complex, 14 = *S. caripense*, 15 = pear melon (*S. muricatum*), 16 = *S. ochrantum*, 17 = *S. radicans*.

Hosts of P. infestans sensu lato

All but one of the hosts we found for *P. infestans* sensu lato are in the genus *Solanum*, including five cultivated *Solanum* species: tomato (*S. lycopersicum*), tree tomato (*S. betaceum*), potato (*S. tuberosum* and *S. phureja*), pear melon (*S. muricatum*); nine wild species: *S. regularifolium, S. paucijugum, S. caripense*, *S. colombianun*, *S. minutifoliolum*, *S. tuquerrense, S. ochranthum, S. radicans* and *S. andreanum*; and one complex of wild species similar to *S. brevifolium* that we refer to as the *Anarrhichomenum* complex, pending further taxonomic clarification. One isolate was collected from *Brugmansia sanguinea* in 2001. All the wild species we studied grow in the highlands of Ecuador, but sometimes at different altitudes (Figure 1). Many tuber-bearing species and some wild species, such as *S. caripense* and the *Anarrhichomenum* complex grow at higher altitudes between 2500 and 4000 m above sea level. Tree tomato is generally grown somewhat lower, between 1500 and 2500 m above sea level. The identification of host species was based on published descriptions (Correll, 1962) and when possible verified by taxonomists.

Phenotypic description of P. infestans sensu lato in Ecuador

Most foliar lesions from which *P. infestans* sensu lato isolates were taken closely resembled those produced on potato after infection with *P. infestans*, although some wild host species showed slightly differing symptoms in the field. Leaves of plants in the *Anarrhichomenum* complex, for instance, which are small and thin (most are less than 3 cm long), blackened rapidly and sporulation was usually visible only at the edges of lesions. Leaflets of *S. ochranthum*, which are up to 20 cm long and fleshy, had extensive chlorosis associated with infection. The morphology of sporangia from all isolates we studied was typical of *P. infestans*: limoniform with a short pedicel (Erwin and Ribeiro, 1996). Sporangial dimensions were consistent with those published for *P. infestans* for all isolates except some from tomato, tree tomato and pear melon (US-1 clonal lineage), which were all larger than those reported previously.

Metalaxyl resistance

Except for the population attacking potato, *P. infestans* sensu lato in Ecuador appears to be generally sensitive or of intermediate resistance to metalaxyl (Table 1). A high proportion of isolates from the population attacking potato was also found resistant to metalaxyl in an earlier study in Ecuador (Forbes et al., 1997). In contrast to the potato-

attacking population, a high proportion of isolates in the US-1 lineage from tomato and *S. caripense* were of intermediate sensitivity, but few were resistant (Table 1). The number of isolates assayed for most hosts was small, making it difficult to do further comparisons. Nonetheless, it is interesting that only 2 of 19 isolates assayed from *S. colombianum* were resistant, a much lower proportion than found in isolates from cultivated potato. *S. colombianum* generally grows close to potato production zones and isolates coming from this wild species could not otherwise be differentiated from those on potato with the markers we used.

Genetic markers

Based on mating type, mtDNA haplotype and RFLP fingerprint, isolates coming from all hosts except the *Anarrhichomenum complex* fell into one of three distinct groups, US-1, EC-1 and EC-3 (Table 2). We consider these groups to be clonal lineages because all are A1 mating type, each has at least one unique marker allele, and we found very little polymorphism within each group. All three lineages were described previously for all markers except AFLP (Erselius et al., 1999; Oliva et al., 2002; Ordoñez et al., 2000) and the recent study did not produce any marker information that was inconsistent with the previous descriptions.

In contrast, isolates collected recently from plants in the *Anarrhichomenum* complex provided data that are not consistent with our previous descriptions. We have reported previously on isolates coming from *S. brevifolium* and *S. tetrapetalum*, for which host identifications were based on published descriptions (Correl, 1962; Ordoñez et al., 2000). It now appears, however, that one or more new host species may be involved and the identification of these hosts is uncertain, although all are vines with roots growing from nodes and belong to the section *Anarrhichomenum* of the genus *Solanum* (Lynn Bohs, personal communication). Pending further taxonomic clarification, we refer to this group of hosts as the *Anarrhichomenum* complex,

It is also now evident that the pathogen population associated with the *Anarrhichomenum* complex is more genetically diverse than we had thought. Some isolates, a total of 57, collected recently are similar to those reported previously as EC-2 in that they are A2 mating type, 100/100 and 76/100 for *Gpi* and *Pep*, respectively, Ic mtDNA haplotype and have one of two similar RFLP fingerprints (Oliva et al., 2002; Ordoñez et al., 2000). However, other isolates collected recently from the *Anarrhichomenum* complex are A1

mating type and Ia mtDNA haplotype, and are therefore quite distinct from earlier EC-2 isolates. Nonetheless, these Ia haplotype isolates have the typical RFLP fingerprint of the previously described EC-2 clonal lineage (Ordoñez et al., 2000). Furthermore, although the sample of isolates from the *Anarrhichomenum* complex is small, there appears to be more polymorphism for *Gpi* (Table 2) than we have found in the other groups of *P. infestans* sensu lato in Ecuador. Two isolates from the *Anarrhichomenum* complex had the 86/100 genotype for *Gpi* and one had the 90/100 genotype. The 86/100 genotype is generally associated with the US-1 lineage which has been found worldwide (Goodwin et al., 1994) and 90/100 is found in the EC-1 lineage and genotypes in Europe (Forbes et al., 1997).

Table 2. Some characteristics of sub-populations of *Phytophthora infestans* sensu lato found in Ecuador since 1995 and host species from which they were isolated

RFLP[a]	Clonal lineage[b]	Mating type	*Gpi*[c]	*Pep*[c]	mtDNA[c]	Hosts
US-1	US-1	A1	86/100	92/100	Ib	Tomato (*S. lycopersicum*), pear melon (*S. muricatum*), *S.caripense, S. ochranthum, S. andreanum*[d]
EC-1	EC-1	A1	90/100	96/100	IIa	Potatoes (*S. tuberosum* and *S. phueja*) and wild potatoes (*Solanum* spp. in section Petota)
EC-2, EC2.1[e]	?	A2	100/100[f]	76/100	Ic	*Anarrhichomenum* complex[g], pear melon (*S.muricatum*)
EC-2	?	A1	100/100[h]	76/100	Ia	*Anarrhichomenum* complex, *Brugmansia sanguinea*
EC-3	EC-3	A1	86/100	76/100	Ia	Tree tomato (*S. betaceum*)

[a] Restriction fragment length polymorphism (RFLP) fingerprints are named according to the clonal lineage with which they were associated in previous publications. [b] Designations for populations with very little variability and for which there is no evidence of sexual reproduction.[c] *Gpi = Glucose-6-phosphate isomerase*; *Pep = peptidase*; and mtDNA = mitochondrial DNA haplotype.[d] *S. andreanum* is a tuber-bearing species in the section Petota. It is also attacked by isolates of EC-1.[e] The EC-2.1 sub-lineage was described previously (Ordoñez et al., 2000). The RFLP fingerprint of EC-2.1 differs from EC-2 by 3 bands. [f] Two isolates of the *Anarrhichomenum complex* with the Ic mtDNA haplotype were 86/100 for *Gpi*.[g] Specific species in this complex have not yet been determined.[h] One isolate of the *Anarrhichomenum complex* with the Ia mtDNA haplotype was 90/100 for *Gpi*.

AFLP analysis

The AFLP analysis demonstrated that all isolates from the Ecuadorian highlands can be classified to one of two major clusters (Figure 2). Isolates from potato and its tuber-bearing wild relatives (EC-1 lineage) and tomato (US-1) formed one cluster, which is genetically distant to isolates from tree tomato (EC-3) and the *Anarrhichomenum* complex, the latter two forming the second major cluster. Each of these major clusters was divided into minor clusters. The first major cluster was clearly divided into EC-1 and US-1 lineages; the second clearly distinguished EC-3 from isolates attacking the *Anarrhichomenum* complex. Furthermore, the AFLP analysis separated isolates coming from the *Anarrhichomenum* complex into two groups, each one associated with a particular mtDNA haplotype and mating type combination.

Both EC-1 and US-1 minor clusters had isolates from several different hosts and there was no evidence of host-related grouping, as *S. tuberosum* was scattered across the EC-1 cluster. The taxonomy of the *Anarrhichomenum* complex is too obscure at this stage to tell whether any particular host species is associated with either of the pathogen groups isolated from the host complex. Results of the cluster analysis of the multilocus genotypes involving isozyme banding patterns, mating type and RFLP fingerprints are not presented here because they were generally similar to those from the AFLP analysis.

The AFLP analysis also indicated that isolates from the *Anarrhichomenum* complex and EC-3 have a low genetic similarity with potato-tomato related groups of *P. infestans* sensu lato, ranging from 0.488 to 0.536 and 0.380 to 0.435 for US-1 and EC-1, respectively (Table 3). High genetic similarities were calculated for isolates within EC-3 and within the two groups attacking the *Anarrhichomenum* complex.

A2 isolates, identical for marker data with the original EC-2 archetypal description (Ordoñez et al., 2000) were isolated from severely infected plants of pear melon. Prior to this, only US-1 isolates had been collected from pear melon. One isolate with Ia haplotype was also isolated from a lesion on flower petals of *Brugmansia sanguinea*. This is the first isolate of *P. infestans* sensu lato we have collected from a host outside the genus *Solanum*.

Table 3. Genetic similarity between and within groups of Ecuadorian isolates of *Phytophthora infestans* sensu lato based on AFLP fingerprinting

Group	US-1	EC-1	Ic	Ia	EC-3
Tomato (*S. lycopersicum*) (US-1)	0.826	…	...	...	...
Solanum spp. section Petota (EC-1)	0.651	0.834	...	...	...
Anarrhichomenum complex Ic (Ic)[a]	0.488	0.380	0.944	...	...
Anarrhichomenum complex Ia (Ia)[a]	0.536	0.435	0.834	0.955	…
Tree tomato (*S. betaceum*) (EC-3)	0.504	0.433	0.787	0.759	0.924

[a] Isolates from a complex of hosts similar to *S. brevifolium*. One group of isolates has a Ic mitochondrial haplotype and another group has the Ia haplotype.

Discussion

This study confirmed earlier studies in several ways, but also provided new insights into the population structure of *P. infestans* sensu lato in Ecuador. The present study confirmed the presence and previous description of clonal lineages EC-1, US-1 and EC-3 (Erselius et al., 1999; Oliva et al., 2002; Ordoñez et al., 2000). Data derived from some isolates collected recently, however, are not consistent with the previous description of the putative clonal lineage EC-2 (Ordoñez et al., 2000). EC-2 isolates collected previously were

all A2 mating type, and had 100/100 and 76/100 for *Gpi* and *Pep*, respectively. Furthermore, all had a new mtDNA haplotype that was subsequently described as Ic (Oliva et al., 2002) and one of two similar RFLP fingerprinting patterns (Ordoñez et al., 2000). Strains with the variant fingerprint were all collected in the valley of Nono and were designated EC-2.1 as a sub-group within the clonal lineage EC-2 (Ordoñez et al., 2000). Genotypes in the EC-2 lineage were believed to indiscriminately attack two host species, which were then identified as S. *brevifolium* and *S. tetrapetalum*. In recent collection trips, we found plants that do not appear to be either *S. brevifolium* or *S. tetrapetalum*, however all are woods vines with roots growing from nodes and belong to the section *Anarrhichomenum* in the genus *Solanum*. Further collection and taxonomic evaluation is needed to clarify which host species in section *Anarrhichomenum* occur in Ecuador and are hosts of *P. infestans* sensu lato

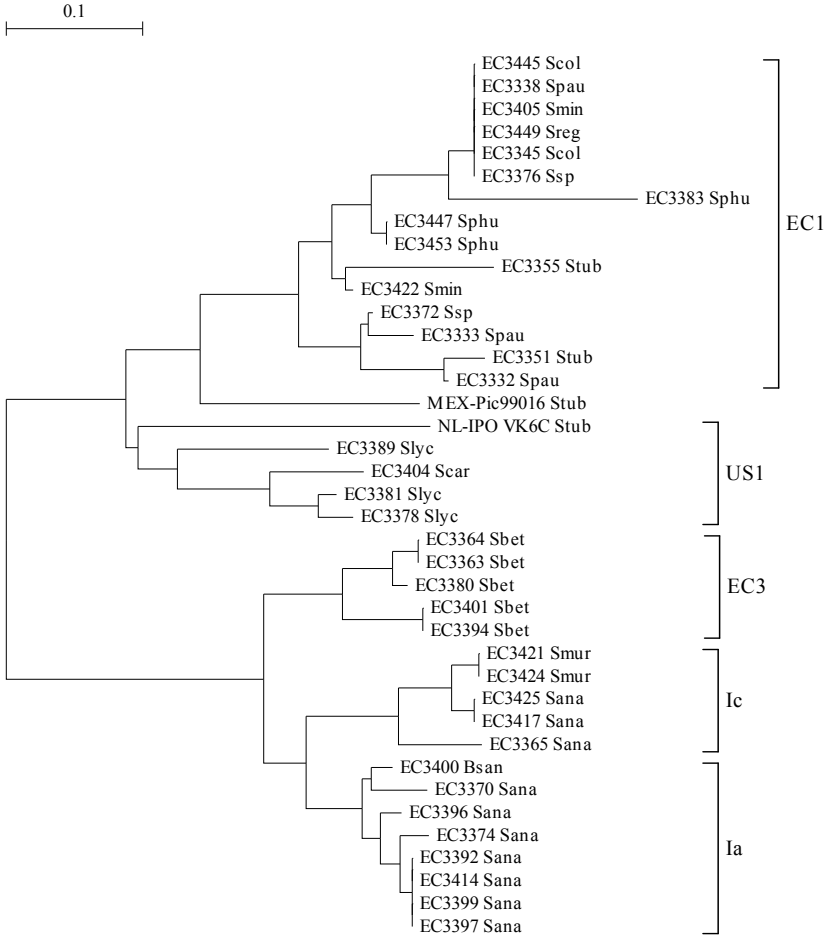

Figure 2. Phenogram of cluster analysis of amplified fragment length polymorphism (AFLP) banding patterns for 37 isolates of *P. infestans* sensu lato collected in Ecuador between 1995 and 2002. EC-1, US-1 and EC-3 refer to putative clonal lineages of the pathogen; anar Ic and anar Ia are two pathogen groups that together attack hosts in the *Anarrhichomenum* complex. Ic and Ia refer to the mitochondrial haplotype of isolates in each group. Mex-Pic99016 and NL-IPO-VK6C are from Mexico and the Netherlands, respectively. The latter was previously determined to be in the US-1 lineage. Bsan = *Brugmansia sanguinea*, Sana = *Anarrhichomenum* complex, Sbet = *S. betaceum* (tree tomato), Scar = *S. caripense*, Scol = *S. colombianum*, Slyc = *S. lycopersicum* (tomato), Smin = *S. minutifoliolum*, Smur = *S. muricatum* (pear melon), Spau = *S. paucijugum*, Sphu = *S. phureja* (potato), Ssp = *Solanum* spp., Stub = *S. tuberosum* (potato), and Sreg = *S. regularifolium.*

Analysis of isolates collected recently from these hosts revealed greater diversity than that previously described for EC-2 (Ordoñez et al., 2000). Some new isolates are A1 mating type and Ia mtDNA haplotype. Although these represent important differences with the EC-2 lineage as described previously (A2 mating type and Ic mtDNA haplotype), the new A1 isolates have RFLP fingerprints identical to the archetype RFLP fingerprint of EC-2. Furthermore, recent isolates from the *Anarrhichomenum* complex that have an A2 mating type and Ic haplotype have the EC-2.1 fingerprint, which we thought previously was only associated with the pathogen populations in the relatively isolated valley of Nono (Ordoñez et al., 2000). To add to the confusion, two isolates with A2 mating type and Ic haplotype have the 86/100 genotype for *Gpi* and one isolate (A1 mating type, Ia haplotype) is 90/100 for *Gpi*. Each of these *Gpi* genotypes occurs in one or more of the 3 clonal lineages found in Ecuador: US-1, EC-1 and EC-3. With our limited sample we cannot speculate on how these *Gpi* genotypes occurred in the *Anarrhichomenum* complex but gene flow between this group and the more common potato and tomato types of *P. infestans* can not be excluded until further studies are carried out.

Previous studies had established a one-host/one-pathogen hypothesis for the host pathogen relationship of *P. infestans* sensu lato in Ecuador. A particular pathogen lineage could be associated with several hosts, but each host species was associated with one primary pathogen lineage. This was particularly true for potato (EC-1 lineage) and tomato (US-1), but also for tree tomato (EC-3), *S. caripense* (US-1), *S. colombianum* and other tuber-bearing *Solanaceae* (attacked by EC-1). Other pathogen genotypes may infect a host as alternative pathogens, but these are weak and not epidemiologically significant (Erselius et al., 1999; Forbes et al., 1997; Ordoñez et al., 2000; Oyarzun et al., 1998). For example, both EC-1 and US-1 had been isolated from *S. ochranthum*, but EC-1 appeared to be a weak pathogen in the field and this was subsequently confirmed by inoculations on detached leaves (Erselius et al., 1999). EC-1 was probably only evident on *S. ochranthum* in the absence of inoculum from the primary pathogen lineage.

Recent observations indicate that cultivated pear melon may be an exception to the one-host/one-pathogen hypothesis. Pear melon is generally attacked by genotypes of the US-1 lineage in all areas where we have collected in Ecuador, including near the town of Baños. However, a few isolates taken from severely infected plants near Baños were identical to those previously described as EC-2 lineage (A2 mating type, Ic haplotype). The RFLP fingerprint was also identical to EC-2.1 (Ordoñez et al., 2000). These isolates were subsequently found to be highly aggressive on pear melon in detached-leaf inoculations. This

observation is important for two reasons. First it is the only case we have documented in Ecuador of major epidemics being caused by two distinct pathogen genotypes on the same host. Second, the two pathogen groups are A1 and A2 mating type, respectively, greatly increasing the potential for sexual reproduction (Adler et al., 2002). Reduced viability and low pathogenicity of offspring from *P. infestans* sensu lato crosses made in Ecuador (Oliva et al., 2002) are consistent with earlier work on oospore viability in *P. infestans* (Flier et al., 2001). The hypothesis that the risk of recombination may be greater between co-existing genotypes that are genetically distant or between local and introduced genotypes (Brasier et al., 1999) is pertinent to the Ecuadorian situation because genotypes infecting pear melon are genetically distant.

Other hosts may also be attacked by more than one pathogen group but we have not found them because of limited sample size. On one occasion we isolated both US-1 and EC-1 lineages from plants of *S. andreanum* (Table 2) all growing within a 50 m radius. Since that time, however, all isolates taken from *S. andreanum* have been EC-1 and we have no clear explanation for the earlier presence of US-1.

The genetic diversity of *P. infestans* sensu lato in wild *Solanum* species found in Ecuador is extremely wide, although more different genotypes are found in sexual reproducing populations (e.g. Mexico and the Netherlands). Three clonal lineages and one heterogeneous group as well as a high diversity in the mitochondrial DNA were found in association with different host species in the genus *Solanum* and beyond. The pathogen groups attacking the *Anarrhichomenum* complex and the EC-3 lineage are quite different from any genotypes of *P. infestans* described to date. It appears highly unlikely that either was introduced on potato seed, which is considered the primary means of long distance transport of this pathogen (Fry et al., 1993). An earlier study on isolates from the *Anarrhichomenum* complex, which were then designated as EC-2 clonal lineage, compared them with genotypes from a global database using multi-locus marker data (Ordoñez et al., 2000). EC-2 was different from any genotype of *P. infestans* reported previously. The AFLP analysis done here confirmed the earlier study in that it showed that isolates from the *Anarrhichomenum* complex are distinct from genotypes of *P. infestans* found on potato and tomato. Therefore, it is extremely difficult to speculate on the time when the *Anarrhichomenum* complex pathogens and EC-3 were possibly introduced into South America, or on the mechanism of introduction. A more plausible alternative hypothesis to explain the presence of these distinctive groups is that they are indigenous to the Andean highlands of South America, surviving in humid refugia during dry seasons. The hypothesis that these pathogen groups represent a

palaeoendemic population is supported by the fact that several of their alleles, including the mitochondrial haplotype of some isolates, have not yet been reported for other populations of the *P. infestans*, not even those studied in Mexico (Forbes et al., 1998). The taxonomic status of the isolates assigned to the *Anarrhichomenum* complex and EC-3 clonal lineage is still unresolved. A detailed taxonomic study including quantitative assessment of gene flow and construction of a molecular phylogenetic tree based on conserved DNA sequences may elucidate their relatedness to populations of the pathogen that attack potato and tomato.

Based on our limited study of resistance to the systemic fungicide metalaxyl, it would appear logical that high resistance was found in isolates coming from cultivated crops which are regularly sprayed with fungicides (potato, tomato and tree tomato). The apparent difference in frequency of resistance between wild tuber bearing hosts and potato is interesting and merits further consideration. However, the difference could be due to sampling in some potato fields where metalaxyl had been used, which would increase the frequency of resistant isolates.

The economic importance and social significance of *P. infestans* have been historically aligned with the disease it causes on potato, and to a lesser extent tomato (Schumann, 1991). The potato and tomato populations, however, appear to represent only two of several specialized forms of this pathogen. In terms of significance for humans, other specialized forms that attack tree tomato and pear melon are extremely important in the areas where these crops are produced and are in fact among the most eminent threats to the survival of these native crops in the Andean highlands of Ecuador.

It is important to note that our study was not exhaustive and even greater genetic diversity in *P. infestans* sensu lato probably exists in Ecuador. Furthermore, because of the relatively small sample size that we have been able to analyze, we do not know to what extent the new diversity we report here is due to geographic sub structuring or is temporal in nature. For that reason, it is difficult to conclude whether the population of *P. infestans* sensu lato in Ecuador is stable or changing. The sub-populations on potato and tomato appear to be stable (Erselius et al., 2000; Forbes et al., 1997; Oyarzun et al., 1998) but we do not know if this is the case for new diversity found in sub-populations on pear melon or the *Anarrhichomenum* complex.

At this time, the diversity of *P. infestans* sensu lato reported in Ecuador is much greater than that reported in other Andean countries. We assume that, to some extent, the diversity exists but has not been described in other countries. We know, for example, that tree tomato is severely affected by late blight in Colombia although we are not aware of reports

demonstrating that the pathogen is EC-3. Furthermore, plant species in the *Anarrhichomenum* complex are found throughout the Andes (Corell, 1962) and these may be attacked by *P. infestans* sensu lato. For these reasons we are unable to comment at this time on the geographic limits of the diversity we have found.

Our ability to manage this important plant pathogen may be greatly enhanced when we better appreciate its genetic potential and further investigation in the Andean highlands is clearly warranted.

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3.3

Phytophthora andina sp. nov., a newly identified heterothallic pathogen of solanaceous hosts in the Andean highlands

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Abstract

A blight disease on fruits and foliage of wild and cultivated *Solanum* spp. was found to be associated with a new species of *Phytophthora*. The proposed novel species is named *Phytophthora andina* Adler & Flier, sp. nov. based on morphological characteristics, pathogenicity assays, mitochondrial DNA haplotyping, AFLP fingerprinting and nuclear and mitochondrial DNA sequence analyses. Isolates of *P. andina* (n=48) from the Andean highland tropics of Ecuador were collected from 1995 to 2006. *P. andina* is closely related to *P. infestans* and has semipapillate, ellipsoidal sporangia borne on sympodially branched sporangiophores. It is heterothallic and produces amphigynous antheridia. The species consists of several clonal lineages, including the EC-2 and EC-3 RFLP lineages, which were described previously as *P. infestans*. Approximately 75% of isolates react as compatibility type A2 when paired with an A1 compatibility type isolate of *P. infestans*. However, when A2 isolates from the *Anarrhichomenum* section of *Solanum* were paired in all combinations, viable oospores were obtained in several crosses suggesting that there is a unique compatibility interaction in *P. andina* that is complementary to that described in *P. infestans.* Nuclear and mitochondrial sequence analysis supported the species designation of *P. andina*. This newly identified heterothallic pathogen shares a common ancestor with *P. infestans* and may have arisen from hybridization events with sister taxa in the Andes.

Introduction

The Andean highlands are considered a centre of origin and diversity for the plant genus *Solanum* (Hawkes, 1990). In addition to potato (*Solanum tuberosum*) and tomato (*S. lycopersicum*), several solanaceous crops have been domesticated there, including *S. betaceum* known as tomate de árbol or tree tomato, and as tamarillo outside the Andes, *S. muricatum* known as pepino dulce, pepino or pear melon, and *S. quitoense* known as naranjilla (Ecuador) or lulo (Colombia). The first two are spreading globally, while the latter are primarily restricted to the Andes (Popenoe et al., 1989). Cultivated *Solanum* crops are often grown in close proximity to wild *Solanum* spp. including *S. caripense, S. hispidum* and *S. ochranthum* and the poorly defined species in the *Anarrhichomenum* section (e.g., *S. tetrapetalum*, *S. sodiroi, S. siphonobasis,* and *S. brevifolium*). Although the taxonomy of tuber bearing *Solanum* spp. and solanaceous fruit crops remains complex, most species have

been described and classified (Spooner et al., 2003). Much less is known, however, about the phylogenetic relationships and taxonomy of non-tuber bearing wild *Solanum* spp.

Phytophthora populations associated with *Solanum* hosts in the Andes appear to be more complex than was originally believed. Until 1994, genetic diversity studies in Ecuador revealed only limited variation in the local populations of *P. infestans* on cultivated species. Late blight epidemics on potato and tomato were exclusively caused by the EC-1 and US-1 clonal lineages of *P. infestans*, respectively (Forbes et al., 1997; Oyarzun et al., 1998). Both lineages belong to the A1 mating type and reproduce clonally. This simple appraisal of the genetic structure of the pathogen population began to change in 1995 when four *Phytophthora* isolates were collected from blighted leaves of a plant in Ecuador then identified as *S. brevifolium*. The isolates from *S. brevifolium* were the A2 mating type when crossed with A1 tester isolates of *P. infestans* and were initially identified as *P. infestans* although they were recognized as novel and characterized by a previously unknown mtDNA haplotype (Ic) and a new RFLP fingerprint (EC-2) (Ordoñez et al., 2000). A total of 53 isolates were collected between 1996 and 1999 from hosts then identified as *S. brevifolium* and *S. tetrapetalum*. Based on a unique RFLP fingerprint, with limited polymorphism, a new mtDNA haplotype and A2 mating type, these isolates were classified in a new clonal lineage of *P. infestans* designated EC-2. Further study of the hosts revealed that the original 53 isolates came from one or more *Solanum* species in the *Anarrhichomenum* section of *Solanum* (Spooner et al., 2003). Ordoñez and co-workers did not rule out the possibility that the EC-2 lineage was indigenous to Ecuador. The identification of the clonal lineage as *P. infestans* remained unsatisfactory so the isolates were classified as *Phytophthora infestans* sensu lato (Ordoñez et al., 2000).

In a subsequent study which included a systematic sampling of foliar *Phytophthora* pathogens from various wild and cultivated solanaceous hosts, Adler et al. (2004) reported the presence of another clonal lineage (EC-3) on *S. betaceum*. These authors also found numerous isolates that were not of the EC-2 lineage, attacking plants in the *Anarrhichomenum* complex. These isolates were the A1 mating type and Ia mtDNA haplotype. In the study by Adler et al. (2004), amplified fragment length polymorphism (AFLP) fingerprinting confirmed the presence of host specific groups of isolates, but the taxonomic status of these *Phytophthora* isolates attacking several non-tuber bearing *Solanum* spp. remained unresolved.

The phylogeny of the genus *Phytophthora* has been the subject of several recent studies in which molecular techniques were used to supplement morphological classification (Waterhouse, 1963) and facilitate phylogenetic inference (Cooke et al., 2000; Kroon et al.,

2004; Martin and Tooley, 2003). Sequence variation for the ITS region of *Phytophthora* species has been shown to be useful for resolving the general relationships within the genus (Cooke et al., 2000), but proved insufficient to discriminate among *P. infestans*, *P. mirabilis*, *P. phaseoli* and *P. ipomoeae*, which are all placed in Group IV (sensu Waterhouse) and clade Ic (sensu Cooke) (Flier et al., 2002). This lack of resolution of the clade Ic species using ITS DNA has led to other studies with more variable DNA regions. Martin and Tooley (2003) sequenced part of the mitochondrial *Cox I* gene while Kroon et al. (2004) used parts of the *β-tubulin*, translation elongation factor 1-alpha, NADH-4 and *Cox I* gene to construct a high-resolution molecular phylogeny for the genus *Phytophthora*. The latter study demonstrated that the EC-2 clonal lineage of *P. infestans* sensu lato was closely related to *P. infestans, P. ipomoeae,* and *P. mirabilis*, yet not identical to them, thus stimulating discussion on the origin of the *Phytophthora* spp. in Ecuador. Although a new species had not been formally described, Kroon et al. (2004) referred to the one isolate from Ecuador they had examined as *P. andina*. That isolate had come from an *Anarrhichomenum* host and corresponded to the EC-2 lineage of Ordoñez et al. (2000), and to our knowledge, this was the first reported use of the name. Wattier et al. (2003) also concluded that isolates from *Anarrhichomenum* complex hosts were distinct from *P. infestans* based on mitochondrial inter-gene sequences, although the species name *P. andina* was not used in their report. Four isolates of the EC-2 lineage from *Anarrhichomenum* hosts were included in a coalescent analysis in which a South American origin was inferred for *P. infestans* and were referred to as *P. andina* (Gomez-Alpizar et al., 2007). The four isolates used in that study shared a common ancestor with *P. infestans* but were derived from a distinct mitochondrial lineage (Ic) that had undergone many mutations.

In an effort to clarify the taxonomy of this group of *Phytophthora* isolates from solanceous hosts in Ecuador, phylogenetic analysis of 11 EC-2 and 8 EC-3 isolates was conducted using *Cox* I gene and intron 1 of *Ras* gene sequences. EC-2 isolates with the Ic mtDNA haplotype were considered distinct and were designated *P. andina* (Gomez-Alpizar et al., 2008). No clear description of the new species was given in that study. Furthermore, the taxonomy of the EC-3 group of isolates from *S. betaceum* remained unclear since few EC-3 isolates were examined and some of these grouped more closely to *P. infestans* than *P. andina* in the mitochondrial genealogy. Using microsatellite (SSR) markers, Oliva et al. (2007), found that EC-2 and EC-3 lineages were clearly separated from the US-1 and EC-1 lineages of *P. infestans*. However, the authors referred to the whole population as *P. infestans*, only

mentioning *P. andina* in reference to earlier papers. In a recent and comprehensive multi-locus phylogeny of the genus *Phytophthora*, two Ecuadorian isolates corresponding to the EC-2 lineage were considered closely related to, but distinct from *P. infestans* (Blair et al., 2008). In reference to an uncertain taxonomic designation for the isolates, the authors referred to the taxon as *P.* sp "*andina*".

All the previous studies examining the genetic relatedness among clade Ic species of *Phytophthora* species and isolates from the putative *P. andina, i.e.,* those originally described as EC-2 and EC-3 lineages of *P. infestans (*sensu lato*)* have suggested a close relationship between this novel species and *P. infestans* and *P. mirabilis* (Blair et al., 2008; Gomez-Alpizar et al., 2008; Kroon et al., 2004; Wattier et al., 2003) but a species description has not been given. Thus, as recognized by Blair et al. (2008) with their use of quotation marks, the taxon sometimes referred to as *P. andina* has not been defined. *Phytophthora* species attacking solanaceous hosts in the Andes cause severe damage on several regionally important crops and provide a very interesting model of host/pathogen coevolution (Oliva et al., 2007). Further studies of these pathogens are to be expected and taxonomic uncertainty of the species could hinder progress.

In this paper, we formally describe the new species *P. andina*, and clarify its taxonomic status and relatedness to *P. infestans.* To achieve this, we investigated a representative sample of blight-causing *Phytophthora* isolates collected between 1995 and 2006 from the highlands of Ecuador. The new species is described on the basis of morphological characteristics, host range, mating behaviour, allozyme genotype, RLFP genotype, and mitochondrial DNA haplotype. Genetic relatedness to *P. infestans* is based on one mitochondrial gene (*Cox II*), ITS sequences and a portion of a single-copy nuclear gene (*Ras*). AFLP fingerprinting was used to explore genetic similarities between host specific groups of *P. andina* and their relatedness to other *Phytophthora* species in the Ic clade that attack *Solanum*, *Ipomoea* and *Mirabilis* spp. Results are discussed in relation to previous studies, particularly for relatedness to *P. infestans* (Oliva et al., 2007) and for host range (Chacón Acosta, 2007).

Materials and methods

Pathogen collection and maintenance

Isolates of *Phytophthora* species used in this study were obtained from diseased host plants collected during sampling trips throughout the Ecuadorian Andes (Figure 1, Table 1). Isolates were collected in almost all provinces of Ecuador between 1995 and 2006, ranging from 800 to 3800 meters over sea level. A map describing the main collection areas was published by Adler et al. (2004). Individual 1 cm^2 pieces of diseased leaves and fruits were transferred to selective Rye B agar (Caten and Jinks, 1968) amended with vancomycin (100 mg), polymyxin-B (500 mg), ampicillin (200 mg), rifampicin (20 mg), pentachloronitrobenzene (67 mg) and benomyl (100 mg) in 1000 ml of distilled water. Colonies growing on selective media were transferred to Rye B agar for initial identification as *Phytophthora* species. All isolates were maintained for short periods on Rye A (RA) or Rye B agar at 18°C in the dark (Ordoñez et al., 2000). Isolates were stored for longer periods on RA slants at 15°C with a 12 h photoperiod in Quito, Ecuador and in liquid nitrogen at Plant Research International in Wageningen, the Netherlands. Isolates were compared with reference isolates of *P. infestans*, *P. mirabilis*, *P. ipomoeae,* and *P. phaseoli* from the *Phytophthora* culture collection at Plant Research International (Table 1).

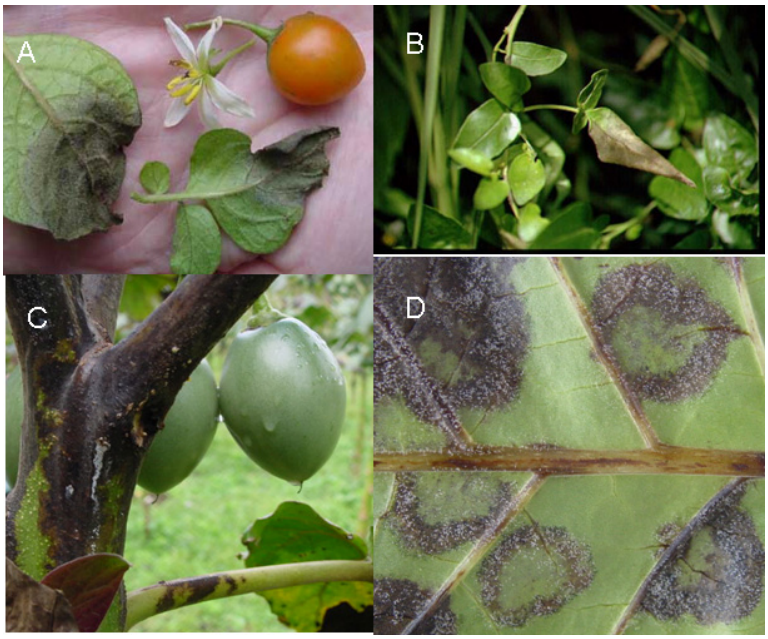

Figure 1. Blight symptoms caused by *P. andina* on *Solanum* species belonging to the *Anarrhichomenum* complex (A,B), and on *S. betaceum* (tree tomato) (C,D).

Table 1. Characteristics of isolates of *Phytophthora andina, P. infestans*, *P. mirabilis*, *P. ipomoeae,* and *P. phaseoli* used in morphology, sexual compatibility, AFLP fingerprinting and analysis of nuclear and mitochondrial DNA sequences.

Isolate	Species	Origin	Host	Allozyme[a] Genotype		MtDNA[b] Haplotype	RG57 RFLP[c]	Mating type[e]	Used in[d]
				Pep	*Gpi*				
EC1836	*P. andina*	Ecuador	*Anarrhichomenum*	76/100	100/100	Ic	EC-2	A2	a, b
EC3163	*P. andina*	Ecuador	*Anarrhichomenum*	76/100	100/100	Ic	EC-2	A2	d
EC3186	*P. andina*	Ecuador	*Anarrhichomenum*	76/100	100/100	Ic	EC-2	A2	a, b
EC3189	*P. andina*	Ecuador	*Anarrhichomenum*	76/100	100/100	Ic	EC-2	A2	a, b
EC3190	*P. andina*	Ecuador	*Anarrhichomenum*	76/100	100/100	Ic	EC-2	A2	a, b
EC3229	*P. andina*	Ecuador	*Anarrhichomenum*	76/100	100/100	Ic	EC-2	A2	a, b
EC3231	*P. andina*	Ecuador	*Anarrhichomenum*	76/100	100/100	Ic	EC-2	A2	a, b
EC3232	*P. andina*	Ecuador	*Anarrhichomenum*	76/100	100/100	Ic	EC-2	A2	a, b
EC3233	*P. andina*	Ecuador	*Anarrhichomenum*	76/100	100/100	Ic	EC-2	A2	a, b
EC3261	*P. andina*	Ecuador	*Anarrhichomenum*	76/100	100/100	Ic	EC-2	A2	a, b
EC3262	*P. andina*	Ecuador	*Anarrhichomenum*	76/100	100/100	Ic	EC-2	A2	a, b
EC3323	*P. andina*	Ecuador	*S. quitoense*	76/100	86/100	Ia	nd	A1	d
EC3358	*P. andina*	Ecuador	*Anarrhichomenum*	76/100	100/100	Ic	EC-2	A2	a, b
EC3363	*P. andina*	Ecuador	*S. betaceum*	76/100	86/100	Ia	EC-3	A1	c
EC3364	*P. andina*	Ecuador	*S. betaceum*	76/100	86/100	Ia	EC-3	A1	c
EC3365	*P. andina*	Ecuador	*Anarrhichomenum*	76/100	100/100	Ic	EC-2	A2	c
EC3369	*P. andina*	Ecuador	*Anarrhichomenum*	76/100	100/100	Ic	EC-2	A2	a, b,e
EC3370	*P. andina*	Ecuador	*Anarrhichomenum*	76/100	100/100	Ia	EC-2	A1	c
EC3371	*P. andina*	Ecuador	*Anarrhichomenum*	76/100	100/100	Ia	EC-2	A1	e
EC3375	*P. andina*	Ecuador	*Anarrhichomenum*	76/100	100/100	Ia	EC-2	A1	e
EC3380	*P. andina*	Ecuador	*S. betaceum*	76/100	86/100	Ia	EC-3	A1	c, e
EC3392	*P. andina*	Ecuador	*Anarrhichomenum*	76/100	100/100	Ia	EC-2	A1	e
EC3394	*P. andina*	Ecuador	*S. betaceum*	76/100	86/100	Ia	EC-3	A1	c
EC3395	*P. andina*	Ecuador	*S. betaceum*	76/100	100/100	Ia	EC-3	A1	e
EC3396	*P. andina*	Ecuador	*Anarrhichomenum*	76/100	100/100	Ic	EC-2	A2	c
EC3399	*P. andina*	Ecuador	*Anarrhichomenum*	76/100	100/100	Ia	EC-2	A1	a, b
EC3400	*P. andina*	Ecuador	*Brugmansia spp.*	76/100	100/100	Ia	EC-2	A1	e
EC3402	*P. andina*	Ecuador	*S. betaceum*	76/100	100/100	Ia	EC-3	A1	e
EC3414	*P. andina*	Ecuador	*Anarrhichomenum*	76/100	100/100	Ic	EC-2	A2	a, b
EC3417	*P. andina*	Ecuador	*Anarrhichomenum*	76/100	100/100	Ia	EC-2	A1	c
EC3421	*P. andina*	Ecuador	*S. muricatum*	76/100	100/100	Ic	EC-2	A2	c, e
EC3423	*P. andina*	Ecuador	*S. betaceum*	76/100	100/100	Ia	EC-3	A1	e
EC3424	*P. andina*	Ecuador	*S. muricatum*	76/100	100/100	Ic	EC-2	A2	c
EC3514	*P. andina*	Ecuador	*S. betaceum*	76/100	86/100	Ia	EC-3	A1	d
EC3540	*P. andina*	Ecuador	*Anarrhichomenum*	76/100	100/100	Ic	EC-2	A2	e
EC3542	*P. andina*	Ecuador	*S. betaceum*	76/100	100/100	Ia	EC-3	A1	e
EC3561	*P. andina*	Ecuador	*S. quitoense*	76/100	86/100	Ia	nd	A1	d
EC3644	*P. andina*	Ecuador	*S. betaceum*	76/100	86/100	Ia	EC-3	A1	d
EC3650	*P. andina*	Ecuador	*Anarrhichomenum*	76/100	100/100	Ia	EC-2	A1	d
EC3658	*P. andina*	Ecuador	*Anarrhichomenum*	76/100	100/100	Ic	EC-2	A2	a, b
EC3674	*P. andina*	Ecuador	*Anarrhichomenum*	76/100	100/100	Ic	EC-2	A2	d
EC3783	*P. andina*	Ecuador	*S. hispidum*	76/100	100/100	Ic	EC-2	A2	d
EC3816	*P. andina*	Ecuador	*Anarrhichomenum*	76/100	100/100	Ic	EC-2	A2	d
EC3818	*P. andina*	Ecuador	*Anarrhichomenum*	76/100	100/100	Ia	EC-2	A1	a, d
EC3830	*P. andina*	Ecuador	*S. hispidum*	76/100	100/100	Ic	EC-2	A2	d
EC3860	*P. andina*	Ecuador	*Solanum sp.*	76/100	100/100	Ic	nd	A2	d
EC3875	*P. andina*	Ecuador	*S. betaceum*	76/100	86/100	Ia	EC-3	A1	d

EC3120	*P. infestans*	Ecuador	*S. ochranthum*	96/100	90/100	IIa	nd	A1	e
EC3241	*P. infestans*	Ecuador	*S. ochranthum*	92/100	86/100	Ib	US-1	A1	d, e
EC3322	*P. infestans*	Ecuador	*S. quitoense*	76/100	86/100	Ib	nd	A1	d
EC3338	*P. infestans*	Ecuador	*S. paucijugum*	96/100	90/100	IIa	EC-1	A1	e
EC3351	*P. infestans*	Ecuador	*S. tuberosum*	96/100	90/100	IIa	EC-1	A1	c
EC3355	*P. infestans*	Ecuador	*S. tuberosum*	96/100	90/100	IIa	EC-1	A1	c
EC3361	*P. infestans*	Ecuador	*S. tuberosum*	96/100	90/100	IIa	EC-1	A1	e
EC3378	*P. infestans*	Ecuador	*S. lycopersicum*	92/100	86/100	Ib	US-1	A1	c, e
EC3379	*P. infestans*	Ecuador	*S. minutifoliolum*	96/100	90/100	IIa	EC-1	A1	e
EC3381	*P. infestans*	Ecuador	*S. lycopersicum*	92/100	86/100	Ib	US-1	A1	c, d, e
EC3382	*P. infestans*	Ecuador	*S. tuberosum*	96/100	90/100	IIa	EC-1	A1	e
EC3383	*P. infestans*	Ecuador	*S. phureja*	96/100	90/100	IIa	EC-1	A1	c
EC3389	*P. infestans*	Ecuador	*S. lycopersicum*	92/100	86/100	Ib	US-1	A1	c, d
EC3390	*P. infestans*	Ecuador	*S. solisii*	96/100	90/100	IIa	EC-1	A1	e
EC3404	*P. infestans*	Ecuador	*S. caripense*	92/100	86/100	Ib	US-1	A1	c, e
EC3413	*P. infestans*	Ecuador	*S. paucijugum*	96/100	90/100	IIa	EC-1	A1	e
EC3415	*P. infestans*	Ecuador	*S. minutifoliolum*	96/100	90/100	IIa	EC-1	A1	e
EC3435	*P. infestans*	Ecuador	*S. colombianum*	96/100	90/100	IIa	EC-1	A1	e
EC3445	*P. infestans*	Ecuador	*S. colombianum*	96/100	90/100	IIa	EC-1	A1	e
EC3447	*P. infestans*	Ecuador	*S. phureja*	96/100	90/100	IIa	EC-1	A1	c
EC3528	*P. infestans*	Ecuador	*S. andreanum*	96/100	90/100	IIa	EC-1	A1	e
EC3529	*P. infestans*	Ecuador	*S. tuberosum*	96/100	90/100	IIa	EC-1	A1	d
EC3531	*P. infestans*	Ecuador	*S. tuberosum*	96/100	90/100	IIa	EC-1	A1	e
EC3532	*P. infestans*	Ecuador	*S. tuberosum*	96/100	90/100	IIa	EC-1	A1	e
EC3534	*P. infestans*	Ecuador	*S. andreanum*	96/100	90/100	IIa	EC-1	A1	e
EC3620	*P. infestans*	Ecuador	*S. ochranthum*	92/100	90/100	Ib	EC-1	A1	d
EC3774	*P. infestans*	Ecuador	*S. ochranthum*	96/100	86/100	Ib	US-1	A1	d
EC3808	*P. infestans*	Ecuador	*S. muricatum*	92/100	86/100	Ib	US-1	A1	d
EC3842	*P. infestans*	Ecuador	*S. habrochaites*	92/100	86/100	Ib	US-1	A1	d
PICWF021	*P. mirabilis*	Mexico	*M. jalapa*[f]	96/96	100/100	*HaPmir*I	nd	nd	c
PICWF031	*P. mirabilis*	Mexico	*M. jalapa*	96/96	100/111	*HaPmir*I	nd	nd	c
PICWF005	*P. mirabilis*	Mexico	*M. jalapa*	96/96	90/111	*HaPmir*I	nd	nd	c
PICWF053	*P. mirabilis*	Mexico	*M. jalapa*	96/96	100/111	*HaPmir*I	nd	nd	c
PIC991-2	*P. ipomoeae*	Mexico	*I. longipedunculata*[f]	78/78	108/108	*HaPipo*I	nd	sf	c
PIC991-1	*P. ipomoeae*	Mexico	*I. longipedunculata*	78/78	108/108	*HaPipo*I	nd	sf	c
PIC993-3	*P. ipomoeae*	Mexico	*I. longipedunculata*	78/78	108/108	*HaPipo*I	nd	sf	c
PIC993-4	*P. ipomoeae*	Mexico	*I. longipedunculata*	78/78	108/108	*HaPipo*I	nd	sf	c
CBS556.88	*P. phaseoli*	nd	*Phaseolus* sp.	nd	nd	*HaPpha*I	nd	sf	c
PiIa[g]	*P. infestans*	AY894835	*S. tuberosum*	nd	nd	Ia	nd	nd	d
PiIIa	*P. infestans*	AY898627	*S. tuberosum*	nd	nd	IIa	nd	nd	d
PiIIb	*P. infestans*	AY898628	*S. tuberosum*	nd	nd	IIb	nd	nd	d
Pmi	*P. mirabilis*	AY129214	*M. jalapa*	nd	nd	nd	nd	nd	d
Pphas	*P. phaseoli*	AY129221	*P. lunatus*	nd	nd	nd	nd	nd	d

[a] Allozyme alleles scored at the putative *peptidase* and *glucose*-6-*phosphate isomerase* loci. [b] Haplotype nomenclature according to Griffith and Shaw (1998) and Oliva et al. (2002). [c] Previously published RFLP fingerprints (Adler et al., 2004; Forbes et al., 1997; Ordoñez et al., 2000). Both Ia and the Ic mtDNA groups of isolates from *Anarrhichomenum* hosts can have either the EC-2 or EC-2.1 fingerprints. [d] Isolates used in a= morphology, b= sexual compatibility, c= AFLP analysis, d= sequence studies, and e = pathological test. [e] Mating type determined after crosses with tester isolates A1 (*P. infestans*) and A2 (*P. andina*) isolates. [f] *M. jalapa* = *Mirabilis jalapa,* and *I. longipedunculata* = *Ipomoea longipedunculata*. [g] PiIa correspond to GenBank accession AY894835, nd = not determined; sf = self fertile.

Morphology

Sixteen isolates were examined in morphological studies (Table 1). Sporangia were observed in an aqueous suspension prepared by flooding a 10 d old culture grown on pea agar (PA), prepared by autoclaving 120 g of frozen peas in 1 liter of water for 20 min at 121°C. The broth was filtered through cheesecloth to remove the peas and then autoclaved again with 17 g agar per liter. Measurements of 40 randomly selected sporangia for each isolate were made at 400x magnification. Oospores of *P. andina* were measured from four in vitro crosses: EC3189 x EC3399, EC3233 x EC3414, EC3658 x EC3358 and EC3818 x EC3261. Diameters of 40 randomly selected oospores for each cross were measured.

Sexual compatibility and mating system

The *P. infestans* mating system is known to comprise two compatibility types, A1 and A2, and has been used as a phenotypic marker and for genetic studies for decades (Gallegly and Galindo, 1958). The following experiment was designed to learn more of the *P. andina* mating system. Ten isolates from *P. andina* (*Anarrhichomenum* complex; mtDNA Ic) were used for oospore production studies (Tables 1 and 2). These isolates, which reacted as A2 compatibility type when paired with A1 isolates of *P. infestans,* were mated in all possible combinations (Table 2). Parental isolates were transferred to RA plates and cultured for 10 d at 20°C. Agar discs (5 mm diameter) taken from margins of fast growing colonies of the parental isolates (1 disc for each parent) were placed 30 mm apart in a plate (9 cm diameter) containing 10 ml RA, amended with 0.05 g l^{-1} ß-sitosterol to stimulate oospore production (Flier et al., 2001). Two plates were prepared for each parental combination. Plates were incubated for 15 d at 20°C in the dark. The presence and density of oospores was determined microscopically at 100 x magnification.

Allozyme analysis

Electrophoretic banding patterns for the allozymes glucose-6-phosphate isomerase (*Gpi*, EC 5.3.1.9) and peptidase (*Pep*, EC 3.4.3.1) were assessed on polyacrylamide gels following a published method (Ordoñez et al., 2000). Polyacrylamide gel electrophoresis (PAGE) was performed using 1 mm thick 7.5 % gels with 25 mM Tris, 0.19 M glycine, pH 8.8 as buffer. Bands were clearer when a 1 cm stacking gel (2.5% acrylamide 0.06 M Tris-HCl, pH 6.7) was used. PAGE gels were run with a constant current of 5 mA for 1 h, and then increased to 10 mA. Voltage rose continuously throughout, from approximately 50 to 280 V.

Allozyme genotypes (inferred from banding pattern phenotypes) were scored as described by Spielman et al. (1990).

Table 2. Average numbers of oospore per mm^2 from *in vitro* pairings of isolates of *P. andina* collected from hosts of the section *Anarrhichomenum*.

	Isolate number									
Isolate	1	2	3	4	5	6	7	8	9	10
EC1836 (1)	0									
EC3186 (2)	0	0								
EC3190 (3)	0	14	0							
EC3229 (4)	5	12	0	0						
EC3231 (5)	1	0	19	3	0					
EC3232 (6)	24	0	0	11	18	0				
EC3233 (7)	19	102	0	41	155	129	0			
EC3261 (8)	0	0	3	56	7	9	41	0		
EC3262 (9)	0	0	63	79	25	46	84	0	0	
EC3369 (10)	0	0	2	2	1	160	7	0	0	0

DNA extraction

Isolates were grown for 10-14 d at 20°C in pea broth. The mycelium grown on pea broth was harvested, lyophilized, and stored at –80°C. Lyophilized mycelium (10-20 mg) was ground in micro-centrifuge tubes with a pestle and sterile sand. Total DNA was extracted using the Puregene kit (Gentra/Biozym, Landgraaf, the Netherlands) according to manufacturer's instructions. DNA was dissolved in 100 µl of TE (10 mM Tris-HCl pH 8.0, and 1 mM EDTA pH 8.0) and stored at –20°C.

MtDNA haplotypes

Mitochondrial haplotypes were determined by amplification of DNA of each isolate using primers designed for 4 specific regions of the mitochondrial genome of *P. infestans* (Griffith and Shaw, 1998). PCR reactions were performed in a PTC200 thermocycler (MJ Research, Gentra/Biozym, Landgraaf, the Netherlands). Digestion of the amplified regions with restriction enzymes *Cfo*I, *Msp*I, and *Eco*RI yielded banding patterns used for classification into mtDNA haplotypes: Ia, Ib, IIa, IIb (Griffith and Shaw, 1998) and Ic (Oliva et al., 2002).

RFLP fingerprinting

RFLP fingerprints were generated according to Ordoñez et al. (2000) using the moderately repetitive clone RG57 (Goodwin et al., 1992). Restriction fragments were detected using the ECL non-radioactive kit (Amersham, Eindhoven, the Netherlands) according to the manufacturer's instructions.

AFLP fingerprinting

All *Phytophthora* isolates from Ecuador and described in a previous study (Adler et al., 2004) were analysed with isolates of *P. ipomoeae*, *P. mirabilis* and *P. phaseoli*. AFLP fingerprinting was performed according to Flier et al. (2003). Selective PCR was performed using Cy5-labeled fluorescent *Eco*21 primer (5' CTCGTAGACTGCGTACC), and *Mse*16 primer (5' GATGAGTCCTGAGTAACC). A total of 172 distinct and reproducible AFLP bands were identified in 29 isolates. Bands were treated as putative single AFLP loci and a binary matrix for presence (1) or absence (0) of these reproducible bands was constructed and used for further analysis. A similarity matrix of AFLP genotypes was constructed using Rogers' modified distance method in the statistical software package GENSTAT version 6.1 (Lawes Agricultural Trust, Rothamstead Experimental Station, UK). Trees were constructed from the distance matrix using the Neighbor Joining algorithm and visualized with TREECON version 1.3b software (Van de Peer and De Wachter, 1997).

Mitochondrial and nuclear sequences

The mitochondrial *CoxII* (cytochrome c oxidase subunit II) gene, the ITS regions and part of the single copy *Ras* gene (Chen and Roxby, 1996) were amplified and sequenced for a subset of 22 *Phytophthora* isolates collected in Ecuador (Table 1). The *CoxII* gene was amplified by primers FM35 (5'CAGAACCTTGGCAATTAGG) and FMPh-10b (5'GCAAAAGCACTAAAAATTAAATATAA) (Martin and Tooley, 2003; Martin et al., 2004). ITS DNA was amplified using ITS primers 4 and 6 (Cooke et al., 2000). Primers Iras F (5'TTGCAGCACAACCCAAGACG) and Iras R (5'TGCACGTACTATTCGGGGTTC) were used to amplify a 223-bp fragment of the intron 1 of the *Ras* gene (Gomez-Alpizar et al., 2007). Reactions were carried out in a 50 µl volume according to authors' description. DNA was sequenced in the forward and reverse direction using a Macrogen system (Macrogen Sequencing System, Korea). Sequences were aligned and chromatograms checked using BioEdit (Hall, 1999) before alignment with other *Phytophthora* species from GenBank.

Phylogenetic relationships were inferred using neighbor-joining analysis based on the Kimura 2-parameter distance method (Kimura, 1980) under MEGA 3 (Kumar et al., 2004). In addition, the Maximum Likelihood (ML) method described in the RAxML web-interface were also performed (Stamatakis et al., 2005).

Pathogenicity

Plant material used for detached-leaf assays came from different sources: host plants collected directly in their natural environments, plants derived from true potato seed (from berries collected in the field) and seed tubers and plants growing near CIP station in Quito, Ecuador. Growing condition and plant accessions were used exactly as previously described by Chacón (2007). Fourteen different inoculation assays were conducted between 2001 and 2004 based on the availability of plant material. In potato, the first pair of fully expanded lateral leaflets of the upper 1/3 of the plant was used. In *S. ochranthum*, the first pair of fully expanded lateral leaflets of mature and well developed leaves was used. In tomato, *S. caripense*, and *Anarrhichomenum* hosts, whole leaves were employed. In the case of *S. betaceum*, pieces of the central part of the leaves were cut out and used. For the sake of simplicity, hereafter we refer to all tissues as leaves. Prior to inoculation leaves were washed with tap water, towel dried and stored abaxial side up in the lids of inverted Petri dishes which contained water agar (4%) in the base. Petri dishes were subsequently used as high humidity chambers for inoculation and incubation.

Before inoculation, the isolates from tuber bearing species (such as potato and wild relatives), were multiplied on leaves of their original hosts to restore aggressiveness after cultivation on rye agar. Isolates from *S. ochranthum* and *B. sanguinea* were washed directly from pure culture plates (Rye-A). Sporangia from either living tissue or Rye A medium were rinsed several times with distilled water over a 12 μm filter before use. Irregular sporulation among the isolates made it impossible to use a common inoculum concentration, and therefore concentrations ranged from 2.0 to 3.0 x 10^4 sporangia/ml. One 10 μl drop of sporangia suspension was placed on the abaxial side of each leaf close to the midrib.

Given the large number of host-pathogen interactions, difficulty in producing consistent inoculum and the further problem of erratic availability of tissue from such wide diversity of hosts, assays were run sequentially, as inoculum and leaf tissue were available. Overall 79 host-pathogen interactions were assessed and the following applied to all cases. Each host genotype by isolate interaction was assessed on six different leaves, distributed into three different Petri dishes. Petri dishes containing inoculated leaflets were incubated by

replication (in blocks) at 15 ± 2°C with 14 h of fluorescent light per day. Inoculated leaves were monitored 5, 6, 7 and 10 (sometimes 11) days after inoculation for disease development and were visually and qualitatively rated for the presence or absence of disease. Infection (compatible reaction) was defined as the presence of expanding, sporulating lesions. Any other type of reaction observed (no reaction at all; macroscopic hypersensitive response (nonsporulating, nonexpanding lesions); and expanding, nonsporulating lesions) was rated as incompatible. In total, 12 *P. andina* and 18 *P. infestans* isolates were tested (Tables 1 and 3).

Table 3. Pathogenicity of isolates of *Phytophthora infestans* and *P. andina* on several Solanaceous hosts in Ecuador.

	Host of origin[a]	RFLP[b]	N[c]	Inoculated host[d]								
				tbr1	tbr2	mur	lyc	car	och	bet	ana1	ana2
P. infestans	*S. caripense*	US-1	1	**0**/+	+	+	--	0	+	--	--	--
	S. lycopersicum	US-1	2	0/+	+	0	+	0	0/+	--	+	0
	S. ochranthum	US-1	2	0/+	+	--	0/+	0	+	0	0	0
	S. andreanum	EC-1	2	+	+	0	+	0/+	0/+	0	+	**0** /+
	S. colombianum	EC-1	2	+	+	0	--	--	+	--	--	0
	S. minutifoliolum	EC-1	2	+	+	0	--	--	+	--	--	0
	S. paucijugum	EC-1	2	+	+	--	--	0	+	--	0/+	**0**/+
	S. solisii	EC-1	1	+	+	0	+	0	0	--	0/+	--
	S. tuberosum	EC-1	4	+	+	0	+	0	+	0	0/+	+
P. andina	*S. betaceum*	EC-3	5	0	0	0	0/+	0	0	+	+	**0**/+
	B. sanguinea[e]	EC-2	1	--	--	--	--	0	0	--	+	--
	Anarrhichomenum 1	EC-2	3	0	**0**/+	**0**	0/+	0	0	--	+	+
	Anarrhichomenum 2	EC-2	2	0	0	0	--	0	0	--	--	--
	S. muricatum	EC-2	1	0	0	0	--	--	--	--	--	--

[a] *Solanum* host species from which *P. infestans* and *P. andina* isolates were collected. [b] Pathogen RFLP profile according to RG57 probe.[c] Number of isolates tested. [d] Inoculated host species: tbr1 and tbr2 = *S. tuberosum* cultivar Cruza 148 and Yungay, respectively; mur = *S. muricatum*; lyc = *S. lycopersicum* cultivar Roma; car = *S. caripense*; och = *S. ochranthum* ; bet = *S. betaceum* ; ana1 and ana2 = taxonomically undefined plants from the section *Anarrhichomenum*. 0 = incompatible reaction. Includes: no reaction at all; macroscopic hypersensitive response (nonsporulating, nonexpanding lesions); and expanding, nonsporulating lesions. + = compatible reaction. expanding, sporulating lesions. -- = not inoculated. In bold face, reaction most commonly observed. [e] *Brugmansia sanguinea* belongs to the Solanaceae family.

Results

Morphology

All 16 isolates of *P. andina* (Table 1) assessed for morphology grew well on RA and PA with a radial colony expansion of 8 to12 mm per day at 20°C. Neither hyphal swellings nor chlamydospores were produced in culture. Sporangia were semipapillate (Figure 2) and although shape and dimension of sporangia varied considerably, no consistent differences among isolates were observed (Figure 2). Length of sporangia ranged from 39.5 to 62.5 µm (average 44.6 µm), and width from 14.0 to 24.8 µm (average 17.8 µm), with a length:width ratio ranging from 2.4 to 2.7. Zoospores were released within 2 h from sporangia in water at 10ºC, but zoospore release was also observed at 20ºC on a few occasions. Oospores were formed following mating between compatible parental isolates on RA Petri plates (Figure 3). No systematic differences in oospores size could be detected among the four crosses of *P. andina* isolates, which ranged overall in diameter from 26.5 to 37.5 µm.

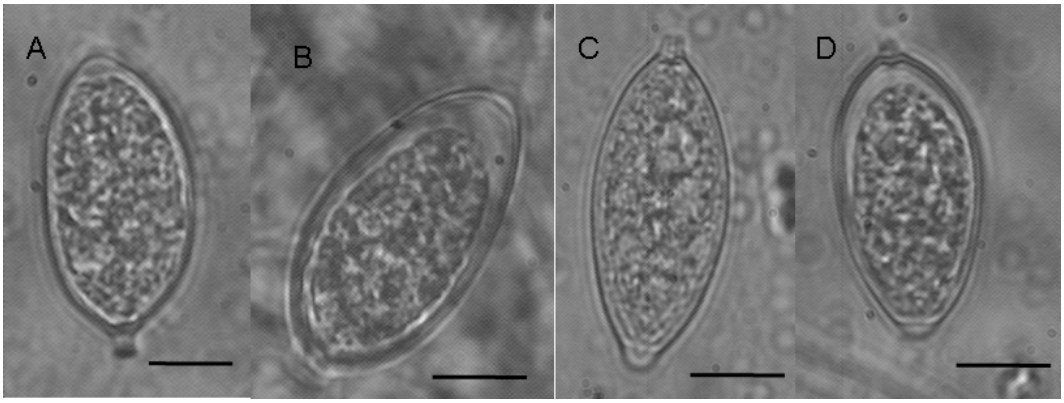

Figure 2. Sporangia of *P. andina* produced on pea agar of isolate EC3189 (A), isolate EC3186 (B) and isolate EC3232 (C,D). Bars represent 10 µm.

Sexual compatibility in P. andina

P. andina is a heterothallic species. No oospores were observed in single cultures, and previous studies had established that *P. andina* isolates interacted with *P. infestans* and could be classified as either A1 or A2 compatibility type (Chacón et al., 2006; Ordoñez et al., 2000). However, we observed here that a complementary system exists in *P. andina,* as oospores were produced in crosses between isolates that had been identified as A2 by a *P. infestans*

tester. Oospores were observed in 29 out of 45 pair-wise crosses within *P. andina* (Table 2) and oospore density at the interaction zone ranged from 1 to 160 oospores mm^{-2} with an average of 39.2 oospores mm^{-2} (Table 2). Oospores had a dark brown and thick outer wall but appeared viable with a large central vacuole and translucent cytoplasm (Figure 3). Crosses of *P. mirabilis* x *P. andina* and *P. infestans* x *P. andina* also yielded oospores of similar diameter, but these were lighter in colour (not shown).

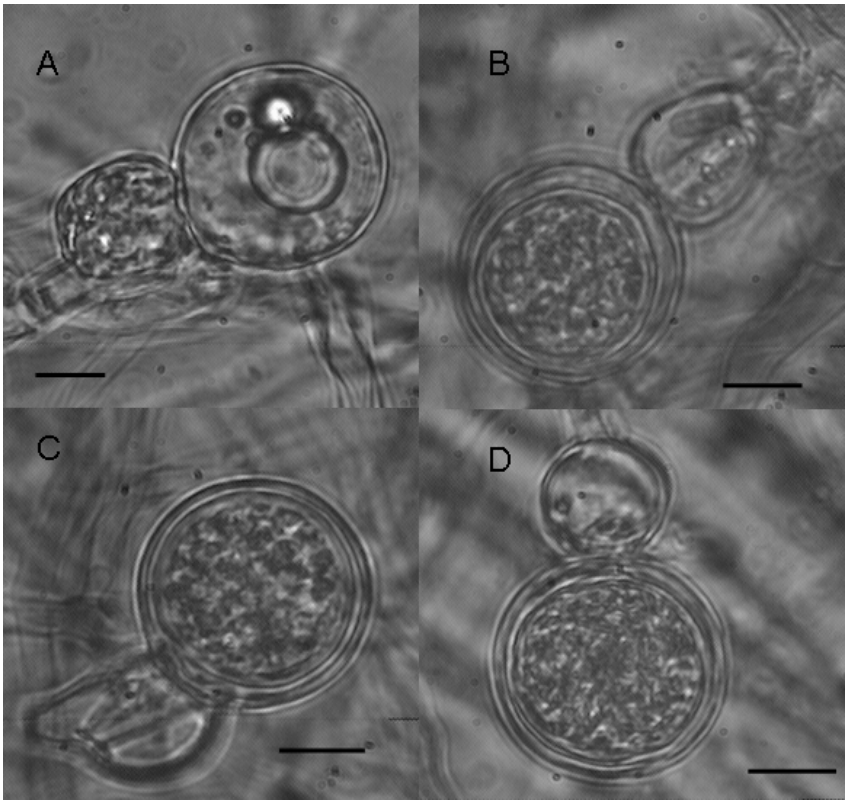

Figure 3. Amphigynous antheridia, oogonia and oospores of different crosses between *Phytophthora andina* isolates produced in pea agar. Crosses from: EC3233 x EC3186 (A), EC3189 x EC3399 (B, C), and EC3818 x EC3261 (D). Bars represent 10 μm.

Allozyme analysis

All isolates of *P. andina* had the *Pep* allele 76 that has not been reported previously for *P. infestans*. *P. andina* alleles for *Gpi* are common in *P. infestans*, although the 86 allele has only been found to date in the US-1 lineage of *P. infestans* (Table 1). Isolates sampled from *Anarrhichomenum* hosts and *S. hispidum* share the *Gpi* 100/100 and *Pep* 76/100 allele combinations. The combination *Gpi* 86/100 and *Pep* 76/100 is typical for EC-3 isolates

collected from *S. betaceum* (Table 1) and also occurs in some isolates from *S. quitoense,* although the latter had an RFLP genotype that differed from that of EC-3 (data not shown).

MtDNA haplotypes

Two mtDNA haplotypes were found among isolates of *P. andina* in Ecuador. Haplotype Ia was associated with isolates from *S. betaceum*, some isolates from *Anarrhichomenum* hosts, and *S. quitoense.* The haplotype Ic was associated with isolates from *Anarrhichomenum* hosts, *S. muricatum*, and *S. hispidum* (Table 1). Haplotypes Ia, Ib and IIa were found in *P. infestans* from *S. tuberosum* and other *Solanum* hosts, which is consistent with previous reports from Ecuador (Adler et al., 2004). Neither the IIb haplotype, previously described for *P. infestans*, nor the mtDNA haplotypes typical for *P. mirabilis and P. phaseoli* (Flier et al., 2002) were found in *Phytophthora* isolates from Ecuador. However, the Ic haplotype associated with isolates from *Anarrhichomenum* hosts was found in isolates of *P. ipomoeae* from Mexico, possibly indicating a shared ancestry (Flier et al., 2002).

RFLP fingerprinting

P. andina isolates had either EC-2 or EC-3 RFLP fingerprints. Limited polymorphism that has been described in more detail previously as EC-2.1 (Adler et al., 2004; Ordoñez et al., 2000) was found within the EC-2 group (data not shown). The EC-2.1 variant occurs with both Ic and Ia mtDNA haplotypes. RFLP fingerprints of *P. infestans* were either EC-1 or US-1, as previously reported (Adler et al., 2004; Forbes et al., 1997; Oyarzun et al., 1998).

AFLP fingerprinting

Based on a Neighbor Joining analysis using 172 AFLP marker loci, 12 isolates of *P. andina* formed a strongly supported clade (bootstrap support of 99%) clearly distinct from isolates of four other *Phytophthora* species included in the analysis (Figure 4). Within the *P. andina* clade, three groups could be distinguished, one associated with the EC-3 lineage from *S. betaceum*, and two additional clusters associated with isolates of the EC-2 lineage that included both Ic and Ia mtDNA haplotypes.

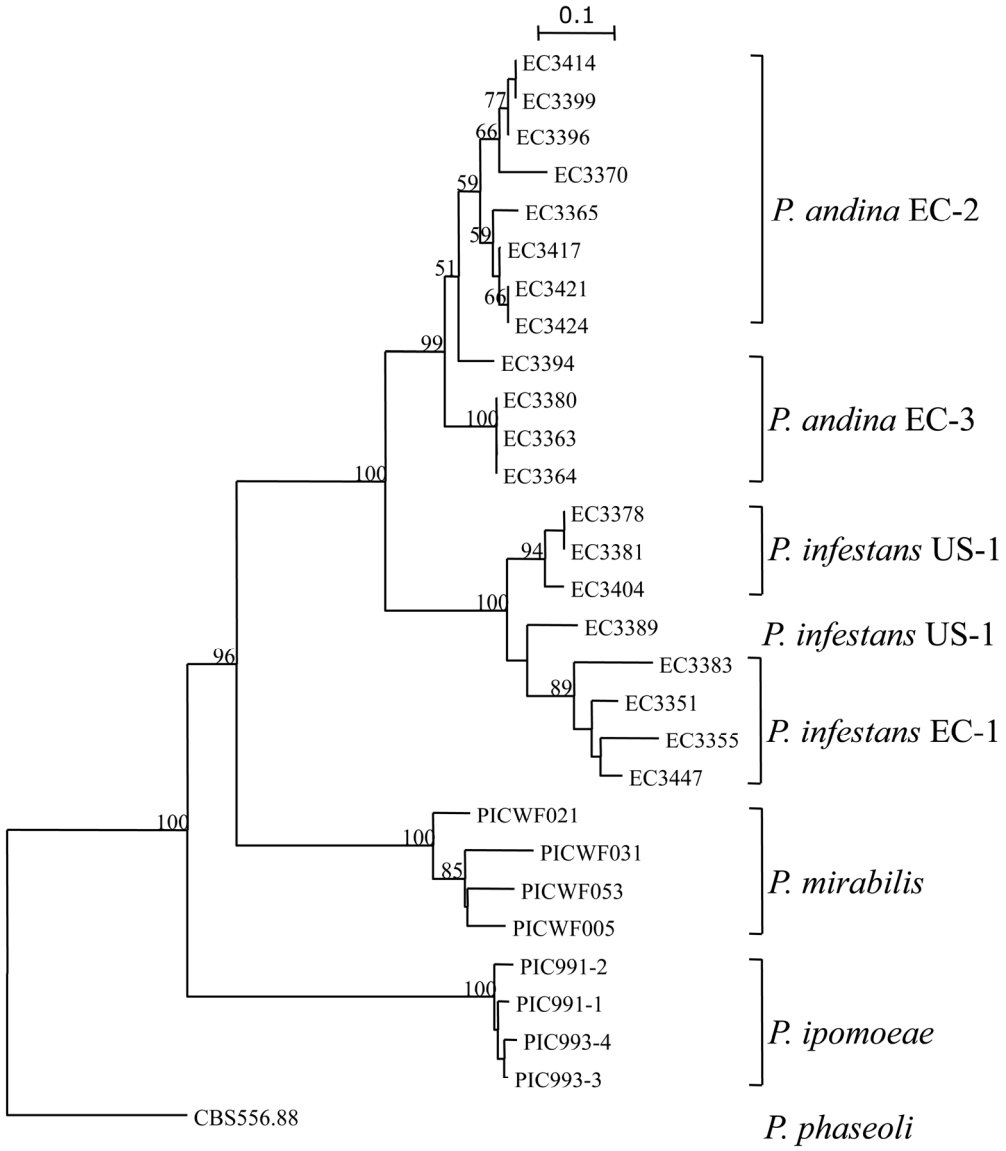

Figure 4. AFLP neighbor joining dendrogram of isolates of *P. andina* and related *Phytophthora* species using Rogers' modified distance. Bootstrap support for tree branches is presented if value exceeds 50% based on 1000 replicates.

Phylogenetic relationships

CoxII sequence data grouped isolates of *P. infestans* in one clade with all isolates of *P. andina* that have the Ia mtDNA haplotype. Isolates of *P. andina* with the Ic mtDNA haplotype formed a distinct branch, as did individual isolates of *P. mirabilis* and *P. phaseoli* (Figure 5). *CoxII* sequences generated in this study were submitted to GenBank (accessions numbers GQ260982 to GQ261004). ITS sequences were identical among isolates of *P. andina* and similar to those of *P. infestans* (GenBank accession AY770741). The sequence

of the *Ras* intron 1 gene was identical for all isolates of *P. infestans*, including GenBank accession U30474. All isolates of *P. andina* had identical sequences as well, but this sequence differed from that of *P infestans* by 9 base pairs (data not shown). In addition to the previous report made by Gomez-Alpizar et al. (2008) who describe six heterozygous sites, we found three additional sites within the *Ras* intron 1 sequence (GenBank accessions GQ261005 to GQ261027). Additional sites correspond to position 551, 610 and 688 according to accession U30474. Within the 223-bp fragment, heterozygous sites were found in all isolates of *P. andina* but these were absent in *P. infestans*.

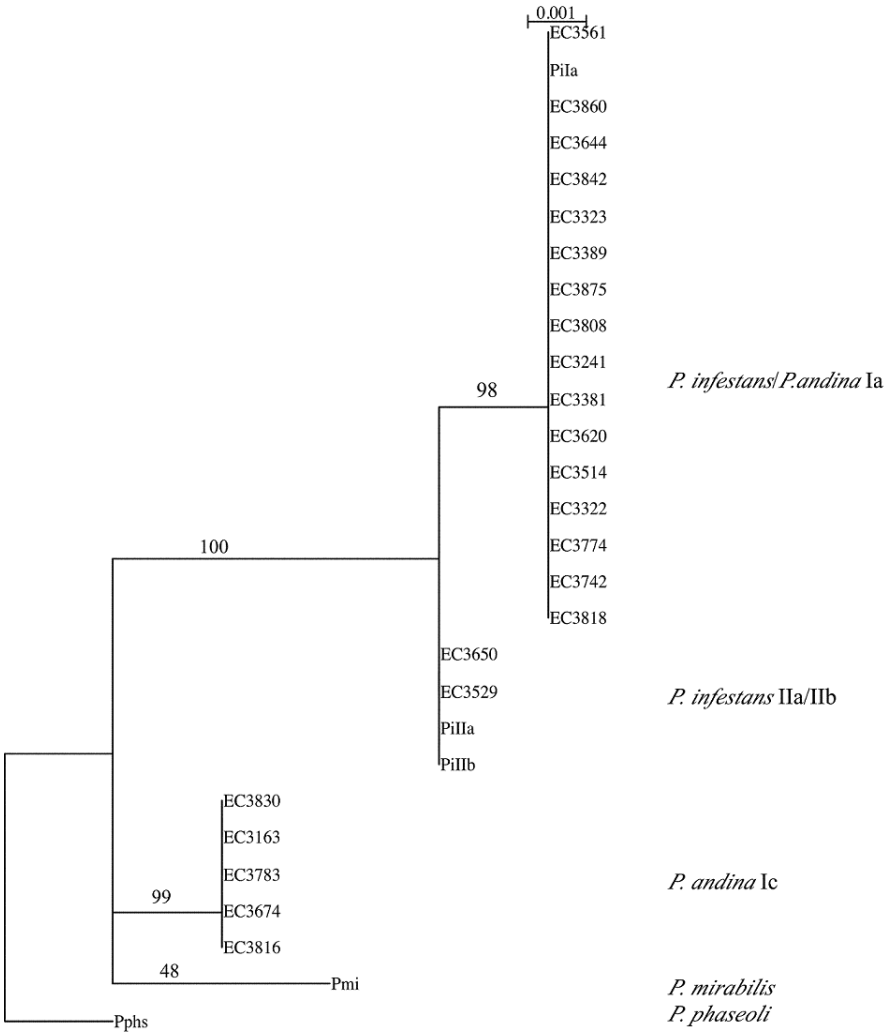

Figure 5. Phylogenetic relationship of isolates of *P. infestans* and *P. andina* using *Cox II* sequence data, based on Maximum Likelihood analysis. Numbers along the branches indicate bootstrap values. PiIa, PiIIa and PiIIb represent *P. infestans* accessions obtained from GenBank (AY894835, AY898627 and AY898628). Pmi and Pphs represent *P. mirabilis* (AY129214) and *P. phaseoli* (AY129221) accessions obtained from GenBank.

Pathogenicity

Isolates of both *P. infestans* RFLP lineages, US-1 and EC-1, caused lesions on most of the solanaceous host species examined, whilst isolates of *P. andina* had a narrower host range (Table 3). Based on qualitative assessment of pathogenicity of the 79 host-pathogen interactions, there was a tendency for isolates to more consistently infect the host they were isolated from (Table 3). None of the *P. andina* isolates were pathogenic on potato cv. Cruza 148 while some did caused weak infections on cv. Yungay, which is more susceptible. Other solanaceous hosts like *S. caripense* and *S. ochranthum* were fully resistant to the *P. andina* strains tested (Table 3). None of the isolates were pathogenic on *S. muricatum.*

Taxonomy

Phytophthora andina **Adler & Flier, sp. nov.** *Coloniis mycelialibus in Secal bene crescentibus. Temperies ad crescendum neccessaria. Minima temperies super 5 ° C, maxima temperies infra 30, optima temperies 24° C. Hyphae eseptatae et copiose ramosae, 4-8 µm diam. Sporangiophori aerii in agaro ramis composito-sympodialibus et indeterminatis, cum tumoribus in loco sporangiis emergentes. Sporangia semipapillata, ellipsoidea, subovoidalibus, caduca cum pedicella brevi, valore medio 44.6 µm longa (variatione 39.5-62.5 µm), ratione longitudinis/latitudinis 2.4-2.7, germinantia directe tubo germinativo vel indirecte cum zoosporis 6-8. Antheridia amphigyna, valore medio 22.0 µm longa, ratione longitudinis/latitudinis 1.4. Oogonia laevitunicata, 34-41 µm diam., basi attenuata. Oosporae laevitunicatae colore luteo, 27-37.5 µm diam.. Segregatus heterothallicis* (Figure 1 and 3).

Typus: Ecuador: Province of Tungurahua: City of Baños isol. Ex *Solanum brevifolium* along road side, July 2001, N. A. Adler, CIP EC3189, deposited in World *Phytophthora* Collection (University of California at Davis). Mycobank number MB514427.

Mycelial colonies grow well on RA. Minimum growth at approximately 5 °C, optimum at 24 °C and maximum at 30 °C. Hyphae nonseptate and freely branching, hyphal diam 4-8 µm, mostly 5.6 µm. Sporangiophore aerial, on rye agar with compound-sympodial and intermediate branches, with swellings where sporangia emerge. Sporangium semipapillate, ellipsoid or semi-ovoid, caduceus with short pedicel, on average 44.6 µm long (ranging from 39.5 to 62.5 µm), with a length/width ratio of 2.4 to 2.7, germinating directly with germ tubes or indirectly with 6-8 zoospores. Antheridia amphigynous, average length 22.0 µm, ratio of length/width 1.4. Oogonia smooth-walled, average diam 34.0-41.0 µm, with

tapered base. Oospores smooth-walled, tinted yellow-brown, almost filling the oogonial cavity, average diam 31.0 μm. Isolates heterothallic.

Discussion

In this paper we provide morphological and molecular evidence for the designation of *P. andina* as a distinct species attacking solanaceous plants in the Andean highlands. This designation is based on examination of a collection of isolates from different hosts made over a ten-year period. Based on AFLP, RFLP, allozyme genotypes and DNA sequence analyses, *P. andina* is closely related to *P. infestans*; however, these same makers indicate very little evidence of gene flow between the two species in nature, even though the potential for sexual reproduction has been established *in vitro* (Oliva et al., 2002). Molecular data presented elsewhere also (Blair et al., 2008; Gomez-Alpizar et al., 2008; Kroon et al., 2004; Oliva et al., 2007;Wattier et al., 2003) supports a new species designation for *P. andina.*

Analysis of genetic variation at the rRNA ITS region (Kroon and Flier, unpublished data) revealed that *P. andina* is very closely related to several other foliar pathogens classified into *Phytophthora* clade Ic (Cooke et al*.,* 2000). The rRNA ITS sequence analysis identified *P. andina* as a putative sister group to *P. infestans*, *P. mirabilis*, *P. ipomoeae* and *P. phaseoli*, a result consistent with the AFLP marker data shown here (Figure 4) and by Adler et al. (2004). Additional support for the classification of *P. andina* as a sister species of *P. infestans* and *P. mirabilis* was provided by the high-resolution molecular phylogeny for the genus *Phytophthora* published by Kroon et al. (2004) and the phylogenies proposed by Gomez-Alpizar et al. (2007, 2008) and Blair et al. (2008).

Phytophthora andina, P. mirabilis and *P. infestans* are morphologically similar in that they have a heterothallic mating system with amphigynous antheridia and produce large semipapillate sporangia on long sporangiophores. Unique features of *P. andina* (other than host range) are the EC-2 and EC-3 RFLP and AFLP fingerprints, heterozygosity in the *ras* intron 1 sequence, the Ic mtDNA haplotype and the *Pep* 76 allele.

The origin of *P. andina* is not directly addressed in this paper, however is possible that *P. infestans* and *P. andina* may have evolved either from a common ancestor or as a consequence of interspecific hybridization. The presence of the Ia haplotype and the *Gpi* 86 allele in both *P. infestans* and *P. andina* suggests that hybridization may have occurred. This finding is consistent with results of Gomez-Alpizar et al. (2008) who found the same

sequence of the *Cox I* gene among isolates comprising both *P. infestans* and isolates of *P. andina* with the Ia mtDNA haplotype. In our study we selected a representative sample of blight-causing *Phytophthora* genotypes with different host preferences, and found that some *P. andina* isolates have a unique *Cox II* sequence but some other share the same sequence with *P. infestans*. The occurrence of similar sequences in both *Cox I* and *Cox II* genes is also consistent with the hypothesis of previous hybridization, in which *P. infestans* must have been involved as one of the parents. On the other hand, both species could also have descended from a common mitochondrial ancestor (Gomez-Alpizar et al., 2007). The contribution of other *Phytophthora* species in the clade to the evolution of *P. andina* remains unclear and needs to be tested further using more rigorous statistical approaches.

Populations of *P. andina* appear to be clonal, with three predominant lineages. One lineage is characterized by the Ic mtDNA haplotype (Table 1) the A2 mating type (using *P. infestans* testers), and has been isolated from hosts of the *Anarrhichomenum* section of *Solanum* and *S. hispidum.* The second lineage has also been isolated from *Anarrhichomenum* hosts but it has the Ia mtDNA haplotype and the A1 compatibility type with *P. infestans* tester isolates. Similar genotypes have also been isolated from *S. quitoense*. A third lineage is characterized by the EC-3 RFLP fingerprint, and the Ia mtDNA haplotype, and to date has only been isolated from the cultivated tree tomato (*S. betaceum*) (Table 1). Two of the three lineages were distinguished in the cluster analysis of AFLP data (Figure 4).

When ten isolates of *P. andina* were crossed among themselves, oospores were produced in a number of the crosses. Since all ten of these isolates react as A2 when tested against an A1 isolate of *P. infestans,* it would appear that while *P. andina* isolates can be classified for mating type with *P. infestans* testers, the mating system within *P. andina* is more complex. This highlights the limited utility of using mating type testers from one species to classify isolates of another species. The incongruence of mating systems (complementary but not identical) is consistent with the designation of *P. andina* as a separate species. We did not attempt at this point to describe the mating system in *P. andina* as our sample was small (ten isolates) and not representative; all isolates came from the *Anarrhichomenum* group. Further work is needed to address mating behaviour in this species.

We are not aware that the complexity we identified in the mating system of this species is unique to *P. andina* with this *Phytophthora* clade. No mating system complementary to that of *P. infestans* has apparently been described for *P. mirabilis*. Goodwin and Fry (1994) were able to cross *P. infestans* with *P. mirabilis* and obtain hybrid oospores, but no such observations on crosses within *P. mirabilis* were reported. In another

study (Oliva, unpublished data) we demonstrated that crosses between *P. andina* and *P. infestans* resulted in oospores that can survive up to one year in soil and hybrid progeny can infect potato in the laboratory. We have not done sufficient studies of viability of oospores resulting from crosses of *P. andina* by *P. andina*. Given the potential implications for genetic recombination, hybridization and disease initiation from oospores, further studies to characterize the mating systems of *P. andina* and oospore viability and epidemiology are needed.

In tree hosts (*S. betaceum, S. quitoense* and *S. hispidum*), *P. andina* causes leaf lesions similar to blight on potato but much larger and with longer infectious periods. The pathogen also causes very serious stem infections (Figure 1) on *S. betaceum*. Blight symptoms in general on the Andean solanaceous hosts appear to be defined more by the host than the pathogen. On the *Anarrhichomenum* hosts (Figure 1), which are viney plants growing in underbrush, disease symptoms are very much like blight of potato or tomato.

Detached-leaf inoculations in the laboratory indicated that *P. andina* can also infect and sporulate on leaves of wild or cultivated potatoes. In nature, however, the pathogen appears to have strong host preferences and through a decade of isolation studies we have not found *P. andina* lesions on potato or tomato plants (Adler et al., 2004; Oliva et al., 2007). However, there are solanaceous hosts which *P. andina* and *P. infestans* may co-infect and, given comments above on the potential for hybridization, this could be important. On two occasions, *P. andina* was isolated from *S. muricatum*, which is generally a host of US-1 genotypes of *P. infestans* (Adler et al., 2002). In each of those cases, *P. andina* appeared to cause disease of epidemic proportions; all isolates were *P. andina.* There are also other hosts where both species have been isolated, including *S. quitoense* and *S. ochranthum* (Oliva, unpublished data).

Similar to *P. infestans* on potato or tomato, *P. andina* appears to be a pathogen of increasing, and potentially great impact on native Andean fruit crops, some of which have or are being traded globally. Although epidemiological studies have not been done, we have noted a marked increase in severity of disease on *S. betaceum.* In the mid 1990s, *P. andina* infection on *S. betaceum* was difficult to find; now for collection we simply have to go to any of the major production areas and disease is always present. The dynamics of *P. andina* also includes *S. quitoense* in Ecuador and Colombia, which is produced in restricted regions at mid level elevations (1000-2000 m above sea level) and occupies a different agro-ecological zone than does *S. betaceum*. Beside the genus *Solanum,* less is known about the occurrence of *P. andina* on other solanaceous crops. Late blight of Cape gooseberry (*Physalis peruviana*)

was recently reported to be caused by *P. infestans* in Colombia (Vargas et al., 2009), although the markers used by the authors would not have distinguished *P. infestans* from *P. andina*.

Some hosts described in this study, which are commonly attacked by *P. andina*, are no longer restricted to the Andes. Perhaps the best example is *S. betaceum*, which is widely cultivated in small orchards around the world, but also considered economically important in some non-Andean countries, particularly New Zealand. Currently, there are no reports of the pathogen outside the Andean highlands.

Analyses of the impact of this pathogen on native fruit crops in the Andean highlands and studies on the evolutionary relationships of this species with other *Phytophthora* pathogens attacking solanaceous hosts are in progress.

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4

Phytophthora diagnostics

4.1

A rapid diagnostic test to distinguish between American and European populations of *Phytophthora ramorum*

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Abstract

A new devastating disease in the United States, commonly known as Sudden Oak Death, is caused by *Phytophthora ramorum*. This pathogen, which was previously described attacking species of *Rhododendron* and *Viburnum* in Germany and the Netherlands, has established itself in forests on the central coast of California and is killing scores of native oak trees (*Lithocarpus densiflora*, *Quercus agrifolia*, *Q. kelloggii*, and *Q. parvula* var. *shrevei*). The phytosanitary authorities in the European Union consider non-European isolates of *P. ramorum* as a threat to forest trees in Europe. To date, almost all European isolates are mating type A1 while those from California and Oregon are type A2. The occurrence of both mating types in the same region could lead to a population capable of sexual recombination, which could generate a new source of diversity. To prevent contact between these two populations, a rapid, reliable, and discriminating diagnostic test was developed to easily distinguish the two populations. Based on a DNA sequence difference in the mitochondrial Cytochrome c oxidase subunit 1 (*Cox*1) gene, we developed a SNP (single nucleotide polymorphism) protocol to distinguish between isolates of *P. ramorum* originating in Europe and those originating in the United States. A total of 83 isolates of *P. ramorum* from Europe and 51 isolates from the United States were screened and all isolates could be consistently and correctly allocated to either the European or the US populations using the SNP protocol.

Introduction

The genus *Phytophthora* comprises over 60 described species, and new species are constantly emerging, either by hybridization of species already known (Bonants et al., 2000; Brasier et al., 1999; Man in 't Veld et al., 1998) or by the discovery of previously undetected species (Flier et al., 2002, Hansen et al., 2003a, Man in 't Veld et al., 2002, Mirabolfathy et al., 2001). One of the newly described species is *P. ramorum*, a pathogen of woody plants, including *Rhododendron*, *Viburnum* and *Quercus* spp. (Rizzo et al., 2002, Werres et al., 2001). Two distinct populations of *P. ramorum* from different regions have been characterized thus far. In the coastal forests of California and Oregon (US), *P. ramorum* has been killing thousands of trees of native oak species (*Lithocarpus densiflora*, *Quercus agrifolia*, *Q. kelloggii*, and *Q. parvula* var. *shrevei* (Rizzo et al., 2002)) while surviving and causing damage on a wide range of other shrubs and trees, including bay laurel (*Umbellularia*

californica), rhododendron (*Rhododendron* spp.), Douglas fir (*Pseudotsuga menziesii*) and coast redwood (*Sequoia sempervirens*). A second population of *P. ramorum* has been found in Europe (EU) on *Rhododendron* and *Viburnum* species used as ornamental plants in nurseries and landscapes, but so far has only rarely been found in forest ecosystems. These populations appear to be distinct based on AFLP-fingerprint patterns (Bonants et al., 2002, Ivors et al., 2002) and differ in mating type (Werres and Zielke, 2003; Zielke and Werres, 2002).

Until recently, only A1 mating type isolates have been found in Europe, while in the USA only A2 type isolates appeared to occur (Hansen et al., 2003b, Werres and De Merlier, 2003; Werres and Zielke, 2003). The presence of two mating types indicates that the pathogen is heterothallic and requires interaction between isolates of opposite mating types for meiotic recombination. These mating types do not correspond to dimorphic forms of the pathogen but are distinguished by the production of specific hormones that induce the formation of gametangia in the opposite mating type. Fusion of male gametangia (antheridia) and female gametangia (oogonia) leads to formation of persistent diploid sexual spores, or oospores (Brasier, 1992, Ko, 1988; Ristaino, 2002). The occurrence of both mating types on a common host has the potential to give rise to meiotic recombination during oospore formation, which could lead to the presence of a sexually reproducing population and greater genetic diversity in the species. The possibility of this occurring is especially poignant given the additional problems encountered with attempts to manage *P. infestans* after introduction of a sexually reproducing population in Europe (Drenth et al., 1995; Hohl and Iselin, 1984; Turkensteen et al., 2000).

It is important that A1 type isolates of *P. ramorum* that occur in Europe at the present time can be differentiated from A2 type isolates that presently occur in the USA. Mating type determination tests for *P. ramorum* are carried out with tester strains of other heterothallic species of *Phytophthora*, e.g. *P. cryptogea*. These tester strains are more suitable for mating type assays, as the ability of *P. ramorum* to form oospores in intra-specific crosses is often impaired (Werres and Zielke, 2003). The mating type of *P. ramorum* isolates is determined by pairing them with tester strains (either of the A1 or the A2 type) and observing the formation of oogonia and antheridia at the interaction zone between the tester strain and the *P. ramorum* isolate. Tester strains of the A2 mating type will induce formation of oogonia and antheridia in *P. ramorum* isolates with mating type A1 (and *vice versa*) while tester strains of the A1 mating type will induce formation of these structures in *P. ramorum* isolates with mating type A2 (and *vice versa*). However, mating type assays are time-consuming and sometimes unsuccessful (Werres and Zielke, 2003) and are therefore not suited for routine diagnostic

procedures in a quarantine laboratory. Moreover, it is not yet clear that differences in mating type between EU and US isolates are sufficiently consistent; only limited numbers of isolates have been tested so far. The underlying assumption for using mating type tests is that both the EU and US populations are the clonal progeny of single introductions (of an A1 and an A2 isolate respectively) from an unknown mother population. Recent findings of A2 type isolates in the European population (Werres and De Merlier, 2003) or A1 type isolates in the US population (Hansen et al., 2003b) will require the use of more solid, preferentially molecular tests to confirm the genetic background and putative origin of these rare findings.

In recent years, molecular techniques have been used for detection and identification of plant pathogenic Oomycetes (Bonants et al., 1997; Cooke et al., 2000b). ITS sequences of rDNA and single-strand-conformation polymorphisms have proven useful in the identification of *Phytophthora* species (Cooke et al., 2000a, Kong et al., 2003). Based upon ITS sequence analysis, a species-specific PCR detection method for *P. ramorum* was developed and validated using stem and leaf material from plants infected with *P. ramorum* (Garbelotto et al., 2002). Real-time detection tests for *P. ramorum* also have been developed using molecular beacons and Taqman probes (Bilodeau et al., 2002, Ivors and Garbelotto, 2002, Kox et al., 2002). However, these tests do not differentiate between A1 and A2 isolates of the pathogen. AFLP DNA fingerprinting showed significant differences between EU and US populations (Bonants et al., 2002, Ivors et al., 2002), but this technique is laborious and results are sometimes hard to analyze consistently.

A phylogenetic study on species of *Phytophthora*, using a multigene sequencing approach (Kroon et al., 2004), revealed sequence differences between EU and US isolates of *P. ramorum* in the mitochondrial Cytochrome c oxidase subunit 1 (*Cox*1) gene. These differences were exploited to develop a diagnostic assay for *P. ramorum* isolates. The objectives of this study were: (i) to test whether EU and US populations of *P. ramorum* can be consistently distinguished by their mitochondrial *Cox*1 gene sequence; (ii) if so, to develop a reproducible Single Nucleotide Polymorphism (SNP) assay to detect and differentiate between EU and US populations of *P. ramorum*; and (iii) to compare sequence-based Restriction Fragment Length Polymorphism (RFLP) profiles of isolates of *P. ramorum* with those of closely-related species and species that occur on common hosts.

Table 1. Isolates of *Phytophthora* used in this study.

Phytophthora	Isolate codes		Isolate origin			
species	PD/IMI/ATCC [a]	Other [b]	Host plant [c]	Country [d]	Year	Isolate source [e]
P. ramorum	PD 93/56	CBS101327	*Rhododendron* 'Catawbiense'	The Netherlands	1993	Hans de Gruyter PD
P. ramorum	PD 98/5233	CBS101330	*Viburnum* sp.	The Netherlands	1998	Hans de Gruyter PD
P. ramorum	PD 99/2855		*Rhododendron* sp.	The Netherlands	1999	Hans de Gruyter PD
P. ramorum	PD 20011060		*Viburnum bodnantense* 'Dawn'	The Netherlands	2001	Hans de Gruyter PD
P. ramorum	PD 20011880-2		*Rhododendron* sp.	The Netherlands	2001	Hans de Gruyter PD
P. ramorum	PD 20018722		*Rhododendron* sp.	The Netherlands	2001	Hans de Gruyter PD
P. ramorum	PD 20019200		*Rhododendron* sp.	The Netherlands	2001	Hans de Gruyter PD
P. ramorum	PD 20019355		*Rhod.* 'Cunningham's White'	The Netherlands	2001	Hans de Gruyter PD
P. ramorum	PD 20019414		*Rhododendron* sp.	The Netherlands	2001	Hans de Gruyter PD
P. ramorum	PD 20019958		*Rhododendron* 'Catawbiense'	The Netherlands	2001	Hans de Gruyter PD
P. ramorum	PD 20019535	CBS109278	*Viburnum bodnantense* 'Dawn'	Germany	1999	Sabine Werres BBA
P. ramorum	PD 20019538	CBS101554	*Rhododendron* sp.	Germany		Sabine Werres BBA
P. ramorum	PD 20019539	CBS101553	*Rhododendron* 'Catawbiense'	Germany	1995	Sabine Werres BBA
P. ramorum	PD 200110053	RH/122/98	*Rhododendron* sp.	Poland		Leszek Orlikowski RIPF
P. ramorum	PD 200110054	RH/2/00	*Rhododendron* sp.	Poland		Leszek Orlikowski RIPF
P. ramorum	PD 200110055	RH/6/00	*Rhododendron* sp.	Poland		Leszek Orlikowski RIPF
P. ramorum	PD 20017607	Pr-13	*Quercus agrifolia*	CA, USA		Matteo Garbelotto UCB
P. ramorum	PD 20017608	Pr-01	*Q. agrifolia*	CA, USA		Matteo Garbelotto UCB
P. ramorum	PD 21008928	Pr-05	*Lithocarpus densiflora*	CA, USA		Matteo Garbelotto UCB
P. ramorum	PD 21008929	Pr-06	*Q. agrifolia*	CA, USA		Matteo Garbelotto UCB
P. ramorum	PD 21008931	Pr-58	*Vaccinium* sp.	CA, USA		Matteo Garbelotto UCB
P. ramorum	PD 21008932	Pr-87	*Arbutus menziessi*	CA, USA		Matteo Garbelotto UCB
P. ramorum	PD 21008933	Pr-88	*Umbellularia californica*	USA		Matteo Garbelotto UCB
P. ramorum		Pr-52	*Rhododendron* sp.	CA, USA		Matteo Garbelotto UCB
P. ramorum		Pr-62	*Q. agrifolia*	CA, USA		Matteo Garbelotto UCB
P. ramorum		Pr-65	*Q. parvula* var. *shrevei*	CA, USA		Matteo Garbelotto UCB
P. ramorum		Pr-67	soil	CA, USA		Matteo Garbelotto UCB
P. ramorum		Pr-70	*Vaccinium ovatum*	CA, USA		Matteo Garbelotto UCB
P. ramorum		Pr-102	*Q. agrifolia*	CA, USA		Matteo Garbelotto UCB
P. brassicae		CBS179.87	*Brassica oleracea*	The Netherlands	1987	CBS
P. cactorum		P6183	*Rubus idaeus*	USA		Hans de Gruyter PD
P. cinnamomi		10A6	Avocado	Italy		Querico Migheli
P. citricola [f]		P1817	*Medicago sativa*	South Africa		Hans de Gruyter PD
P. citrophthora [f]	PD94/353	CBS274.33	*Citrus limonium*	Cyprus		Hans de Gruyter PD
P. cryptogea		HR1/ss/pp/99	*Lycopersicon esculentum*	UK		Joeke Postma PRI
P. drechsleri		CBS292.35	*Beta vulgaris* var. *altissima*	CA, USA	1935	CBS
P. gonapodyides		P245	*Salix matsudana*	UK	1972	David Cooke SCRI
P. heveae [f]	IMI180616	CBS296.29	*Hevea brasiliensis*	Malaysia	1929	David Cooke SCRI
P. hibernalis	ATCC64708	CBS522.77	*Aquilegia vulgaris*	New Zealand		CBS
P. hybrid alder	PD92/1471		*Alnus cordata*	The Netherlands	1992	Hans de Gruyter PD
P. ilicis [f]	PD91/595		*Ilex aquifolium*	The Netherlands	1991	Hans de Gruyter PD
P. infestans		Pic99186	*Solanum stoloniferum*	Mexico	1999	Wilbert Flier PRI
P. lateralis	IMI040503	CBS168.42	*Chamaecyparis lawsoniana*	OR, USA	1942	David Cooke SCRI
P. megasperma	IMI133317	MEG23	*Malus sylvestris*	Australia	1968	David Cooke SCRI
P. nemorosa [g]	ATCC MYA-2948	P7	*L. densiflora*	CA, USA		Matteo Garbelotto UCB
P. nemorosa [g]		P13	*L. densiflora*	CA, USA		Matteo Garbelotto UCB
P. nicotianae [f]		P582	*Nicotiana tabacum*	USA		Hans de Gruyter PD
P. palmivora		CBS236.30	*Cocos nucifera*	India		CBS
P. pseudosyringae [g]		P8	*Q. agrifolia*	CA, USA		Matteo Garbelotto UCB
P. pseudosyringae [g]		IFB PSEU16	*Fagus sylvatica*	Germany		Thomas Jung
P. pseudotsugae	IMI331662	PSE1	*Pseudotsuga menziesii*	USA		David Cooke SCRI
P. syringae	IMI045169	CBS364.52	*Prunus armeniaca*	New Zealand		CBS

[a] PD; isolate code used by the Plant Protection Service, The Netherlands, IMI; isolate code used by the Commonwealth Agricultural Bureaux, ATCC; isolate code used by the American Type Culture Collection [b] CBS; isolate code used by the Centraal Bureau voor Schimmelcultures, The Netherlands, BBA; isolate code used by the Biologische Bundesanstalt, Germany [c] Plant species of which the isolate was recovered [d] RIPF; Research Institute for Pomology and Floriculture, Poland, UCB; University of California, Berkeley, USA, PRI; Plant Research International, The Netherlands, SCRI; Scottish Crop Research Institute, UK [e] Country from which the isolate was initially recovered, CA; California, OR; Oregon [f] These species of *Phytophthora* are only included in the sequence analysis, not in the restriction fragment length polymorphism (RFLP) assay.[g] These species of *Phytophthora* are only included in the restriction fragment length polymorphism (RFLP) assay, not in the sequence analysis.

Materials and Methods

Isolates and cultures

Stems and leaves from plants of *Rhododendron* spp. and *Viburnum* spp. colonized by *P. ramorum* were collected during a national survey of nurseries and gardens in the Netherlands in 2001. Infected leaf and stem material, taken from the periphery of lesions, was surface-sterilized in 50% ethanol and rinsed in water. Plant pieces were placed on selective P_5ARPH medium (per liter: 17 g of Cornmeal agar [Difco, Detroit, USA], 5 mg of pimaricin, 250 mg of sodium ampicillin, 10 mg of rifampicin, 100 mg of PCNB [75% a.i.], and 50 mg of hymexazol [70% a.i.]) and incubated in the dark at 22°C (Jeffers and Martin, 1986). Individual isolates of *P. ramorum* were obtained by transferring agar pieces containing growing mycelium from colonies on P_5ARPH plates to plates of cherry agar (per liter: 100 ml of cherry pulp and 15 g of agar [Oxoid, Basingstoke, UK]) and incubated in the dark at 22°C for 1 to 2 weeks. Isolates were stored on V8-slants (per liter: 200 ml of V8-juice, 2 g of $CaCO_3$, 0.05 g of β-sitosterol, and 15 of agar) at 4°C in the dark. Researchers from Europe and the USA provided us with a diverse range of additional samples, covering the known genetic and geographic diversity in *P. ramorum*. In total, 83 isolates of *P. ramorum* from Europe and 51 isolates from the USA were included in this study (Table 1 and 2). In addition, isolates of 21 other species of *Phytophthora* were included in the analysis to evaluate the species-specificity of the assay (Table 1).

DNA extraction

Isolates were grown in the dark for 10 to 14 days at 20°C in pea broth. This medium was prepared by autoclaving 120 g of frozen peas in one liter of tap water. The broth was filtered through cheesecloth to remove the peas and then was autoclaved again (Flier et al., 2003). Mycelium was harvested and lyophilized, and total DNA was extracted using the Puregene kit (Gentra/Biozyme, Landgraaf, The Netherlands) according to the manufacturer's instructions. The DNA pellet was dissolved in 100 µl of TE (10 mM Tris-HCl [pH 8.0]; 1 mM EDTA), and the solution was stored at –20°C. For infected plant material, DNA was extracted directly from tissue on the periphery of lesions on stems and leaves. From infected host plants found in the Netherlands, 20 samples of infected *Rhododendron* spp. and 4 samples of infected *Viburnum* spp. were analyzed. In addition, infected leaf material (12 samples) of plants inoculated with either an EU- or US-type isolate in greenhouse

experiments were analyzed (De Gruyter et al., 2002). Small pieces (100 to 200 mg) of surface-sterilized (15 seconds in a 1% sodium hypochlorite solution) diseased plant tissue were homogenized in 300 µl of extraction buffer (0.02 M PBS, 0.05% Tween T25, 2% polyvinylpyrrolidone, 0.2% bovine serum albumine) using a cell disrupter (RiboLyzer, Hybaid Thermo, Ashford, UK). The resulting suspension was centrifuged at 13200 rpm (16100 g) in a microcentrifuge (Eppendorf, Hamburg, Germany) for 5 seconds, and 75 µl of the supernatant was transferred to a new 1.5-ml microcentrifuge tube. Total DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Helden, Germany) according to the manufacturer's instructions. The DNA was eluted with 50 µl of AE-buffer, further purified on a Micro Bio-Spin chromatography column filled with polyvinylpolypyrrolidone (PVPP) (Bio Rad, Hercules, USA) and was stored at –20°C.

Table 2. Isolates of *Phytophthora ramorum* used in this study: country and host plant from which isolates were recovered initially.

Country	Host plant [a]							total
	Rhododendron spp.	*Viburnum* spp.	*Quercus* spp.	*L. densiflora*	*V. ovatum*	*U. californica*	other	
Belgium	2	3	...	...	...	...	...	5
Germany	3	4	...	...	...	...	...	7
France	4	3	...	...	...	...	...	7
The Netherlands	30	11	...	...	...	...	...	41
Poland	3	...	...	...	...	...	...	3
Spain	3	1	...	...	...	...	...	4
Sweden	2	...	...	...	...	...	...	2
UK	6	8	...	...	...	...	...	14
total isolates Europe:								83
USA (California)	2	...	10	7	4	6	16	45
USA (Oregon)	1	...	...	1	2	1	1	6
total isolates USA:								51

[a] *Quercus* spp.; *Q. agrifolia*, *Q. parvula* var. *shrevei*, *Q. chrysolepis*, *L. densiflora*; *Lythocarpus densiflora*, *V. ovatum*; *Vaccinium ovatum*, *U. californica*; *Umbellularia californica*, other; *Acer macrophyllum*, *Aesculus californica*, *Arbutus menziesii*, *Corylus cornuta*, *Heteromeles arbutifolia*, *Lonicera hispidula*, *Psuedotsuga menziesii*, *Rhamnus cathartica*, *Rhamnus purshiana*, *Sequoia sempervirens*, soil, *Trentalis latifolia.*

PCR amplification

For amplification of the *Cox*1 gene, the complete mitochondrial DNA-sequence U17009 of *P. infestans* (Paquin et al., 1997) was used as a template to develop primers. CoxF4N 5' GTATTTCTTCTTTATTAGGTGC 3' (bp 9126 to 9147) and CoxR4N 5' CGTGAACTAATGTTACATATAC 3' (bp 10076 to 10097) were chosen to amplify a fragment of 972 bp. DNA from 49 isolates of *P. ramorum* (42 EU and 7 US) was used as template. Representative isolates from each of 21 other *Phytophthora* spp. also were included

in the analysis (Table 1). These species were chosen based on two criteria: pathogenicity on *Rhododendron* spp. or *Quercus* spp. (hosts of *P. ramorum*) or a close clustering to *P. ramorum* in phylogenetic analyses (Kroon et al., 2004). The reaction mix consisted of 10 to 20 ng of template DNA, 200 µM of dNTP's, 1 U of *Taq* DNA polymerase (Roche, Indianapolis, USA), 3.5 mM of $MgCl_2$, and 25 ng of each primer (CoxF4N and CoxR4N) in a reaction volume of 25 µl. Amplifications were run in a PTC200 thermocycler (MJ Research, Waltham, Massachusetts, USA), with an initial denaturation at 94°C for 2 min, followed by 35 cycles consisting of denaturation at 94°C for 30 seconds, annealing at 52°C for 30 seconds, and extension at 72°C for 60 seconds. A final extension at 72°C for 10 min followed.

Based on a comparison of *Cox*1 DNA sequence information for *P. ramorum* isolates with the *Cox*1 sequences of 19 other *Phytophthora* spp., a nested PCR primer pair was developed that was specific for *P. ramorum*. Primers PrnestF 5' TAGCTACTTTATGGGGTGGTTCA 3' (bp 508 to 530 of the 972 bp fragment) and PrnestR 5' CATTCCAACCACTCATAGCATCA 3' (bp 869 to 891) were chosen to amplify a fragment of 383 bp that included the SNP site. DNA from 99 isolates of *P. ramorum* (52 EU and 47 US) was used as template. Amplification conditions were identical to the procedure described for the CoxF4N and CoxR4N primer pair, with the exception of the annealing temperature, which was raised to 69°C. The detection limit for the nested PCR primer pair was determined using a dilution series (1 ng to 0.1 fg of pure *P. ramorum* DNA per reaction). DNA from other *Phytophthora* spp. (Table 1) was used to confirm species-specificity of the nested PCR primer pair.

For amplification of the ITS region in samples from infected plants, the reaction mix consisted of 5 µl of template DNA (unknown concentration), 200 µM of dNTP's, 1 U of HotStar *Taq* DNA polymerase (Qiagen, Hilden, Germany), 1.5 mM of $MgCl_2$ and 0.4 µM of each primer ((Phyto1 5' CATGGCGAGCGCTTGA 3' and Phyto4 5'GAAGCCGCCAACACAAG 3') Garbelotto et al., 2002). Amplifications were conducted in a PTC200 thermocycler, with an initial denaturation at 95°C for 15 min, followed by 35 cycles consisting of denaturation at 94°C for 15 seconds, annealing at 62°C for 60 seconds, extension at 72°C for 45 seconds, and a final extension at 72°C for 10 min. The presence of *P. ramorum* was confirmed by observing a diagnostic band at 687 bp using gel electrophoresis (2 h at 100 volts) on 1.0 % agarose gels (MP, Roche, Mannheim, Germany) in 0.5 x TBE buffer. DNA fragments were visualized after staining with ethidium bromide and illuminating under UV light.

Sequence analysis

PCR products were purified on Sephadex plates (Multiscreen HV, Millipore, Bedford, USA) to remove excess primers and nucleotides. Products were sequenced with the primers CoxF4N or CoxR4N using the BigDye sequencing kit (Applied Biosystems, Foster City, USA) and an ABI3700 DNA Analyzer (Applied Biosystems, Foster City, USA). The trace files originating from the ABI-sequencer were transferred to the SeqMan 5.0 module of DNASTAR (DNASTAR Inc., Madison, WI, USA). Forward and reverse sequences were coupled in Editseq 5.0 (DNASTAR). The sequences were aligned using the ClustalW algorithm in the program MegAlign 5.0 (DNASTAR). Restriction patterns of isolates from the USA and Europe were compared in MapDraw 5.00 (DNASTAR) and differential restriction sites were identified. This program also was used to compare restriction profiles of isolates of *P. ramorum* with those of isolates of 21 other species of *Phytophthora* included in this study (Table 1).

SNP analysis

The 972-bp PCR fragment of the *Cox*1 gene, or the 383-bp nested fragment, was digested with the restriction enzyme *Apo*I ([A/G]AATT[C/T]) (New England Biolabs, Beverly, MA, USA) for 1 h at 50°C according to the manufacturer's instructions. The resulting fragments were separated by gel electrophoresis on 3% REsult LE agarose gels (Biozym, Hess. Oldendorf, Germany) in 0.5x TBE buffer run at 100V for 3 h. The DNA fragments were visualized after staining with ethidium bromide and illuminating under UV light.

Results

PCR amplification

Using primers CoxF4N and CoxR4N, a 972-bp fragment from the *Cox*1 gene was amplified from a total of 33 isolates of *P. ramorum*, including 27 isolates from Europe and 6 isolates from the USA. With inner primers PrnestF and PrnestR, a 383-bp fragment was amplified in 99 samples of DNA from isolates of *P. ramorum*, including 52 isolates from Europe and 47 isolates from the USA. Of these 99 samples, 11 samples from Europe and 3 samples from the USA were tested in both the 972-bp and 383-bp protocol. Specificity of the primers PrnestF and PrnestR was tested for 22 isolates of 17 different *Phytophthora* spp.

(Table 1). By increasing the annealing temperature to 69°C, amplification of the 383-bp product was restricted to isolates of *P. ramorum*. Only *P. hibernalis* showed limited cross-amplification under these stringent conditions (data not shown). By using the inner primers in a second PCR amplification (nested PCR), the sensitivity of detection was increased considerably. A dilution series of DNA from *P. ramorum* amplified with single or nested PCR detected 100 pg and 10 fg of DNA, respectively.

Figure 1. Sequence alignment of part of the 972-bp amplicon of the Cytochrome c oxidase subunit 1 gene for five isolates of *Phytophthora ramorum* from the USA and Europe. The shaded area denotes the additional *Apo*I restriction site present in isolates from the United States. Isolate information is listed in Table 1.

SNP analysis

The *Cox*1 fragment that was amplified in DNA of isolates of *P. ramorum* contained a single-nucleotide polymorphism at position 773 of the 972-bp amplicon (position 266 of the 383-bp amplicon) that distinguished between isolates from Europe and the USA (Figure 1). Isolates from Europe have a cytosine residue at position 773 while US type isolates show a thymine residue at this site. The restriction enzyme *Apo*I was used to develop an SNP assay that can discriminate between isolates of *P. ramorum* from Europe and those from the USA. In a blind test of 18 arbitrarily selected isolates of *P. ramorum* from Europe and the USA, five isolates were found to share the additional *Apo*I restriction site characteristic of the USA population (Figure 2A) while the other 13 isolates had the restriction profile characteristic of the European population. All isolates of *P. ramorum* were correctly and consistently assigned to their geographic region of recovery based on the SNP assay. For the smaller 383-bp amplicon a less complex RFLP pattern was obtained, clearly distinguishing isolates from Europe and the USA (Figure 2B). Thirty-three isolates were tested using the 972-bp *Cox*1 amplicon for the *Apo*I digest (27 from Europe and 6 from the USA) while 99 isolates were analyzed using the 383-bp amplicon (52 from Europe and 47 from the USA).

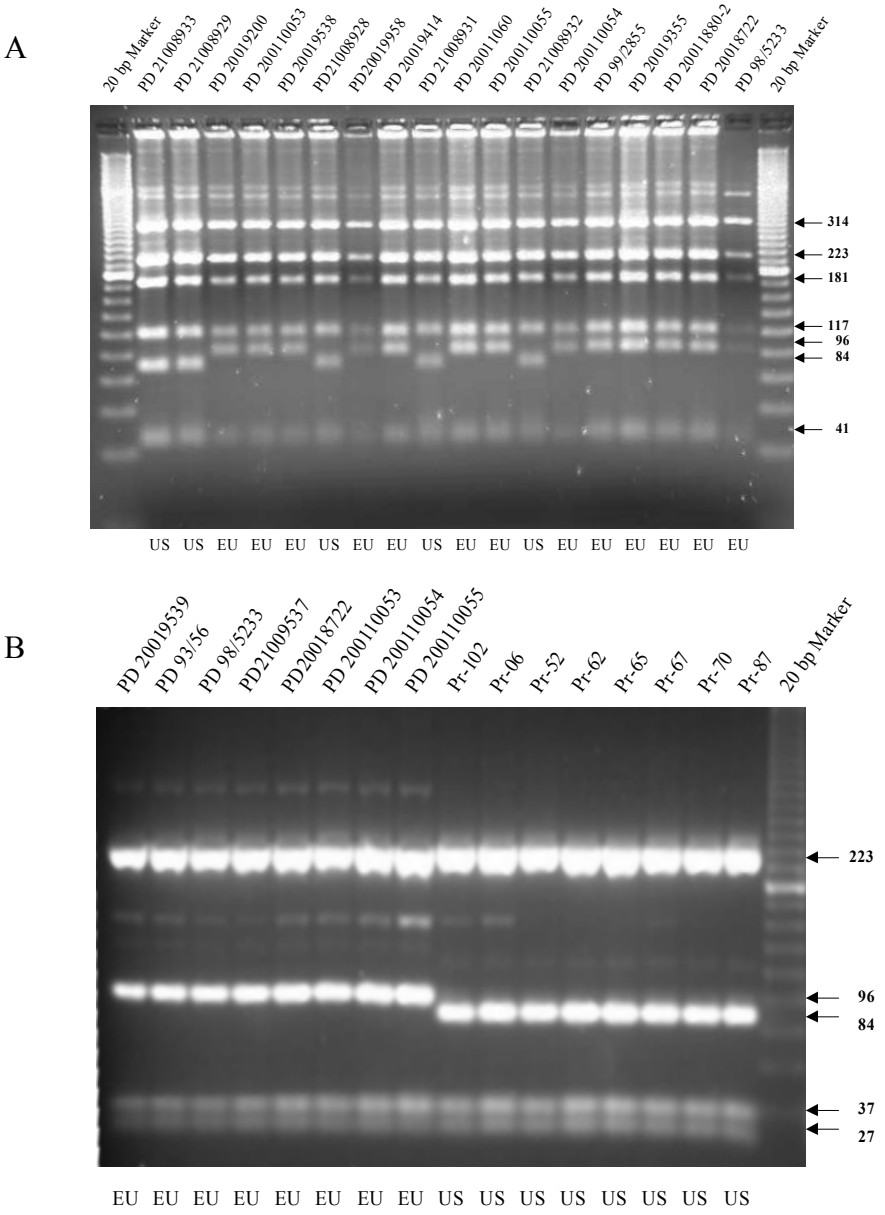

Figure 2. Restriction Fragment Length Polymorphism patterns for the 972-bp amplicon (Figure 2A) and the 383 bp amplicon (Figure 2B) of the Cytochrome c oxidase subunit 1 gene for 18 (Figure 2A) and 16 (Figure 2B) arbitrarily chosen isolates of *Phytophthora ramorum*. Isolates in lanes 1, 2, 6, 9, and 12 (Figure 2A) and isolates in lanes 9-16 (Figure 2B) came from the United States (US); isolates in the other lanes came from Europe (EU). Details on isolates are listed in Table 1. A 20-bp marker is used as size reference.

Infected material

DNA extracted from inoculated or naturally infected plants also was analyzed with this SNP assay. SNP restriction patterns from 8 inoculated plant samples are shown in Figure 3. DNA from plants artificially infected with isolates of *P. ramorum* from Europe produced an RFLP pattern similar to the one produced by European isolates of *P. ramorum* and DNA samples from plants artificially infected with isolates from the USA gave the US RFLP pattern. DNA samples from naturally infected plants from Europe first were screened for the presence of *P. ramorum* using the ITS species-specific primers and 36 samples were identified as positive (data not shown). In 21 out of these 36 samples the presence of EU-type isolates was determined using the SNP assay on the 972-bp fragment. No cross-amplification of *Q. rubra*, *Rhododendron* spp., or *Viburnum* spp. DNA was observed in experiments on infected plant material.

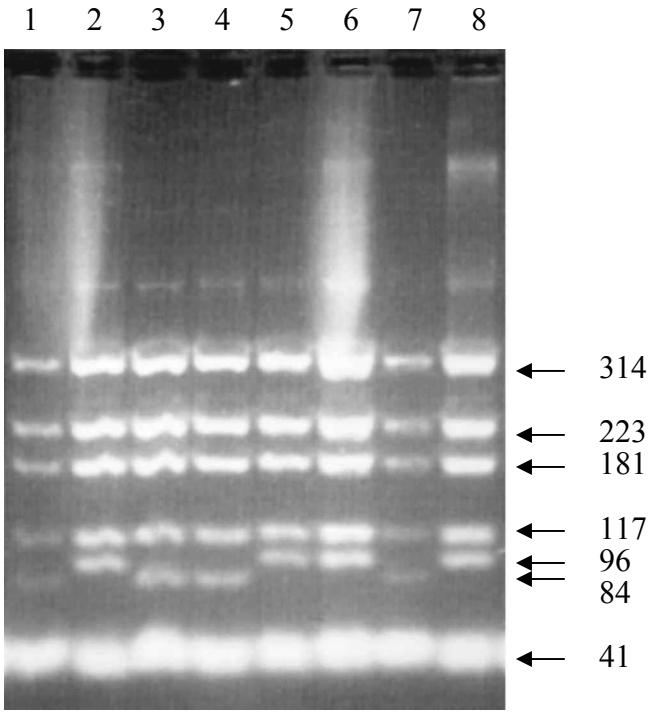

Figure 3. Restriction Fragment Length Polymorphism patterns for the 972-bp amplicon of the Cytochrome c oxidase subunit 1 gene for eight samples of DNA from infected plants inoculated with *Phytophthora ramorum* isolate PD20017608 (US-type, lanes 1, 3, 4 and 7) or PD20019539 (EU-type, lanes 2, 5, 6, and 8). Lane 1, 2 = *Quercus rubra*; lane 3 to 6 = *Rhododendron* sp.; lane 7, 8 = *Vaccinium* sp.

Table 3. *Apo*I restriction fragment lengths for the 972-bp amplicon of the Cytochrome c oxidase subunit 1 gene for isolates of *Phytophthora ramorum* from Europe (EU) and the United States (US) and additional species of *Phytophthora*.

	Restriction fragment size (bp) [a]																	
				235		199				117 119								
Phytophthora spp.	436	355	314	237	223	201	181	153	143	120	96	84	80	41[b]	36[b]	28[b]	24[b]	12[b]
P. ramorum EU	...	...	+	...	+	...	+	...	...	+	+	...	...	+	...	...	...	...
P. ramorum US	...	...	+	...	+	...	+	...	...	+	...	+	...	+	...	...	...	+
P. brassicae	...	...	+	...	+	...	+	...	...	+	...	+	...	+	...	...	...	+
P. cactorum	...	+	...	...	...	...	+	...	...	+++	...	...	+	...	...	...	...	...
P. cinnamomi	...	...	+	+	...	+	+	...	...	...	...	...	...	+	...	...	...	...
P. citricola	...	...	+	...	+	+	+	...	...	...	...	...	...	+	...	...	...	+
P. citrophthora	...	...	+	...	...	...	+	...	...	++	...	+	+	+	...	...	+	+
P. cryptogea	...	...	+	...	+	+	+	...	...	...	...	...	...	+	...	...	...	+
P. drechsleri	...	...	+	+	...	+	+	...	...	...	...	...	...	+	...	...	...	...
P. gonapodyides	...	+	...	...	...	+	+	...	...	+	...	...	+	...	...	...	+	+
P. heveae	...	...	+	...	+	++[c]	+	...	...	...	...	...	...	+	...	...	+[c]	+
P. hibernalis	...	...	+	+	...	...	+	...	...	+	...	+	...	+	...	...	...	...
P. hybrid alder	...	...	+	...	...	+	...	+	...	+	...	...	+	+	+	+	...	...
P. ilicis	...	...	+	...	...	...	+	...	+	+	+	...	+	+	...	...	...	...
P. infestans	...	+	...	...	...	+	+	...	...	+	...	...	+	...	...	...	+	+
P. lateralis	...	+	...	...	+	...	+	...	...	+	...	+	...	...	...	...	...	+
P. megasperma	...	...	+	...	...	+	+	...	...	+	+	...	...	+	...	...	+	...
P. nemorosa	...	...	+	...	...	...	+	...	+	+	...	+	+	+	...	...	...	+
P. nicotianae	...	+	...	...	...	+	+	...	...	+	...	+	...	...	+	...	...	...
P. palmivora	...	+	...	...	...	+	+	...	...	+	...	+	...	...	+	...	...	...
P. pseudosyringae	...	...	+	...	...	...	+	...	+	+	+	...	+	+	...	...	...	...
P. pseudotsugae	...	+	...	...	...	...	+	...	...	+++	...	...	+	...	...	...	...	...
P. syringae	+	...	+	...	...	...	+	...	...	...	...	...	...	+	...	...	...	...

[a] Restriction fragments are pooled in one column if the size difference between the fragments is not distinguishable on gel.
[b] These fragments are difficult to visualize due to low intensity or co-migration of primers in the gel.
[c] Alternate restriction profile because of a variable *Apo*I restriction site in the 223-bp fragment.

Comparison of P. ramorum ApoI restriction profiles with other Phytophthora spp.

Using primers CoxF4N and CoxR4N, the 972-bp fragment was amplified in isolates of 21 other species of *Phytophthora* (Table 1) that are closely related to *P. ramorum* or are pathogenic on hosts common with *P. ramorum* (data not shown). Although amplification efficiency varied considerably among species, *Apo*I restriction profiles could be determined for most species and these profiles corresponded with predicted fragment size from sequence analysis for all species analyzed (data not shown). For five species for which no clear RFLP-profile could be obtained (Table 1), restriction profiles were based on sequence information only. In total, 9 *Apo*I sites were found throughout the sequences and 16 distinct RFLP profiles were observed (Table 3).

European isolates of *P. ramorum* possessed a unique profile, but isolates from the United States had a profile similar to that of *P. brassicae*. The latter species, however, consistently showed additional fragments resulting from partial digestion.

Discussion

Historically, identification of species of *Phytophthora* has been based on morphological characteristics and growth on selective media which can be time-consuming and laborious. In addition, considerable expertise is needed to clearly differentiate among closely related species. When dealing with quarantine pathogens, suspicious material should be evaluated quickly and reliably for the presence of the quarantined organism. Quick evaluation methods will avoid deterioration of product quality as well as expensive storage of arboriculture trade materials. Reliable evaluation methods are essential to take adequate phytosanitary measures in trade and forestry, and may prevent the spread of quarantine organisms to new areas. More and more molecular techniques are being deployed for rapid detection and identification of plant pathogens (Cooke et al., 2000b, Kong et al., 2003). For *Phytophthora* species, ITS sequences of ribosomal DNA have been shown to be useful for species identification, although some related species share identical ITS sequences (Flier et al., 2002). For *P. ramorum*, an ITS-based PCR detection method is available (Garbelotto et al., 2002), but it cannot discriminate between isolates from Europe and isolates from the US.

The present test based on the mitochondrial *Cox*1 gene is the first to differentiate between European and American isolates of the pathogen. A single point mutation in this gene distinguished isolates of *P. ramorum* from Europe from those from the USA. Based on this point mutation we developed a single nucleotide polymorphism-based method, in which a multi-copy marker is amplified in *P. ramorum*, followed by a digestion with restriction enzyme *Apo*I. Although the SNP assay was initially designed to be used on *in vitro* cultures of *P. ramorum*, additional tests demonstrated the potential for using this assay to detect *P. ramorum in planta*.

In order to be reliably used in a regulatory setting, the potential for false positives, caused by the presence of other *Phytophthora* species, must be ruled out. Therefore, *Phytophthora* species with a similar host range as *P. ramorum,* and closely related species (based on a phylogeny of the species inferred from parsimony analysis of mitochondrial and nuclear DNA (Kroon et al., 2004)) were screened for RFLP-patterns of the *Cox*1 gene product

with the *Apo*I restriction enzyme. Most species possessed unique RFLP profiles that could differentiate them from *P. ramorum*. While European isolates of *P. ramorum* shared a unique and distinct profile, isolates from the USA had a different profile that was the same as the predicted profile for *P. brassicae*. However, it is unlikely that *P. brassicae* and *P. ramorum* would be found in the same ecological niche; *P. brassicae* is typically only found on non-woody hosts that mainly are in the family Amaryllidaceae (Man in 't Veld et al., 2002). Moreover, in RFLP-analysis the *P. brassicae* isolate showed additional, uncut *Apo*I fragments and was thus distinguishable from *P. ramorum* isolates. The recently described species *P. nemorosa* and *P. pseudosyringae*, which share an overlapping geographic distribution with *P. ramorum* in California (Hansen et al., 2003a, Martin and Tooley, 2003), were also included in this analysis. These species were clearly distinguishable from *P. ramorum* in RFLP-analysis, and thus the presence of these species in California will not compromise the accuracy of the assay. Intra-specific sequence variation may result in altered *Apo*I restriction profiles. For some species showing a similar profile as *P. ramorum*, this could potentially lead to mis-identification as *P. ramorum* isolates. However, a combination of the SNP-assay with the species-specific ITS-assay Garbelotto et al., 2002), or usage of the nested *Cox*1-PCR will overcome this lack of specificity.

Preventing the introduction of US-type isolates of *P. ramorum* into Europe, or European-type isolates into the USA, is of key importance. Although progeny between the two populations is hard to obtain, it recently has been demonstrated that there is a chance of the establishment of a sexual reproducing population (Werres and Zielke, 2003). However, the vitality and genetic content of the oospores produced in these experiments has not been tested. Studies with other species of *Phytophthora* (e.g. *P. infestans*) (Drenth et al., 1995; Turkensteen et al., 2000) indicate that oospores can survive for several years, even under unfavorable conditions. The occurrence of oospores could lead to additional problems, by providing the pathogen with new opportunities for survival and dispersal, thus thwarting eradication and control strategies for *P. ramorum*. If the two populations differ greatly in genetic content and virulence factors, giving them the opportunity for meiotic recombination would open the way for the generation of new genotypes of the pathogen.

In Europe, one isolate has been recovered that combined the A2 mating type with the European SNP-profile for *Cox*1 (Werres and De Merlier, 2003). This isolate clustered with European isolates in AFLP-analysis (L.P.N.M. Kroon and P.J.M. Bonants, unpublished). This observation may be explained by the process of selfing in A1-type isolates under influence of other species of *Phytophthora* (Ko, 1994), or chemicals (Groves and Ristaino, 2000). In this

way, A2-type isolates may be formed with a similar genetic content and SNP-profile as the European A1-type isolates. The reported presence of A1-type isolates in the USA (Hansen et al., 2003b) with the European type SNP-profile may be explained by a secondary introduction of *P. ramorum* isolates, which show similarity with the European population based on AFLP analysis.

In the event that the populations of *P. ramorum* from the United States and Europe occur in the same region and form sexual progeny, the SNP assay described in this paper can no longer be applied to distinguish these populations. The mitochondrial DNA, on which the *Cox*1 gene is situated, will be randomly distributed in the offspring and the association between mating type and mitochondrial type will be lost. Findings of both populations in Oregon nurseries has resulted in extensive eradication measures and monitoring at these sites (Hansen et al., 2003b).

In Europe, *P. ramorum* has recently been isolated from oak (*Q. rubra*) and this isolate was characterized as EU-type with the A1 mating type. This finding may indicate that the host range of the European population is not limited to *Rhododendron* and *Viburnum* species. Inoculation experiments using isolates from European and US populations on various host plants are in progress (De Gruyter et al., 2002). Further studies will be performed to investigate the populations on both continents in more detail. The present *Cox*1-based SNP-assay offers an attractive test for differentiating isolates at the population level, both for import quarantine purposes and for national surveys for *P. ramorum* biotypes.

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5

General Discussion

Phytophthora diseases worldwide

Plant diseases caused by *Phytophthora* species will remain an ever increasing threat to agriculture and natural ecosystems. The more than 100 species that have been described thus far may represent only a fraction of the species existing in nature. New species emerge continuously, either novel plant pathogens or variants from existing species, and the means to control them are limited. On the one hand, increased international trade will bring plants infected with *Phytophthora* pathogens into new areas, thereby exposing the indigenous flora to these potential pathogens. On the other hand, as many crops and ornamentals are grown in areas different from their centre of origin, they become exposed to the endemic pathogen populations. New methods of cultivation in greenhouses create unique environments in which *Phytophthora* species can interact and adapt to new hosts. With hindsight, could control measures have been taken to prevent or limit the spread and the resulting damage from *Phytophthora* species?

It is hard to change the nature of man. Plant breeders are constantly looking for new plant genotypes with beneficial traits to improve their crops. These wild relatives of our crop plants often harbour resistance genes to counteract plant pathogens, or may contain genes of interest for drought resistance, increased yield or more colourful flowers. The genetic basis of many of our major crops has eroded over time, with the shift from land races that were only grown locally to globally introduced varieties (Frankel, 1974; van Heerwaarden et al., 2009). For banana *(Musa acuminate*) for instance, almost all banana plantations worldwide are planted with one and the same variety, Cavendish (Marin et al., 2003). This variety is susceptible to the Black Sigatoka disease caused by *Mycosphaerella fijiensis* and to the wilt disease caused by *Fusarium oxysporum* f.sp. *cubense* 'Tropical Race 4' (TR4) (Agrios, 2004; Dita et al., 2010). The global production of banana is under great threat due to these diseases, and introduction of resistance genes from wild relatives is troublesome because of the triploid and nearly sterile nature of the cultivated banana.

During the last decades, plant breeders have become aware of the dangers of the narrow genetic basis in their crops, and have scouted the continents for wild relatives to enrich the gene pool of food and ornamental crops. Care should be taken that new diseases are not imported along with these wild varieties. National Plant Protection Services and breeding companies should cooperate efficiently to negate this risk. Part of this risk can be avoided by ensuring that only plant material is brought back that is mostly free of pathogens, like seeds for instance, although even seeds can contain fungi, oomycetes, viruses and bacteria. Proper

phytosanitary measures, molecular and visual diagnostic tests and disinfection are required to ensure that the plants that are used for the introduction of new traits are free of pathogens before they are used in breeding programs.

The quest for new ornamentals is an even greater risk. In history, there have been numerous examples of outbreaks of fungal and oomycete diseases following the introduction of a new ornamental plant species (Brasier, 2008; Cacciola et al., 2009; Moralejo et al., 2009). The root system of transplanted ornamentals, and the adhering soil, is a source of potentially dangerous pathogenic organisms for the destined habitat of the transplanted ornamental. Bacteria, nematodes, insects, fungi and oomycetes that are present in the adhering soil can cause much damage in their new environments where antagonists or natural enemies are lacking. As plant communities evolve, pathogenic microorganisms, viruses and insects have co-evolved with their hosts. These organisms hardly cause damage to their hosts in the centre of origin; they are part of a natural balanced ecosystem that evolved through concerted evolution. However, major problems may arise if a pathogen escapes – or is introduced – to another region of the world where the native plants have little resistance and the pathogen has no natural enemies or antagonists (Brasier, 2008).

The most illustrative and devastating example is the introduction of the root pathogen *Phytophthora cinnamomi* in Australia. It is able to infect more than 3000 plant species (Hardham, 2005) and has probably been spread from its presumed centre of origin in south east Asia. The pathogen has become a threat to some of the world's richest plant communities in south west Australia since the 1950s and continues to damage forests and other ecosystems world-wide. Another example is the introduction of *P. ramorum* into tree nurseries in Europe and the USA. This species, which is thought to originate from the eastern parts of Asia, is now causing Sudden Oak Death in the western part of the USA. Well known invasive fungal species are *Cryphonectria parasitica* and *Ophiostoma ulmi*. *C. parasitica* also originates in Asia and was first detected in the US in the early twentieth century. By the 1950s almost all American chestnut trees in the eastern part of the US had disappeared because of chestnut blight (Anagnostakis, 1987). *Ophiostoma ulmi*, the causal agent of the Dutch elm disease, is also believed to be native to Asia. From there, *O. ulmi* has been accidentally introduced into America and Europe, where it has devastated native populations of elms. These native populations had no opportunity to evolve resistance to the Dutch elm disease and mainly perished (Brasier and Kirk, 2001).

Classifying *Phytophthora* species; Waterhouse versus phylogeny

The first accepted classification system for *Phytophthora* species that is solely based on morphological traits was developed by Waterhouse (1963). In the last decade, the Waterhouse classification has been replaced by a system based on DNA sequences that are used as input for constructing phylogenetic trees, in which species are clustered in clades. However, the Waterhouse traits are still useful for comparing species within a clade or between clades. In most clades, species share the same character states for papillae on zoosporangia, or amphigynous or paragynous attachment of antheridia to oogonia. Several groups of isolates were identified as misfits according to the Waterhouse criteria. Isozyme analysis and DNA sequence analysis have clarified the status of these isolates as new species or species hybrids. Combination of mycological analyses with phylogenetic analysis will lead to interesting new insights in the distribution of species in the evolutionary tree of *Phytophthora* and in the evolution and transition of morphological, pathogenic and reproductive traits.

Hybridization events

There is increasing evidence that hybridization between different *Phytophthora* species plays a major role in the generation of new species. This reticulation fuels rapid evolution in the genus (Ersek and Nagy, 2008). Since this interspecific hybridization could be responsible for accelerated generation of new, potentially destructive pathogens it is of utmost importance to elucidate the mechanisms and to measure the relative occurrence of interspecific hybridization in *Phytophthora*. The incidence of interspecific hybridization is believed to be on the rise, as contact between previously geographically isolated *Phytophthora* pathogens increases due to intensifying trade and traffic of host plant species (Man in ' t Veld et al., 2007).

The occurrence of natural hybrids between *P. nicotianae* and *P. cactorum* in The Netherlands has been described by Man in ‘t Veld et al. (1998), soon followed by reports on similar findings of these hybrids in other countries (Hurtado-Gonzales et al., 2009). Hybrids between other species of *Phytophthora* have also been found. Four isolates which were previously identified as *P. porri* were shown to be hybrids between *P. porri* and *P. primulae*,

and these hybrids had an altered host range when compared to the parental species (A. de Cock, personal communication).

The introduction of an exotic *Viburnum* species in a commercial nursery in the Netherlands coincided with the appearance of *P. hedraiandra* in the same plot. *P. hedraiandra* is not endemic in the Netherlands, so this species probably hitchhiked along with its natural host. Hybrid progeny was found between the introduced species *P. hedraiandra* and the native species *P. cactorum.* The progeny was found to infect numerous new hosts. Moreover, second generation offspring was also detected, presumably resulting from intercrossing between hybrid isolates or backcrossing of hybrid isolates with *P. cactorum* (Man in 't Veld et al., 2007). It is striking that the species *P. cactorum* appears in more than one interspecific hybridization event. This could be due to the omnipresence of this species, or because of an increased ability to interact with other *Phytophthora* species.

Based on molecular data of the ITS region, the sympatric species *P. infestans* and *P. mirabilis* appear indistinguishable. Analysis of nuclear and mitochondrial genes (chapter 2.2) indicated that there is slight sequence variation between *P. infestans* and *P. mirabilis*, but this could easily be intraspecific variation. As *P. infestans* and *P. mirabilis* both originate from the same geographic location, it could be possible that they can still interact and exchange genetic material. To test for this possibility, interspecific hybrids were created between *P. infestans* and *P. mirabilis* isolates in the laboratory and the resulting F1 isolates were intercrossed and backcrossed to *P. infestans* and *P. mirabilis* isolates (chapter 3.1). The hybrid nature of the progeny was verified by DNA fingerprinting, and when the hybrids were tested for virulence in detached leaflet assays on the hosts of the parental isolates -*Mirabilis jalapa* as a host of *P. mirabilis*, and *Solanum tuberosum* and *S. lycopersicum* as hosts of *P. infestans*-, they showed increased vigour and a shared host range when compared to parental isolates. The *Phytophthora* population in Toluca Valley in Mexico has been extensively sampled, but no hybrid isolates between *P. infestans* and *P. mirabilis* were found (Flier et al., 2002; 2003). So despite the fact that sexual recombination between two co-existing *Phytophthora* species is feasible, there is no guarantee that this will happen in nature.

A recent example of interspecific hybridization between *P. infestans* and an as yet unknown clade 1c *Phytophthora* species is described in chapter 3.2 and 3.3 of this thesis. The *P. andina* isolates possess all characteristics of a recent hybridization event in the Andes. As described previously for *P. hedraiandra*, interspecific hybridization can occur if a species is introduced into a new environment. As the local *P. infestans* population consists of only a few

clonal lineages with limited variation within the lineage, the parental species that is introduced into the Andes is probably *P. infestans*.

Centre of origin of *P. infestans*

To understand the population dynamics and the plant pathogenic potential of *P. infestans* populations, both at present and in the future, one needs to understand the history of this pathogen. One of the issues that need to be resolved is the question on the origin of *P. infestans*. In the centre of origin, local *Solanum* populations carrying late blight resistance co-evolved with *P. infestans*. Breeders have exploited these resistant *Solanum* species for introgression of resistance genes into commercial potato varieties, in the hope to generate durable and stable resistance to *P. infestans*.

Many evolutionary biologists and phytopathologists conducting research on *Phytophthora* species in clade 1c consider the Toluca Valley in Central Mexico as the most likely centre of origin of *P. infestans* (Goodwin and Drenth, 1997; Grünwald et al., 2001). In that region, much variation is found in the *P. infestans* population, both mating types occur in equal frequencies, and sexual reproduction is taking place within the *P. infestans* population (Flier et al., 2001). Some closely related species, like *P. mirabilis* and *P. ipomoeae*, also reside in this geographic region. Based on morphology, markers, and sequence analysis these species are very similar to *P. infestans* (Blair et al., 2008; Flier et al., 2002; Goodwin et al., 1999; Kroon et al., 2004) but they cannot infect Solanaceous hosts. Their hosts are *Mirabilis jalapa* (for *P. mirabilis)* and *Ipomoea longipedunculata* (for *P. ipomoeae*), two non-Solanaceous species that are not infected by *P. infestans*.

The discovery of a novel *Phytophthora* species in the Andean highlands in Ecuador has stirred new life into the discussion on the possible origin of *P. infestans*. This new species, named *P. andina* (chapter 3.3), was isolated from *Solanum* species endemic to Ecuador and Peru (chapter 3.2). Although it is described as a new species, it seems more of interspecific hybrid origin. In the gene sequences, a hybrid pattern can be recognized. One parent appears to be *P. infestans*; the other shares character states with *P. ipomoeae* and *P. mirabilis* and hence resembles other clade 1c species. Based on phylogenetic studies, the other parent of this hybrid is likely to be more ancestral than *P. mirabilis* and *P. ipomoeae* (Kroon et al., 2004). These observations raised the thought that the Andes is the centre of origin of *P. infestans* whereas Toluca Valley is merely a centre of diversity.

In the last decade several papers appeared that argue in favour of the Andes being the centre of origin of *P. infestans* (Abad and Abad, 1997; Gomez-Alpizar et al., 2007; Ristaino et al., 2001). These studies are mainly based on mitochondrial DNA as a marker to distinguish between isolates or populations of *Phytophthora* species. For *P. infestans*, four mitochondrial DNA haplotypes have been detected in global populations (Griffith and Shaw, 1998), three of which regularly occur in pathogen populations in the Toluca Valley (Flier et al., 2003). Before the introduction of the A2 mating type of *P. infestans* in Europe in the 1970s, worldwide populations consisted of one single clonal lineage named US1, that has the Ib mitochondrial haplotype and the A1 mating type. This clonal lineage was believed to be responsible for the initial *P. infestans* infections in the 1840s in the US and Europe. However, analysis of herbarium samples collected and archived during the potato famine (1845-1847) in France, Britain and Ireland proved this theory to be wrong; the isolates found on these herbarium samples were of the Ia haplotype (Ristaino, 2002). This would mean that the population that caused the initial outbreaks of potato late blight in Europe was replaced by another, clonally propagated population. This replacement can occur when the newly arrived pathogen population has a fitness advantage over the established population. This advantage may consist of a higher level of resistance to fungicides, virulence factors that can counter resistance genes introduced in potato crops or an elevated aggressiveness to the host.

The absence of the mitochondrial haplotype Ib in *P. infestans* isolates from Toluca Valley in Mexico was considered one piece of evidence that this region is likely not the centre of origin of *P. infestans* (Gomez-Alpizar et al., 2007). In July 2007, however, the occurrence of isolates with the Ib haplotype was reported in Mexico. In these isolates the mating type and isozyme patterns were distinctly different from the US1 genotype, and hence it is likely they are part of a sexually propagating population (Garay-Serrano et al., 2007). Apparently all known mitochondrial haplotypes of *P. infestans* are present in Mexico, albeit in low frequencies in the case of haplotype Ib, and therefore these findings undermine the claim that Toluca Valley cannot be regarded as the centre of origin of *P. infestans* because of the absence of the Ib haplotype.

All four mitochondrial haplotypes of *P. infestans* have been sequenced and the level of variation between and within the haplotypes has been investigated (Avila-Adame et al., 2006; Lang and Forget, 1993). Type I and type II haplotypes have diverged significantly from each other, but as yet the most ancestral line could not be designated. Comparisons between the four mitochondrial haplotypes for *P. infestans*, and the mitochondrial loci that were sequenced for *P. mirabilis*, *P. phaseoli*, *P. ipomoeae*, *P. andina* and *P. nicotianae* (Kroon et

al., 2004; Martin and Tooley, 2003; Oliva et al., 2010), suggest that type I mitochondria are the ancestral type because haplotype Ia and Ib share character states with these species. However, comparison of the mitochondrial DNA sequence of *P. infestans* with those of *P. ramorum* and *P. sojae* indicates that for other Single Nucleotide Polymorphisms (SNPs) between the four different haplotypes, haplotype II seems to be the ancestral type. Sequencing of the complete mitochondrial DNA of *P. mirabilis*, *P. phaseoli*, *P. ipomoeae*, and *P. andina* could shed light on the evolution of mitochondrial DNA in clade 1c species. To resolve the question on the centre of origin, Goméz-Alpizar et al. (2007) included the mitochondrial haplotype of *P. andina* into the *P. infestans* group, stating that the nucleotide diversity in the Andean population was higher than in the Toluca Valley. However, the inclusion of *P. andina* may be questioned, as it is a separate species with many distinct characteristics (chapter 3.2 amd 3.3).

For nuclear diversity, the level of genetic variability in *P. infestans* is much higher in the Toluca Valley than in the Andean Highlands (Adler et al., 2004; Flier et al., 2001, 2003; Forbes et al., 1997; Grünwald, 2006; Grünwald et al., 2001). In the Andes, the population of *P. infestans* seems to consist of clonal lineages, with little gene flow between the groups (chapter 3.2/3.3). According to Goméz-Alpizar et al. (2007), the high levels of genetic variability in the Toluca Valley are the result of sexual reproduction rather than ancestry. This observation seems plausible, but another aspect of population genetics needs to be taken into account. Although sexual reproduction leads to new combinations of alleles, the number of alleles present at a random locus may be higher in clonally reproducing populations. In a clonally propagated isolate, the number of mutations will rise to a certain saturation level. Since *P. infestans* is a diploid organism, mutations at each locus are needed at both alleles to have a negative or deleterious effect. The chance of acquiring two deleterious mutations at one locus is quite low. In a sexually reproducing population, recessive deleterious mutations within the population can be transmitted by both parental isolates. Part of the progeny will be homozygous for the deleterious mutation and will suffer a fitness penalty. Alleles with a negative fitness effect will slowly disappear from the population as a result of this negative effect on fitness.

Alleles that inherit neutrally can also disappear from a sexually reproducing population by random genetic drift. The only accurate way to measure the genetic variability between and within populations in the Andes and in the Toluca Valley, and to define the most likely centre of origin of *P. infestans*, is by measuring allelic variability and allelic frequency. This could be accomplished by whole genome sequencing for a substantial number of isolates

from *P. infestans*, and its close relatives *P. andina*, *P. mirabilis*, *P. ipomoeae* and *P. phaseoli*, and by measuring allele frequency in Andean and Mexican populations of clade 1c species.

Another question that remains to be resolved is the initial source of infection for the devastating late blight epidemics in the USA and in Europe in the early 1840s. The first infection is thought to originate from infected tubers of wild potatoes introduced for breeding purposes (Fry et al., 1992, 1993; Goodwin et al., 1994). Abad and Abad (1997) state that, based on research by Rivera-Peña (1988) this source of infection is unlikely, as infected tubers of wild *Solanum* species have never been found in the Toluca Valley. It is however feasible that oospores could have been present in or on tubers with adhering soil that were transported to the US or Europe (Fernandez-Pavia et al., 2002, 2004; Flier et al., 2001). A latent infection or presence of inoculum in the soil on the transported tuber would lead to an infected plant in the following growing season, and the start of an epidemic.

When taking all the facts into consideration, the centre of origin of *P. infestans* is likely to be the Toluca Valley rather than the Andes. The *P. infestans* population in Toluca Valley in Mexico is the most diverse population found worldwide, whereas the *P. infestans* population in the Andes consists of clonal lineages that may have been introduced only in the last 150 years. Interspecific hybridization can occur when a species is introduced into a new habitat and it is plausible that such an introduction has lead to the interspecific hybridization event between the unknown parental species of *P. andina* and *P. infestans* in the Andes. The centre of origin of the ancestral clade 1c species remains unknown.

Future perspectives

Improved understanding of the genetic variability of *P. infestans* may facilitate precise predictions on stability of host resistance and fungicide efficacy. In that light, it is important to assess the plant pathogenic potential and dynamics of global populations of *P. infestans*. In the last decade, genome initiatives have resulted in whole genome sequences of several oomycete pathogens including *P. infestans*, *P. ramorum*, *P. capsici* and *P. sojae*, and of *Hyaloperonospora arabidopsis* and *Pythium ultimum* (Haas et al., 2009; Levesque et al., 2010; Tyler et al., 2006). Additionally, Illumina survey sequencing of *P. mirabilis*, *P. phaseoli*, *P. andina* and *Albugo candida* has been carried out, and four genotypes of *P. sojae* have been sequenced using the 454 sequencing technology (Chi, 2008). The comparison of these sequence data has produced a wealth of information on toxin and effector

genes that may be involved in interactions with host plants. The enormous numbers of potential effectors identified in the genomes of *Phytophthora* species show their versatility and potential to infect many different host plants by employing those effectors. It will be a challenge to designate functions for all those effectors during infection, colonization, survival in the absence of a host plant, and reproduction (Tyler et al., 2009b).

Effector genes encode proteins that are capable of entering host cells by virtue of their RXLR-motif (Whisson et al., 2007). This motif facilitates entry into plant cells seemingly independent of a specific microbe-derived machinery (Govers and Bouwmeester, 2008). Once the effectors are in the cell, they potentially target any host protein or process to the microbe's own needs. Suppression or mitigation of effector-triggered R protein-mediated immunity or PAMP-triggered immunity are obvious targets for these effectors (De Wit, 2007; Jones and Dangl, 2006). In the *P. sojae* genome, 400 effector candidates have been identified, and nearly all of the 200 candidates that have been tested so far for suppression of host defence had an effect on either effector- or PAMP-triggered immunity, or on both (Tyler et al., 2009a). In addition, the interaction between *P. sojae* and soybean (*Glycine max*) has been studied at the transcriptome level. The changes in mRNAs levels in both host and pathogen during infection are dramatic and partly concern genes related to the mechanisms required for suppression of defence (Tyler et al., 2009b).

For *P. infestans* and *Solanum* species, much information has become available on the key components determining the outcome of a host-pathogen interaction (Haas et al., 2009; Jacobs et al., 2010). The comparisons of sequences available for these components and their regulation in host-pathogen interactions will advance research on host specificity in *P. infestans* in an unprecedented way.

With the application of the third generation sequencing technologies (Pennisi, 2010), the feasibility of sequencing large numbers of isolates, and their corresponding hosts, will increase within the next few years. Not all information can be read from the DNA though; there remain some mysteries to be solved. Processes like epigenetic gene regulation or RNA interference (RNAi) can strongly affect biological processes and can not be studied by sequencing alone. Expression studies are needed in addition to the sequence information, and the theories on the mechanisms underlying pathogenicity have to be tested experimentally in infection assays. The dissection of the various roles of effectors by functional gene analyses remains a bottleneck for *Phytophthora* research.

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SUMMARY

Plant diseases caused by *Phytophthora* species are an ever increasing threat to agriculture and natural ecosystems. Hundred and thirty-four years ago Anton de Bary founded the genus *Phytophthora* and named the causal agent of potato late blight *Phytophthora infestans*. Since then, over one hundred species have been added to the *Phytophthora* genus and especially during the last decade, the number of characterized and taxonomically described *Phytophthora* species has rapidly increased. Lately, descriptions of new *Phytophthora* species appear almost on a monthly basis. This stunning increase is, on the one hand, due to the availability of more sophisticated tools for species determination and on the other hand to large scale surveys for the presence of novel *Phytophthora* species in natural and agricultural settings. Phylogenetics has paved the way for a detailed and comprehensive analysis of the evolution of Eukaryotes. In the tree of life the genus *Phytophthora* is classified in the Stramenopile lineage within the supergroup Chromalveolates, and in the class of Oomycetes. **This thesis** describes studies on phylogeny, speciation and host specificity in the genus *Phytophthora*.

The general introduction **(section 1)** gives some insight in the biology of *Phytophthora* and the damage caused by a few notorious *Phytophthora* species. Subsequently, literature concerning the emergence of novel *Phytophthora* species by means of sexual interspecific hybridization and somatic hybridization is summarised and the scope of this thesis is presented.

The *Phytophthora* phylogeny is covered in **section 2**. **Chapter 2.1** provides an overview of the evolution of species identification and classification throughout the last five decades, from classical mycology to multi-locus sequence analysis. The impact of large scale surveys on presence, niche preference and species diversity of *Phytophthora* species in diverse habitats is being discussed. **Chapter 2.2** describes the first phylogenetic analysis of the *Phytophthora* genus based on both nuclear and mitochondrial DNA sequences. The analysis included in total 113 isolates from 48 *Phytophthora* species that were classified into eight clades. The evolution and transition of morphological, pathogenic and reproductive traits were inferred from the cladogram and the genus *Phytophthora* was confirmed to be monophyletic. **Chapter 2.3** presents an overview of over one hundred *Phytophthora* species that are currently known to exist. It includes the distribution of the species over ten clades and details on their preferred host(s), spore morphology and niche preference.

In **section 3** the focus is on interspecific hybridization, host specificity and speciation in clade 1c of the *Phytophthora* genus. The main representative in this clade is *P. infestans*. In **chapter 3.1**, sexual interspecific hybridization is examined in the closely related species *P. infestans* and *Phytophthora mirabilis*. Crosses between *P. infestans* and *P. mirabilis* can occur in nature, as both species occupy the same ecological niche. Interspecific progeny generated in the laboratory was tested for aggressiveness and host specificity on the plant species infected by either parent. This revealed that tomato may not only act as a bridging host for the interspecific hybrids, but may also provide the right conditions for reproduction and selection of viable and virulent intra- and interspecific hybrids that are pathogenic on *Mirabilis jalapa*, tomato and potato. The Andes in South America is the centre of origin of many Solanaceous plant species. As described in **chapter 3.2**, field surveys were carried out in the Andean Highlands, and a large collection of samples of diverse Solanaceous hosts was collected and characterized by molecular and mycological techniques. This resulted in the identification of distinct lineages of *P. infestans* and a number of aberrant *P. infestans*-like lineages. **Chapter 3.3** sheds more light on the status of these *P. infestans*-like lineages. Based on phylogenetic and biomarker analyses we classified these lineages as a new species named *Phytophthora andina,* which may well be a species of hybrid origin. As mentioned in the general discussion of this thesis **(section 5)**, the presence of this putative species hybrid in the Andes, and the role of *P. infestans* in this hybridization event supports the hypothesis that the Toluca Valley in central Mexico is the centre of origin of *P. infestans.*

Section 4 describes a diagnostic assay that was developed to distinguish between *Phytophthora ramorum* isolates that were found in the USA on oak trees and in Europe on rhododendrons. This assay was a spin-off of the phylogenetic analysis covered in **section 2**. The test was used by National Plant Protection Agencies to screen nurseries and plant materials that are shipped between countries for the presence of *P. ramorum*, the causal agent of Sudden Oak Death.

In the general discussion **(Section 5)** the influence of man on natural plant communities and local pathogen populations is discussed. By introducing exotic plant species and the adhering plant pathogenic microflora into ecosystems, the delicate balance between host plants and pathogens may be broken, which in turn can lead to devastating epidemics of the newly introduced pathogen species. The introduction of exotic pathogens into a new habitat can also lead to interspecific hybridization between introduced and endemic pathogens, a process that has been found to occur regularly in *Phytophthora* species, both in natural systems and in agriculture. Phylogenetic analyses that were used to define the parental

species involved in interspecific hybridization events have provided insight into reticulate evolution events and suggest that *Phytophthora* species hybridization may produce unique offspring capable of infecting an expanded range of host plants. Moreover, this offspring has the potential to evolve rapidly, leading to accelerated speciation. Finally, the role of next generation sequencing in studies on *Phytophthora* speciation and population dynamics, and the impact of the wealth of information that has become available since the public release of genome sequences of three *Phytophthora* species, is discussed. Increased insight in the way effectors produced by *Phytophthora* species modulate host defence will shed new light on the genetic and molecular basis of host specificity in *Phytophthora*.

SAMENVATTING

Plantenziekten die veroorzaakt worden door *Phytophthora* soorten vormen een steeds toenemende bedreiging voor zowel de landbouw als natuurlijke ecosystemen. Anton de Bary was degene die honderdvierendertig jaar geleden het geslacht *Phytophthora* in het leven riep en die de veroorzaker van de aardappelziekte de naam *Phytophthora infestans* (verwoestende plantenvernietiger) gaf. Sindsdien zijn meer dan honderd soorten toegevoegd aan het geslacht *Phytophthora* en vooral in het laatste decennium is het aantal soorten dat gekarakteriseerd en taxonomisch beschreven is, snel toegenomen. In de literatuur verschijnen bijna maandelijks beschrijvingen van nieuwe *Phytophthora* soorten. Deze snelle toename is enerzijds toe te schrijven aan de beschikbaarheid van meer verfijnde methoden voor soortsdeterminatie en anderzijds aan grootschalige monitoring op de aanwezigheid van nieuwe *Phytophthora* soorten, zowel in natuurgebieden als in de land- en tuinbouw. Met de opkomst van de moleculaire genetica is een grondige en gedetailleerde analyse van de evolutie van de Eukaryoten mogelijk geworden. In de "tree of life" valt het geslacht *Phytophthora* binnen de Stamenopiles in de supergroep Chromalveolaten, en in de klasse van de Oomyceten. **Dit proefschrift** beschrijft onderzoek naar de fylogenie, soortsvorming en waardplant-specificiteit in het geslacht *Phytophthora*.

De inleiding **(sectie 1)** geeft inzicht in de biologie van *Phytophthora* en in de schade die wordt veroorzaakt door een aantal beruchte *Phytophthora* soorten. Dan volgt een samenvatting van literatuur op het gebied van het ontstaan van nieuwe *Phytophthora* soorten door sexuele interspecifieke hybridisatie en somatische hybridisatie, en tenslotte worden de doelstellingen en opzet van het onderzoek beschreven die in dit proefschrift worden gepresenteerd.

De fylogenie van het geslacht *Phytophthora* wordt behandeld in **sectie 2. Hoofdstuk 2.1** geeft een overzicht van ontwikkelingen op het gebied van soorts-identificatie en -classificatie gedurende de laatste 50 jaar, van klassieke mycologie tot multi-locus sequentie-analyse, en belicht de impact van grootschalige monitoring op aanwezigheid, soorts-diversiteit en niche-preferentie van *Phytophthora* soorten in diverse habitats. **Hoofdstuk 2.2** beschrijft de eerste fylogenetische analyse van het geslacht *Phytophthora* gebaseerd op zowel nucleaire als mitochondriële DNA-sequenties. De analyse omvatte 113 isolaten van 48 *Phytophthora* soorten, die gegroepeerd werden in acht clades. De evolutie en overerving van morfologische en reproductieve eigenschappen, en van pathogeniteit konden worden afgeleid uit het cladogram. Deze studie bevestigde dat het geslacht *Phytophthora* monofyletisch is.

Hoofdstuk 2.3 geeft een overzicht van de meer dan honderd *Phytophthora* soorten die nu bekend zijn. De soorten zijn gerangschikt in tien clades en van iedere soort worden de belangrijkste waardplant(en), de morfologie van de sexuele en asexuele sporen en de natuurlijke niche vermeld.

In **sectie 3** ligt de focus op interspecifieke hybridisatie, waardplant-specificiteit en soortsvorming in clade 1c van het geslacht *Phytophthora*. De meest belangrijke *Phytophthora* soort in deze clade is *P. infestans*. In **hoofdstuk 3.1** wordt onderzoek aan interspecifieke hybridisatie tussen de nauw verwante soorten *P. infestans* en *Phytophthora mirabilis* beschreven. Aangezien beide soorten dezelfde ecologische niche bezetten, is het mogelijk dat onder natuurlijke omstandigheden beide soorten kruisen. Interspecifieke hybride nakomelingen die in het laboratorium werden gegenereerd, zijn getest op agressiviteit en virulentie op de waardplanten van beide afzonderlijke oudersoorten. Dit onderzoek bracht aan het licht dat tomaat niet alleen een overbruggings-waardplant kan zijn voor de interspecifieke hybriden, maar ook een waardplant waarop virulente en levensvatbare intra- en interspecifieke hybride nakomelingen gevormd kunnen worden die pathogeen zijn op *Mirabilis jalapa*, tomaat en aardappel. De Andes in Zuid-Amerika is het oorsprongsgebied van veel plantensoorten uit de familie van de nachtschades (Solanaceae). In **hoofdstuk 3.2** wordt beschreven hoe in veldonderzoek in de hooglanden van de Andes een grote collectie van isolaten, die op verschillende soorten binnen de Solanaceae voorkomen, is verzameld, en hoe deze collectie gekarakteriseerd is met moleculaire en mycologische technieken. Dit heeft geresulteerd in de identificatie van een aantal afgebakende groepen binnen de *P. infestans* populatie in de Andes, en van een aantal onderscheidbare groepen die wel sterke gelijkenis vertonen met *P. infestans.* **Hoofdstuk 3.3** werpt nieuw licht op de status van deze nieuwe groepen. Op basis van fylogenetische analyse en biologische merkers konden deze groepen worden geclassificeerd als een nieuwe soort, *Phytophthora andina*, met een mogelijke hybride oorsprong. In de algemene discussie **(sectie 5)** worden argumenten aangedragen hoe de aanwezigheid van deze mogelijke hybride in de Andes, en de rol van *P. infestans* in de hybridisatie-gebeurtenis die aan deze soort ten grondslag ligt, de hypothese dat de vallei van Toluca in Mexico het werkelijke oorsprongsgebied van *P. infestans* is, kunnen ondersteunen.

Sectie 4 beschrijft een diagnostische toets die is ontwikkeld om *Phytophthora ramorum* isolaten die in de Verenigde Staten op eik voorkomen te onderscheiden van isolaten die in Europa voorkomen op rhododendron. Deze diagnostische toets is een direct resultaat van de fylogenetische analyse beschreven in **sectie 2** en is gebruikt door

Plantenziektenkundige Diensten in verschillende landen om boomkwekerijen en internationale plantenhandelswaar te controleren op aanwezigheid van *P. ramorum*.

In de algemene discussie **(sectie 5)** wordt de invloed van de mens op de natuurlijke flora en locale pathogeenpopulaties bediscussieerd. De introductie van exotische plantensoorten, en de plantenpathogene microflora die daar op aanwezig is, kan resulteren in verstoring van de gevoelige balans die in ecosystemen bestaat tussen plantenpopulaties en pathogenen. Introductie van exotische pathogenen in een nieuwe habitat kan verwoestende epidemieën tot gevolg hebben en kan ook leiden tot interspecifieke hybridisatie tussen geïntroduceerde en endemische pathogenen. Dit proces kan met zekere regelmaat worden waargenomen in *Phytophthora* soorten, zowel in de natuur als in agrarische systemen. Fylogenetische analyses zijn gebruikt om de soorten te identificeren die als ouder betrokken zijn bij interspecifieke hybridisatie, en geven inzicht in reticulaire evolutie. De resultaten van dit onderzoek suggereren dat hybridisatie in *Phytophthora* soorten leidt tot de vorming van unieke nakomelingen die de eigenschap hebben om een meer uitgebreide waardplant-reeks aan te tasten dan de afzonderlijke oudersoorten. Bovendien zijn er aanwijzingen dat deze nakomelingen sneller evolueren hetgeen weer kan leiden tot versnelde soortsvorming. Tenslotte wordt de rol van "next generation sequencing" in het onderzoek naar soortsvorming en populatie-dynamica in *Phytophthora* belicht, en de betekenis van de schat aan informatie die beschikbaar is gekomen uit de genoom-sequenties van drie *Phytophthora* soorten. Meer kennis over de wijze waarop effectoren die geproduceerd worden door *Phytophthora* soorten afweer-mechanismen van waardplanten kunnen ondermijnen, zal nieuwe inzichten geven in de genetische en moleculaire basis van waardplant-specificiteit in *Phytophthora*. Het ophelderen van de rol van deze virulentie-factoren van *Phytophthora* soorten zal op termijn hopelijk ook kunnen leiden tot meer duurzame methoden om hun waardplanten te beschermen.

DANKWOORD

Het is gedaan! Na jarenlang “bijna klaar” te zijn geweest, is het nu toch echt zo ver. Het was er bijna niet meer van gekomen, maar na lang (en stevig) aandringen van collega’s, professoren en familie is de knoop doorgehakt, en heb ik toch genoeg tijd en energie gevonden voor het afronden van mijn proefschrift. Met het prikken van de promotiedatum is alles in een stroomversnelling gekomen en toen ik eenmaal iedereen verteld had dat in juni 2010 een feestje gevierd kon worden, was er geen weg meer terug. Gelukkig maar, want als ik het niet afgemaakt zou hebben, was er denk ik altijd iets blijven knagen.

Het had ook niet veel gescheeld of ik was niet verder gegaan in het onderzoek, maar beroepsmilitair geworden. In de zomer van 1998 werd ik toegelaten op de Koninklijke Militaire Academie in Breda. Jan van Kan heeft flink op me ingepraat om dat niet te doen en toch verder te gaan op mijn vakgebied. Uiteindelijk ben ik overstag gegaan en dat is een goede keuze geweest. Bedankt, Jan!

Eind 1998 kon ik bij het Instituut voor Plantenziektenkundig Onderzoek aan de slag, eerst in het *Fusarium*-onderzoek en daarna met *Phytophthora infestans*. Het laatste pathogeen werd direct mijn favoriet. Deels omdat ik de aardappelziekte van huis uit al kende, deels omdat ik er met een groep ontzettend enthousiaste en leuke collega’s werkte. Wilbert, jouw passie voor het onderzoek (en voor ongein) werkte aanstekelijk. Ik heb veel van je geleerd, al mag ik jouw methode voor urenregistratie niet meer toepassen in mijn huidige baan. Trudy, als iemand het bewijs is dat gezelligheid en hard werken heel goed samen kunnen gaan, ben jij dat wel. Henry en Maarten waren prima partners in crime voor het BlightMOP project, en ook Marieke, Petra en Bert zorgden voor een goede sfeer in het *Phytophthora* lab. Geert, ook van jou heb ik een hoop opgestoken en ik snap nu waar die grijze haren bij het afronden van jouw proefschrift vandaan kwamen. Mijn kapster heeft me gelukkig gerustgesteld met de opmerking dat het “erotisch grijs” is. Nicky, the times you were with us in Wageningen were very “gezellig”, thanks for the good time!

Daarnaast zijn er natuurlijk nog vele andere collega’s waar ik bij het IPO en bij Plant Research International met veel plezier mee gewerkt heb. Het gaat te ver om ze allemaal te noemen, maar Els, Ineke, Peter, Marjanne, Marga, Odette, Theo, Thamara, Cees, Gert en Piet wil ik toch op deze manier speciaal bedanken voor de leuke tijd, en de kans om een goed onderzoeksproject neer te zetten. Ook wil ik Arthur de Cock van het CBS bedanken voor de leuke discussies en de ondersteuning.

Na zes jaar bij het IPO en Plant Research International ging ik terug naar mijn roots in West-Friesland en kreeg ik een baan bij Bejo Zaden in Warmenhuizen bij de groep Merkertechnologie en Genomics. Ook hier heb ik het geluk dat ik in een enorm gezellige en sociale groep terecht ben gekomen. Henk, als "pater familias" van de groep ben je niet meer in het lab te vinden, maar ik loop graag bij je binnen voor wat goede raad over het onderzoek, of om nieuwe technieken te bespreken. Dorien, Wendy, Cees, Alie, Rosa, Wijnand, Jan-Dick, Mirelle, Mariëtte, Ralf, Simone, Gerda en Femmy, jullie zijn super-collega's. Bert, jij bent vanuit Bejo een goede stimulans en hulp geweest, en Astrid, je hebt me geweldig geholpen met het samenstellen en inbinden van de leesversie.

Francine en Pierre, ik weet niet of jullie veel andere PhD-studenten gehad hebben voor wie een deadline zo'n rekbaar begrip was. Ik hoop dat jullie toch begrijpen dat een keuken, verwarming of badkamer veel tastbaardere begrippen zijn dan een dokterstitel, en op de eerste plaats kwamen. Zeker als je een hele winter lang stoomwolkjes geblazen hebt in een keuken van vijf graden moet je prioriteiten stellen. Toch wisten jullie het werk weer vlot te trekken en kreeg ik snel feedback op mijn ingeleverde stukken. Het vlotte commentaar en het enthousiasme werkten aanstekelijk en gaven me weer lol in het schrijven. Rolf, ook jouw inbreng bij de start van het project heb ik erg gewaardeerd.

Naast het werk had ik natuurlijk ook wat (ont)spanning nodig, en dat kon ik vinden bij het peloton van de Nationale Reserve in Ede. Mannen van Bravo 2, het afronden van mijn proefschrift betekent gelijk ook het einde van een hele mooie periode van ruim 10 jaar bij een geweldig peloton. Ik hoop dat de sfeer en de inzet in Bergen op hetzelfde niveau ligt, maar daar vertrouw ik wel op. Matthew en Jacco, ik hoop nu dat ik tijdens de 25-uurs oefeningen wel af en toe wat nachtrust krijg, al ga ik jullie eindeloze gesprekken 's nachts misschien nog wel missen ook.

Mijn familie is de afgelopen jaren een echte steunpilaar geweest waar ik op kon bouwen, ze zijn mij blijven stimuleren om dit proefschrift af te ronden. De foto op de voorzijde lijkt weinig met soortsvorming en *Phytophthora* soorten te maken te hebben, maar toch vond ik hem wel op mijn boekje passen. Het draait in dit proefschrift toch om ouders, nakomelingen die eigenschappen van beide ouders meekrijgen en die zelf hun niche weten te vinden. Ik heb de liefde voor de landbouw van mijn vader en moeder meegekregen, en daarnaast hebben ze er altijd voor gezorgd dat mijn zussen en ik de kansen kregen (en pakten) om verder te komen. Veel eigenschappen van mijn ouders zie ik in ons terug. Helaas kan mijn vader er vandaag niet meer bij zijn, maar met de foto op de voorzijde wil ik laten zien dat ik dit proefschrift ook voor hem geschreven heb.

Als laatste wil ik Lidwien bedanken. Lidwien, ik denk dat de afronding van dit proefschrift op jou wel het meeste invloed heeft gehad. Ik kan niet zeggen dat de deadlines en vormgevings-stress heel positief voor mijn humeur geweest zijn in de afgelopen periode. Na vier jaar samen in een klushuis gewerkt en gewoond te hebben, was dit nog even de kers op de taart. Gelukkig kunnen we er samen altijd weer om lachen, en ben jij degene die me er doorheen getrokken hebt. Zonder jou was het me niet gelukt, en ik ben blij met jou!

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Kroon, L.P.N.M., Van Raaij, H.M.G., and Flier, W.G., 2010. Inheritance of host specificity in hybrid progeny of the oomycete pathogens *Phytophthora infestans* and *P. mirabilis*. European Journal of Plant Pathology (under revision).

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Kema, G.H.J., Köhl, J., **Kroon, L.P.N.M.**, Löffler, H.J.M., Meekes, E.T.M., Schepers, H.T.A.M., Scholten, O.E., Van Harmelen, M. and Waalwijk, C., 2002. *Fusarium* in granen. Nota 142 Plant Research International B.V. Wageningen.

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CURRICULUM VITAE

Laurentius Petrus Nicolaas Martinus (Laurens) Kroon is geboren in Midwoud op 13 juli 1974 en behaalde in 1992 zijn VWO diploma aan het Oscar Romero in Hoorn. Daarna begon hij de opleiding "Plantenveredeling en Gewasbescherming" aan de Wageningen Universiteit, waarbij hij heeft gekozen voor de specialisatie moleculaire biologie. Tijdens deze studie heeft hij afstudeeropdrachten gedaan bij de Laboratoria voor Erfelijkheidsleer en Fytopathologie, op het gebied van mechanismen van fungicide-resistentie en -gevoeligheid in de schimmels *Aspergillus niger* en *Erysiphe graminis*. Voor zijn stage heeft hij bij het National Institute of Agro-Environmental Sciences in Tsukuba (Japan) onderzoek gedaan aan *Venturia nashicola*, een pathogeen van de Nashi-peer. In juni 1998 rondde Laurens zijn studie aan de Wageningen Universiteit af, en begon hij later dat jaar als junior onderzoeker bij het Instituut voor Plantenziektenkundig Onderzoek (IPO-DLO) te werken aan *Phytophthora infestans*, de veroorzaker van de aardappelziekte. Na de overgang van het IPO in Plant Research International werkte Laurens aan het Europese BlightMOP project, een onderzoek naar het effect van biologische en conventionele aardappelteelt op de veldpopulaties van *Phytophthora infestans*. Naast dit project kon hij zich verder verdiepen in de fylogenie, waardplant-specificiteit en soortsvorming in het genus *Phytophthora*. Dit onderzoek is in samenwerking met het Laboratorium voor Fytopathologie omgezet naar een promotie-project, en heeft uiteindelijk geresulteerd in dit proefschrift. Sinds juni 2005 werkt Laurens bij het Research Centrum van Bejo Zaden in Warmenhuizen als onderzoeker Merkertechnologie en Genomics.

Education Statement of the Graduate School
Experimental Plant Sciences

Issued to: Laurens Kroon
Date: 16 June 2010
Group: Plant Research International & Laboratory of Phytopathology, Wageningen University and Research Centre

1) Start-up phase	date
► **First presentation of your project**	
Plant Specificity in *Phytophthora* species	January 09, 2001
► **Writing or rewriting a project proposal**	
Host plant specificity in Phytophthora species; broadening of host range by means of interspecific hybridisation and adaptation	2001
► **Writing a review or book chapter**	
► **MSc courses**	
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*4.5 credits**

2) Scientific Exposure	date
► **EPS PhD Student Days**	
EPS PhD Student Day 2003, Utrecht University	March 27, 2003
EPS PhD Student Day 2004, Vrije Universiteit Amsterdam	June 03, 2004
► **EPS Theme Symposia**	
EPS Theme 2 Symposium 'Interactions between Plants and Biotic Agents', University of Amsterdam	January 10, 2003
► **NWO Lunteren days and other National Platforms**	
ALW / EPW Annual Meeting, Lunteren	April 15-16, 2002
ALW / EPW Annual Meeting, Lunteren	April 07-08, 2003
Willie Commelin Scholten day, Royal Botan. Society Netherlands (KNBV), section Phytopathology, Utrecht	January 30, 2003
Willie Commelin Scholten day, Royal Botan. Society Netherlands (KNBV), section Phytopathology, Utrecht	January 22, 2004
PhD day Phylogenomics	May 10, 2004
► **Seminars (series), workshops and symposia**	
Seminars: Jane Ristaino, Pieter van West, Ricardo Oliva, Francine Govers	2001-2010
Plant Research International seminars	2000-2004
BlightMOP meetings	2001-2003
KNPV workgroup meetings	1999-2003
► **Seminar plus**	
► **International symposia and congresses**	
European Conference on Fungal Genetics Copenhagen, Denmark	April 17-20, 2004
CBS Centenary Symposium	May 13-14, 2004
Plant and Animal Genome XVII Conference, San Diego, USA	January 10-14, 2009
► **Presentations**	
Oral presentation BlightMOP Ploudaniel, France	June 29-30, 2001
Oral presentation BlightMOP Newcastle, UK	February 14-17, 2002
Oral presentation BlightMOP Witzenhausen, Germany	July 08-10, 2002
Poster for autumnschool: Disease Resistance in Plants	October 14-16, 2002
Oral presentation BlightMOP Darmstadt, Germany	January 22-24, 2003
Oral presentation BlightMOP Driebergen	July 02-04, 2003
Oral presentation *Phytophthora / Pythium* workgroup KNPV	September 27, 2001
Oral presentation *Phytophthora infestans* workgroup KNPV	November 21, 2002
Oral presentation *Phytophthora / Pythium* workgroup KNPV	September 18, 2003
Oral presentation for autumnschool: Disease Resistance in Plants	October 14-16, 2002
Oral presentation WCS day	January 22, 2004
Poster presentation ECFG Copenhagen, Denmark	April 16-20, 2004
► **IAB interview**	
► **Excursions**	
Subtotal Scientific Exposure	*18.1 credits**

3) In-Depth Studies	date
► **EPS courses or other PhD courses**	
AFLP markers in plant systematics and breeding (Prof. Van den Berg)	May 16-18, 2001
Disease resistance in plants (Dr. Kormelink)	October 14-16, 2002
The analysis of natural variation within crop and model plants (Prof. Koornneef)	April 22-25, 2003
Molecular basis of microbe-plant interactions (Prof. Lugtenberg)	June 05-07, 2003
Molecular phylogenies: reconstruction and interpretation (Prof. Van den Berg)	November 04-07, 2003
► **Journal club**	
study group "Tracks of Evolution"	2002-2004
► **Individual research training**	
Subtotal In-Depth Studies	*7.1 credits**

4) Personal development	date
► **Skill training courses**	
Projectmatig werken, Kern Consult	November-December 2001
Techniques for writing and presenting a scientific paper	June 18-20, 2003
► **Organisation of PhD students day, course or conference**	
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*1.9 credits**

TOTAL NUMBER OF CREDIT POINTS*	**31,6**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 credits

** A credit represents a normative study load of 28 hours of study*