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The expansion of *Phytophthora* clade 8b: host adaptation and speciation through hybridization and polyploidy

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Cover illustration: Visualization of *Phytophthora porri* nuclei in hyphae using DAPI (4',6-diamidino-2-phenylindole).

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List of Abbreviations

CHEF	Contour-clamped homogeneous electric field (electrophoresis)
CV	Coefficient of variation
DAPI	4',6-diamidino-2-phenylindole
DPI	Days post inoculation
DI	Disease index
EtBr	Ethidium bromide
ETI	Effector-triggered immunity
FCM	Flow cytometry
FL	Fluorescence
ITS	Internal transcribed spacer
LOH	Loss of heterozygosity
MS	Murashige and Skoog medium
NGS	Next generation sequencing
PFGE	Pulsed field gel electrophoresis
PAMP	Pathogen associated molecular pattern
PI	Propidium iodide
PTI	PAMP-triggered immunity

1
Problem statement

The genus *Phytophthora* is an important group of filamentous, plant pathogenic eukaryotes (kingdom Stramenopiles). Based on sequence data from seven informative DNA loci, the genus is divided into ten well supported phylogenetic 'clades'. A clade (or monophylum) can be defined as a group of organisms or species that are derived from a single ancestor (monophyletic). In this work, we studied the phylogenetic clade 8b as defined by Grünwald et al. (2011). At the start of this work, clade 8b contained three species: *P. porri*, *P. brassicae* and *P. primulae*. In general, very little research had been done on the diseases caused by these pathogens. Of this little attention, most had been given to *Phytophthora porri*, the causal agent of white tip disease in leek (*Allium porrum*; Smilde (1996); Smilde and VanNes (1992); Smilde et al. (1995, 1997, 1996); Declercq et al. (2009); Declercq (2009)). This research mainly focused on the integrated disease management of white tip disease in leek. Studies of *P. brassicae* were largely limited to fundamental studies on the interaction with *Arabidopsis thaliana* (Man in 't Veld et al., 2002; Hermansen and Hoftun, 2005; Mauch et al., 2009; Bouwmeester and Govers, 2009; Schlaeppi et al., 2010; Si-Ammour, 2002; Belbahri et al., 2008; Jiang et al., 2006). Several reports had been published describing *Phytophthora* clade 8b-like organisms causing diseases in other crops such as lettuce, carrot, onion and parsley. From previous research carried out at our laboratory (Declercq, 2009), there were indications that some of these isolates might represent new, host-specific species. Next to this, indications of interspecific hybridization between *P. porri* and *P. primulae* had been detected in some isolates Declercq et al. (2009). Moreover, in the years preceding the start of this work, a new disease had caused important yield losses in Brussels sprouts cultivation in Flanders. Preliminary research in our lab had indicated that the disease might be caused by a disease complex containing *P. brassicae* and *Botrytis*.

The cultivation of field-grown vegetable crops is an economically important sector in Belgium. The region of Flanders is responsible for almost half the production of frozen vegetables in Europe (Vandermeulen and Van Huylenbroeck, 2006). For these reasons, a detailed study of this group of pathogens was needed.

The project initiated from the following hypotheses:

1. *Phytophthora* clade 8b contains a group of cold tolerant, homothallic, soil-borne species that are specialized in causing disease in winter grown vegetables.
2. Within *Phytophthora* clade 8b, host adaptation is occurring to specific plant families and species such as *Brassica* spp. (Brussels sprouts, head cabbages, etc.), *Allium* spp.

(leek, onion, etc.), *Apiaceae* (carrot, parsley, etc.).
3. Interspecific hybridization is possible in clade 8b, possibly causing shifts in host range.
4. Recent problems with *Botrytis* in Brussels sprouts cultivation are due to primary infections with *P. brassicae* or a related species.
5. The *Phytophthora* clade 8b species have a similar infection cycle starting with oospore germination in the soil and release of zoospores in rain puddles whereafter they can be splashed upon the host plant by splash dispersal. Secondary infections through sporangium formation on the leaves does not occur, as has been shown by Declercq (2009); Declercq et al. (2009).

To investigate these hypotheses, our goals were threefold:

1. Build a collection of isolates from different host plants and analyze the genetic diversity by multilocus sequencing (rDNA ITS region and other informative loci).
2. Gain insight into the new disease on Brussels sprouts, probably caused by *P. brassicae* and *Botrytis*.
3. Gain insight into the infection cycles of the *Phytophthora* clade 8b species on their respective hosts with the aim of developing a suitable crop protection strategy.

In **Chapter 2**, we provide an introduction to the matter discussed in the following chapters by giving an overview of the most important and most recent literature.

We built a collection of more than one hundred *Phytophthora* clade 8b isolates, containing isolates from the known hosts (leek, cabbages and *Primula* spp.) and from many other hosts such as onion, garlic, parsnip, lettuce, chicory, carrot, etc. In **Chapter 3**, we assessed the genetic diversity of the clade using multilocus sequencing (ITS and *Cox1*). We found multiple taxonomic entities that differed from the known species and there was a clear correlation with the host plant, i.e. almost all taxonomic entities (or clades) in the phylogenetic analysis could be correlated to a specific host plant or plant family. To investigate whether these taxonomic novelties represented separate species, a detailed morphological and physiological study of the new isolates was performed. This led us to describe three new species in this group: *Phytophthora cichorii* sp. nov., *Phytophthora dauci* sp. nov. and *Phytophthora lactucae* sp. nov., named after their respective host plants chicory, carrot and lettuce. Two other taxonomic novelties were found that possibly also represent new species, but could not be officially described as such yet due to several reasons. Therefore, we tentatively named them *Phytophthora* taxon parsley and

Phytophthora taxon castitis.

Several isolates were found to show signs of interspecific hybridization (specifically: dimorphic positions in the ITS region) and we describe these isolates in **Chapter 4**. Using multilocus sequencing, cloning and DNA content estimation using flow cytometry, we concluded that three different types of hybrids have formed in clade 8b. The three hybrid types originated from interspecific hybridization between different clade 8b species and represent a group of evolving allopolyploid genotypes.
DNA content measurements were also done for the non-hybrid species. Intra-specific ploidy variation in *P. porri* and *P. brassicae* indicated that polyploidy is the prevalent cytotype in these species and most likely also in the other clade 8b species.
From these observations, we formulated a hypothesis of host adaptation and speciation through hybridization and polyploidy in *Phytophthora*. Moreover, our results show that the footprints of hybridization are easily lost and hence the remnants of older hybridization events are masked. Therefore, we speculated that interspecific hybridization in *Phytophthora* might be much more common than is acknowledged today. Moreover, the occurrence of polyploidy in *Phytophthora* might point to ancient hybridization events.

In **Chapter 5**, we tested the pathogenic potential of the eleven clade 8b species, taxa and hybrids and of *P. syringae* (clade 8d) on four different plant hosts: leek, onion, Chinese cabbage and lettuce. From these experiments, it can be concluded that all three hybrid types can infect multiple hosts and generally show higher virulence than the non-hybrid clade 8b species. Moreover, we confirmed the hypothesis formulated in Chapter 3 that onion could serve as a hot spot for interspecific hybridization between the clade 8b species in nature.

In **Chapter 6**, we describe the experiments that were done to evaluate if the prevalence of polyploidy in *P. porri* can be linked to an ecological advantage of polyploidy over diploidy. We found significant differences in cold and frost tolerance between the diploid and polyploid isolates. However, since only one diploid isolate was available for study, no strong conclusions could be drawn. We found no correlation between ploidy level and fungicide (Metalaxyl-M) resistance or pathogenic potential on leek.

In **Chapter 7**, we reviewed the literature on polyploidy in *Phytophthora* and postulate that polyploidy could be an important mechanism contributing to the genomic flexibility that has made *Phytophthora* species such well equipped and notorious pathogens.

In **Chapter 8**, we summarize the main findings of this project and discuss these findings in a broader context. In the last part, we formulate the most important questions that remain after this work and put together some suggestions for future work.

The second goal was to investigate the disease caused by *P. brassicae* and *Botrytis* on Brussels sprouts. Despite numerous attempts, we were not able to isolate *Phytophthora* from the diseased Brussels sprouts. Also, in an early stage it was confirmed that *Botrytis* could infect Brussels sprouts independently from *P. brassicae.* Moreover, during the last five years, the disease was not as prevalent compared to the previous years. For these reasons, we decided to abandon this research topic and focus on others.

In the third goal we wanted to gain insight in the infection cycles of the clade 8b pathogens. No experiments were conducted on this topic. However, the information gathered in this thesis supports the hypothesis that the infection cycles of the different clade 8b species are similar, with infection always initating from oospore germination in the soil.

If I have seen further than others, it is by standing upon the shoulders of giants.

Isaac Newton

2
Literature review

2.1 The genus *Phytophthora*

Plant diseases can be caused by a variety of organisms such as bacteria, viruses, fungi and oomycetes and form a continuous threat to our supply of food and natural resources. The most widespread and devastating plant diseases belong to the Fungi and Oomycota (also called 'water molds'), and to this last class belongs the genus *Phytophthora* (Greek for 'plant destroyer').

Few micro-organisms have had such an impact on mankind as *Phytophthora*. The infamous *Phytophthora infestans*, causing potato late blight disease, led to the Great Irish Famine in the middle of the nineteenth century. *Phytophthora infestans* is still causing enormous yearly losses in worldwide potato production, and is therefore one of the best studied filamentous plant pathogens in the world.

2.1.1 Phylogeny of the genus

The first *Phytophthora* species (*P. infestans*) was officially described by Heinrich Anton de Bary in 1876. After this, the number of *Phytophthora* species increased steadily until the turn of the previous century, with around 50 species described by the year 2000 (Erwin and Ribeiro, 1996). During the last 14 years, however, more than 70 new species have been described (Kroon et al., 2012). This sudden explosion in species number is due to several reasons. First and most importantly, the advances in molecular techniques such as DNA sequencing played an important role. DNA sequence data together with the availability of more advanced tools for species delimitation (such as molecular phylogeny) caused many species complexes to be split up into separate species. For example the *Phytophthora megasperma* complex was split up in no less than six species, namely *P. megasperma* sensu stricto, *P. sojae*, *P. trifolii*, *P. medicaginis*, *P. sansomeana* and *P. rosacearum*. Next to this, the use of specific selective media and proper isolation techniques also played a role. Thanks to these methods, large scale surveys in natural and agricultural settings can now be performed (Scibetta et al., 2012) and many new species have been discovered in this way.

At the time of writing, this flood of new species descriptions was still going on, with new species being described monthly. An estimate of around 600 extant *Phytophthora* species has been made (Brasier, 2009), so the flood is expected to go on for more years yet. Questions can be raised about the definition of a species in the genus *Phytophthora*. The definition of a species is a tough one throughout the whole discipline of biology and many species concepts have been proposed (Hey, 2006; De Queiroz, 2007). Species concepts are

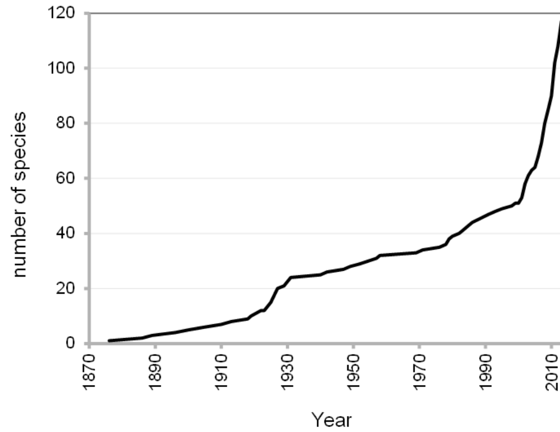


Figure 2.1: Increase of described *Phytophthora* species over time.
The figure is adapted from Kroon et al. (2012). The 18 new species described since October 2011 (until January 2014) were added.

especially difficult in fast evolving micro-organisms like *Phytophthora* (Taylor et al., 2000).

Recently, an updated phylogeny of the whole genus was published (Martin et al., 2014) and it contains 92 recognized and 17 provisional species. The genus is divided into ten well-supported clades, of which most are subdivided into one or more subgroups. The phylogenetic tree (based on DNA sequence information from 7 nuclear and 4 mitochondrial loci) is shown in Figure 2.2.

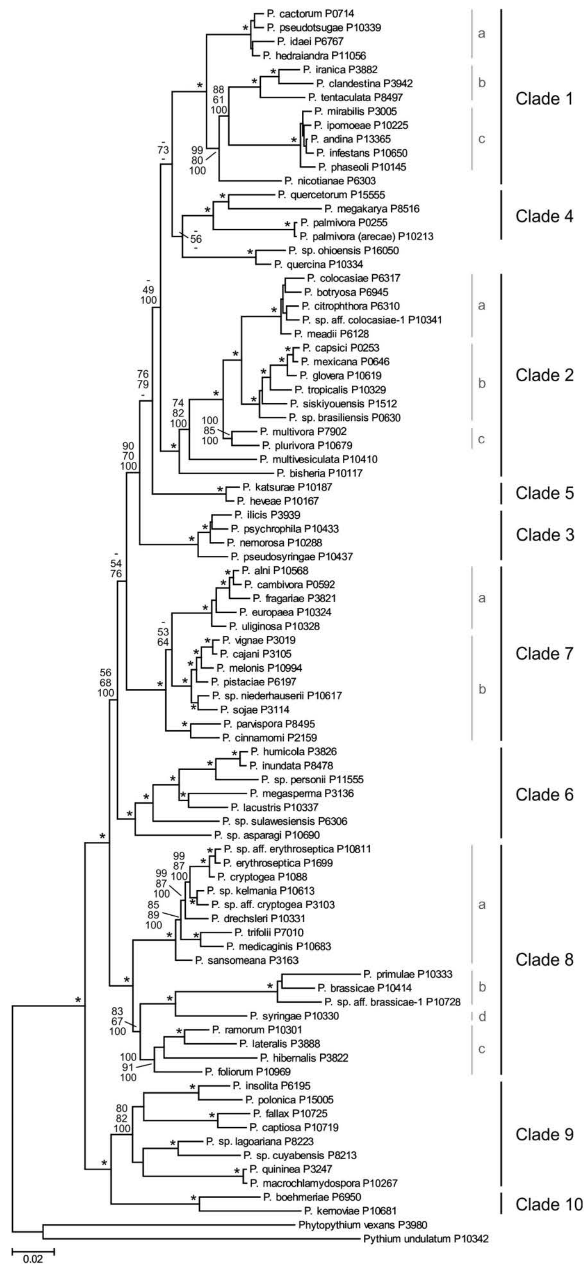


Figure 2.2: Genus wide phylogeny for *Phytophthora* using seven nuclear and four mitochondrial loci (10,828 nucleotides in total). Maximum likelihood branch lengths shown. Numbers on nodes represent bootstrap support values for maximum likelihood (top), maximum parsimony (middle) and Bayesian posterior probabilities as percentages (bottom). Nodes receiving significant support (>95%) in all analyses are marked with an asterisk (*). Scale bar indicates number of substitutions per site. Reprinted from (Martin et al., 2014) with permission from the publisher.

2.1.2 Higher level taxonomic classification of *Phytophthora*

Up until a few decades ago, the oomycetes were classified as Fungi because of their similar morphology, ecology and heterotrophic nutritional lifestyle (see section 3.2.6 and Table 2.1). Recent advances in molecular phylogeny have shown that oomycetes are very distantly related to the true Fungi in evolutionary terms and instead belong to the lineage of biflagellate 'heterokont' organisms, commonly termed Stramenopiles (Beakes et al., 2012). The Stramenopiles are a large group of eukaryotes containing an extremely diverse assemblage of over 100 000 species. Many of these organisms are autotrophic, chlorophyll-c containing algae (such as brown algae and diatoms), while the oomycetes and multiple other groups are heterotrophs.

For a detailed phylogeny of the oomycetes, we refer to (Beakes et al., 2012). The oomycetes are divided into two large taxonomic 'galaxies'. The first galaxy includes the largely saprophytic 'saprolegnian water mould orders' and the other contains the often plant pathogenic 'peronosporalean orders' containing the genus *Phytophthora* along with other genera of plant pathogenic species such as *Pythium*, *Bremia*, *Peronospora* and *Albugo*. Recent sequencing projects have found many other genera that also belong to the oomycetes but are outside these two main galaxies. These genera (which are mainly found in marine habitats) represent the early-diverging clades from which all other oomycetes evolved.

The higher taxonomic classification of the Stramenopiles is still heavily disputed. Not very long ago, Stramenopiles were classified together with Alveolates, Cryptophyta and Haptophyta in a monophyletic supergroup termed Chromalveolata (Adl et al., 2005; Cavalier-Smith, 1999, 2003). This grouping is based on the hypothesis that the last common ancestor of these lineages acquired its plastid from a single event of endosymbiosis with a red alga that has been subsequently inherited strictly vertically. This controversial grouping was mainly proposed to reduce the number of endosymbiotic events, since many independent endosymbiotic events would be an overly complex and therefore unlikely evolutionary scenario (Keeling, 2013). The Chromalveolate hypothesis therefore implies many independent plastid losses in the heterotrophic lineages such as the oomycetes.

Although initial support for the monophyly of Chromalveolata was found through phylogenetic studies of the different plastid genomes, recent phylogenomic studies of nuclear genomes are not in agreement with the plastid data (Baurain et al., 2010; Burki et al., 2007, 2012; Kim and Graham, 2008). Therefore, most authors have now dismissed the Chromalveolate hypothesis, although a good alternative has not been proposed. In recent work (Petersen et al., 2014), the invalid kingdom Chromalveolata has been replaced by the informal term 'CASH lineages' (Cryptophyta, Alveolates, Haptophyta and Stra-

menopiles).
The hypothetical ancient presence of a plastid in oomycetes has been supported by the detection of genes from algal origin in *Phytophthora* genomes (Tyler et al., 2006), and this was interpreted as support for the Chromalveolate hypothesis. However, other authors (Stiller et al., 2009) find no proof that these genes are linked with photosynthetic ancestry. Alternatively, the presence of algal genes in oomycete genomes can be explained by lateral gene transfer.
An alternative explanation for the presence/absence of plastids within these lineages is probably to be sought in complex scenarios of serial eukaryotic-eukaryotic endosymbiosis events (SEEE hypothesis) (Baurain et al., 2010; Petersen et al., 2014; Stiller et al., 2009).

2.1.3 Morphology, lifestyle and disease cycles

The similarities between oomycetes and Fungi (such as their filamentous heterotrophic lifestyle) are an example of convergent evolution, i.e. the independent evolution of similar features in species of different lineages. However, recent work also underscores the importance of horizontal gene transfer from Fungi in the evolution of plant pathogenesis mechanisms in oomycetes (Keeling and Palmer, 2008; Richards et al., 2006, 2011).
For management of plant diseases, it is important to recognize the large differences in morphology and biochemistry between Fungi and oomycetes. An overview of the most important differences is given in Table 2.1.

Table 2.1: Differences between oomycetes and true Fungi.
Table adapted from Judelson and Blanco (2005).

Feature	oomycetes	Fungi
Neighbouring taxonomic groups	Diatoms and heterokont algae	Animals
Hyphal architecture	Aseptate and coenocytic tubular hyphae	Either single cell or septated hyphae, with one or more nuclei per compartment
Ploidy of vegetative hyphae	Diploid (at least), except for transient haploid nuclei in gametangia	Haploid or dikaryotic; often with a stable or semi-stable diploid stage following mating
Typical genome size	40-250 Mb	10-40 Mb
Major glucans in cell wall	Cellullose and β-1,3- and β-1,6-linked glucose polymers	Usually chitin (β-1,4-linked N-acetylglucosamine), and/or chitosan (β-1,4-linked glucosamine), often with other β-1,3 and β-1,6 glucans
Pigmentation	Usually unpigmented	Very common in hyphae or spores, or secreted (f.e. melanin, carotenoids, etc.)
Presence of motile asexual spores	Nearly universal, biflagellate zoospore	Uncommon, only in chytrids, which are monoflagellate
Sexual spores	Oospores, formed on hyphae (intercalary or terminal) after fertilization of an oogonium (female) by an antheridium (male).	Various types, often formed in large numbers within complex enclosures (f.e. perithecia, mushroom caps, etc.)
Type of mitochondria	With tubular cristae	With flattened cristae
Major energy reserves used by spores	Mycolaminarin and lipid	Glycogen and trehalose, also sugar alcohols and lipid

Virtually all *Phytophthora* species hitherto described are plant pathogens, and hence their lifestyle is tightly linked to their respective hosts. *Phytophthora* species are hemibiotrophic pathogens, meaning that they have a biotrophic phase in which they feed from living host plant cells, after which they switch to a necrotrophic phase, killing the host plant cells. Some *Phytophthora* species, such as *P. cinnamomi* and *P. ramorum*, show a very broad host range. However, most *Phytophthora* species show some degree of host specificity to certain plant families or species, for example *P. infestans* infects solanaceous hosts like potato and tomato, *P. sojae* infects soybean and the *Phytophthora* clade 8b species infect specific winter-grown vegetable species (as will be discussed in Chapter 3). The general life cycle of *Phytophthora*, with all possible life stages, is presented in Figure 2.3.

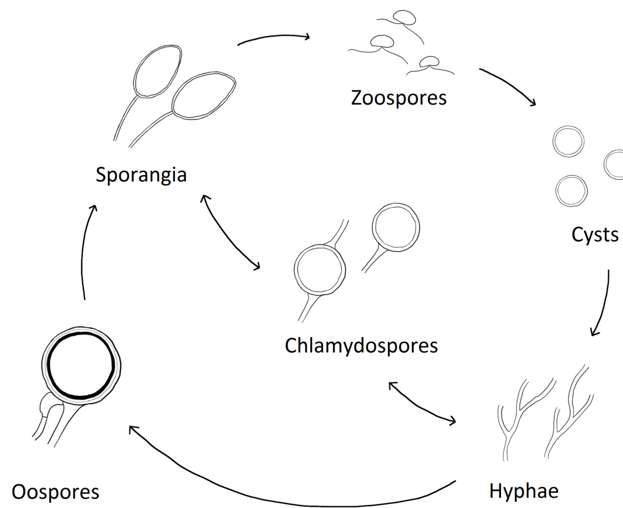


Figure 2.3: Basic *Phytophthora* life cycle. *Phytophthora* species can reproduce either asexually through chlamydospores and zoospores (that are formed inside sporangia), or sexually through the formation of oospores. After a motile phase, zoospores shed their flagella and encyst. Cysts germinate through the formation of a germ tube which initiates hyphal growth.

Phytophthora pathogens can reproduce both sexually through the formation of oospores and asexually through the production of zoospores and chlamydospores. The production of zoospores, the 'weapons' of the plant destroyer (Judelson and Blanco, 2005), plays an essential part in plant infection. Zoospores have two flagellae, enabling them to swim in aqueous substances (like rain puddles). It has been demonstrated that zoospores are attracted chemotactically to the roots of their host plants (Morris and Ward, 1992)(see `http://www.youtube.com/watch?v=PxF8OwDtJh0` for a video on chemotaxis of *Phytophthora nicotianae* zoospores to roots of tobacco). In case of leaf-infecting *Phytophthora* species, zoospores can reach the leaves by rain splash (like in *P. porri*) or through the dispersal of sporangia by air-flow (like in *P. infestans*).
Chlamydospores are formed by some but not all species and are mainly formed as a means to survive harsh environmental conditions (McCarren et al., 2005).
In their sexual reproduction, *Phytophthora* species can be either homothallic (self-fertile) or heterothallic. Homothallic species can engage in sexual reproduction more easily than

heterothallic species, since in the latter form, close presence of two different mating types (A1 and A2) is necessary. Details about the molecular basis of heterothallism have recently been elucidated (Lee et al., 2012; Ojika et al., 2011; Qi et al., 2005; Yajima et al., 2007). Sexual reproduction of heterothallic species does not necessarily mean genetic exchange between the two organisms. A single *Phytophthora* isolate, homothallic or heterothallic, is always bisexual and can produce both female oogonia and male antheridia. In heterothallic species, close presence of an organism of the other mating type of the same species (and in some cases even of another species) is enough to trigger oospore production in an isolate as it is regulated through the production of conserved mating type hormones (Billiard et al., 2012). It is important to mention that, although at low frequency, outcrossing does occur in natural populations of homothallic species (Tyler et al., 1995).

The multinucleate hyphae and mononucleate zoospores are diploid and meiosis takes place in the gametangia. In Figure 2.4, a detailed description of the sexual system in *Phytophthora* is given. The haploid products of male meisosis (gametes) move from the antheridium to the oogonium where one haploid nucleus fuses with a female gamete, forming a diploid zygote. The other products of meiosis abort and the oogonium then transforms into a long-lived, thick-walled oospore that can survive unfavorable environmental conditions for years.

The possession of a mixed reproduction system (the possibility of sexual reproduction, together with the ability to reproduce clonally) plays an important role in a pathogen's adaptive potential and hence in the risk that a certain pathogen poses of becoming a threat to our food supply (Heitman, 2006; McDonald and Linde, 2002).

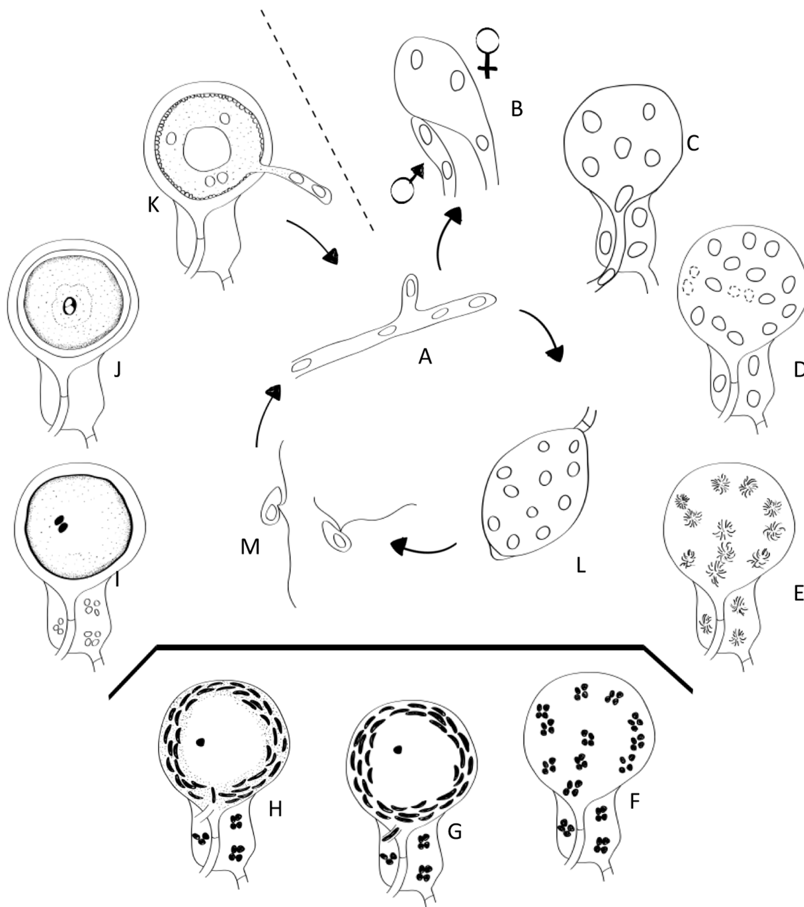


Figure 2.4: The nuclear cycle in *Phytophthora*, as seen in *P. infestans* (not to scale). A, somatic mycelium with diploid nuclei (A1 or A2 mating type in heterothallic species). B, formation and association of antheridial (male) and oogonial (female) incepts. C, emergence of the young oogonium (following penetration of the amphigynous antheridium by the oogonial incept); migration of nuclei into the developing oogonium. D, abortion of nuclei in pairs in the expanded oogonium; increase in size of remaining nuclei. E and F, meiosis in the oogonium and antheridium; near-synchronous first division with a long prophase followed by a very short second division with no increase in nuclear size between the two divisions, thus resulting in formation of tetrads of haploid nuclei. G, beginning of oosphere delimitation; migration of the male gametic nucleus into the oogonium from the antheridium. H, penetration of the oosphere by the male nucleus. I, association of the male and female gametic nuclei in the oosphere. J, formation of the thick-walled oospore with a diploid zygotic nucleus in the "pellucid" spot. K, germination of the oospore to give the diploid vegetative stage. L, diploid multinucleate sporangium. M, diploid uninucleate zoospores. Redrawn from Brasier and Sansome (1975) with permission from the publisher.

Phytophthora species can be divided into three main categories based on the mode of infection. The majority of species attack belowground plant parts (51% of species based on data from Kroon et al. (2012)). Another 31% specifically infect aboveground plant parts like leaves and fruits and the remaining 18% can infect both or have been found in soil or marine habitats and have not been tested for pathogenic potential.
The disease cycle of a typical root-infecting species (*Phytophthora sojae*) is presented in Figure 2.5. It should be noted that in this type of infection cycle, the disease always initiates from the primary inoculum that is present in the soil (oospores). Hence, this type of pathogen will cause a monocyclic disease progress, meaning that the proportion of disease increases in a linear way over time during a specific growing season.

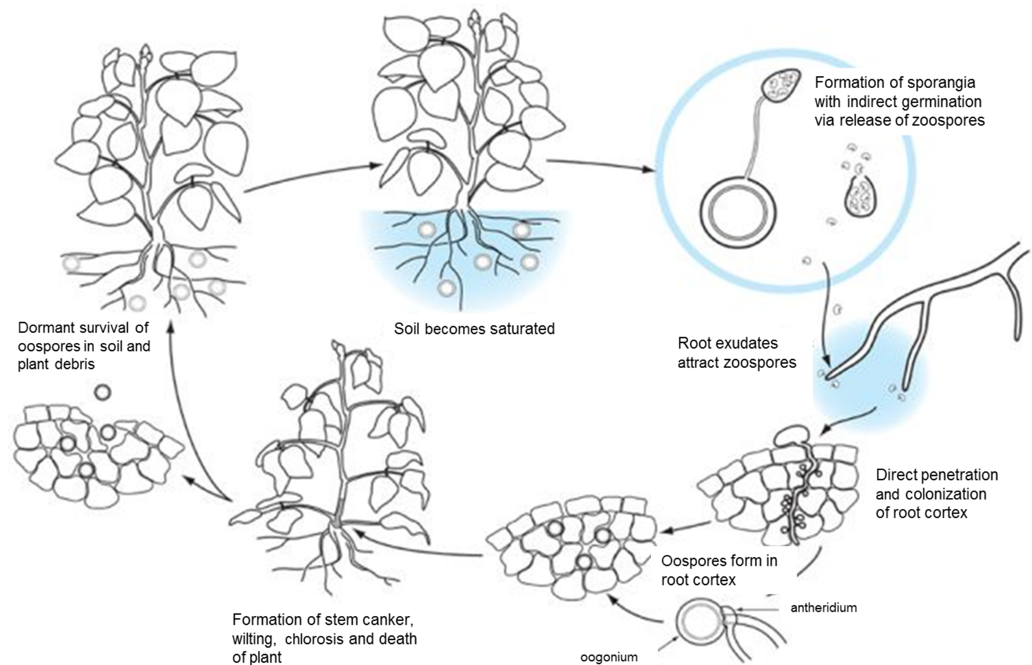


Figure 2.5: Disease cycle of *Phytophthora sojae* on soybean. When humidity is high, resting oospores present in the soil can germinate and form sporangia. Zoospores are released by the sporangia and are attracted by the root exudates through chemotaxis. Zoospores encyst and form a germ tube which directly penetrates the root. After a period of root colonization, causing wilting, stem canker and chlorisis in the plant, oospores are formed inside the root tissue. When plant debris is left in the soil, these oospores form a source of inoculum for the next round of infection. Reproduced from APSnet educational center (Dorrance et al., 2007)

In contrast, leaf-infecting diseases like potato late blight caused by *P. infestans* are mainly spread through air-borne sporangia and zoospores. The disease initiates from latently infected tubers that are mistakenly planted. From the time of germination, *Phytophthora infestans* can start growing inside the plant tissue and when the weather conditions are favourable, sporangia will be formed on the leaves, as visualized in Figure 2.6. Under optimal conditions, these sporangia can be released and blown onto healthy plants where they can start a new round of infection. This mode of infection causes a polycyclic disease progress, meaning that the proportion of disease increases in an exponential way during the growing season.

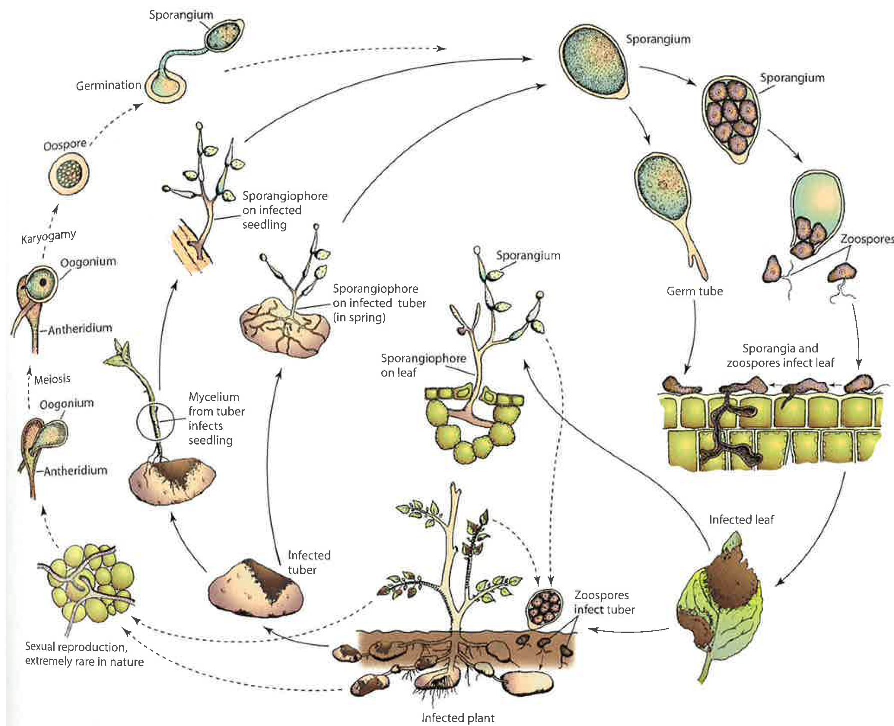


Figure 2.6: Disease cycle of *Phytophthora infestans* on potato.
Disease mainly initiates from latently infected tubers that are planted. The pathogen can spread through all parts of the plants and cause secondary infections through the formation of sporangiophores on leaves, tubers and seedlings. Alternatively, sexual reproduction can occur, giving rise to oospores that can survive for several years in the soil. The figure states that sexual reproduction is extremely rare in nature. However, as discussed in section 8.2, this is probably not accurate (anymore). Image from Agrios (2005).

Other types of infection cycles exist, such as the atypical infection strategy of *Phytophthora porri* on leek. *Phytophthora porri* is a leaf infecting pathogen but disease is always initiated through oospore germination in the soil (monocyclic disease progress). In Figure 2.7, the disease cycle as determined by Declercq et al. (2012) is shown. In brief, after a rain shower, the humidity in soil is high and the germination of oospores is triggered. Germinating oospores mostly form a sporangium inside which up to 20 zoospores are formed. In areas of standing water like rain puddles, zoospores are released and can swim around for several days (especially in colder weather). During the next rain shower, zoospores are splashed upon the leaves where they encyst and can start infection through the formation of a germ tube. These hyphae typically penetrate the stomata, after which they start to ramify within the intercellular spaces between epidermal cells. After a biotrophic phase (typically a couple of days under optimal conditions), *Phytophthora porri* switches to a necrotrophic phase, hereby killing the plant tissue. By this time, oospores have been formed inside the tissue. When leaves are left to decay in the soil, which is a common practice in Flanders, this oospore-containing plant debris is a new source of inoculum for the next growing season.

An important characteristic of (hemi)biotrophic filamentous plant pathogens is the formation of specialized structures called haustoria. These haustoria penetrate the plant cell wall and invaginate the host plant plasma membrane, forming a close-contact zone in which exchange of proteins is possible. It has been postulated that these organs play an important role in nutrient uptake from the plant host. This has been clearly demonstrated for fungal haustoria (Voegele et al., 2001; Voegele and Mendgen, 2003), but evidence for oomycetes remains absent. Another important function of haustoria is the secretion of effector proteins from pathogen to plant. Clear evidence for this function has been provided for both Fungi (Kemen et al., 2005; Rafiqi et al., 2010; Voegele and Mendgen, 2003) and oomycetes (Stassen and Van den Ackerveken, 2011).

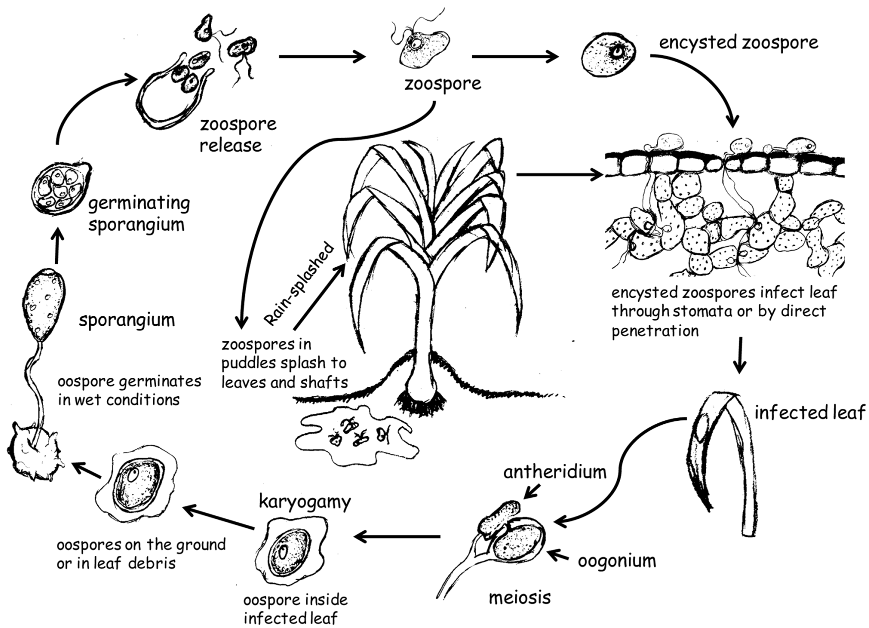


Figure 2.7: Disease cycle of *Phytophthora porri* on leek as determined by Declercq et al. (2012). Oospores (from previous plant debris that is left in the soil) survive the crop-free period and constitute the primary inoculum. Oospores germinate in the soil when free water is available and generate a sporangium filled with zoospores that are released in puddles in the field. During heavy rainfall, zoospores are splashed on healthy leek plants, where they survive in water in the leaf axils, attach to the leaf surface and infect.

2.1.4 *Phytophthora*-plant interaction

When a plant is challenged by a pathogen, it can protect itself by mounting different levels of immune responses (Dodds and Rathjen, 2010; Jones and Dangl, 2006). Firstly, a plant can activate its basal defense responses by the perception of pathogen-associated molecular patterns (PAMPs). This first layer of defense is termed PAMP-triggered immunity (PTI) (Schwessinger and Zipfel, 2008). Examples of PAMPs in oomycetes include cell wall associated proteins such as beta-glucans (Daxberger et al., 2007), but also secreted proteins such as transglutaminases and cellulose-binding proteins (Kamoun, 2006).
Some pathogens, including *Phytophthora*, can suppress these basal defense mechanisms by expressing specific pathogen effector proteins. Upon recognition of these effector proteins by the plant cell, a second layer of defense is triggered, termed effector-triggered immunity

(ETI). This often results in a rapid localized host cell death (the hypersensitive response or HR), hereby limiting disease progression (incompatible interaction). Alternatively, in a compatible interaction, the plant genotype will not be able to recognize the secreted effector proteins and disease will progress, and this is termed effector triggered susceptibility (ETS). As a result, effector genes are under dynamic selection pressures, depending on the host plant genotype that the pathogen interacts with (Michelmore et al., 2013).
The term 'effector' has been broadly used in the field of plant-microbe interactions but different scientific communities attribute different meanings to it. In its broadest sense, effectors include all pathogen proteins and small molecules that alter host-cell structure and function (Hogenhout et al., 2009), thereby facilitating infection (virulence factors or toxins) and/or triggering defense responses (avirulence factors or elicitors) (Kamoun, 2006). We will use this definition throughout this thesis. In *Phytophthora*, different types of effectors exist. Some effectors act in the extracellular space and interact with apoplastic plant proteins involved in pathogen defense (apoplastic effectors). Other effectors (cytoplasmic effectors) are translocated into the host cells where they interact with the products of the so-called resistance (R) genes, which are immune receptors of the nucleotide-binding leucine-rich repeat (NB-LRR) family (Schornack et al., 2009). When an effector protein is recognized by the plant immune system, it is termed an avirulence (Avr) protein. This interaction between Avr proteins from the pathogen and R proteins from the plant has been recognized long before the molecular era as the gene-for-gene concept (Flor, 1971).
Thanks to laborious cloning efforts, 18 of the genes corresponding to Avr proteins have been cloned and characterized, especially in species that cause great economic losses such as *P. infestans* and *P. sojae*. However, most of our current understanding of the mechanisms and evolution of virulence in oomycetes has come from genome sequencing projects completed during the last decade (Jiang and Tyler, 2012). Presently, six *Phytophthora* species have their genomes completely sequenced and annotated, namely *P. sojae* (95 Mb; (Tyler et al., 2006)), *P. ramorum* (65 Mb; (Tyler et al., 2006)), *P. infestans* (240 Mb; (Haas et al., 2009)), *P. capsici* (64 Mb; (Lamour et al., 2012)), *P. parasitica* (53 Mb; *Phytophthora parasitica* Assembly Dev initiative, Broad Institute (`broadinstitute.org`)) and *P. cinnamomi* (78 Mb; `http://genome.jgi-psf.org/Phyci1/`).

The most important notion that came out of this research is that oomycete genomes display a strong dual organization in which conserved housekeeping genes are concentrated in syntenic, gene-rich blocks that evolve slowly, whereas effectors are predominantly located in highly dynamic, repeat-rich regions which show a fast evolution: the so-called '2-speed genome' (Raffaele et al., 2010c). These effector genes can be divided into apoplastic ef-

fectors (which code for different types of proteins such as extracellular toxins, hydrolytic enzymes and inhibitors), and cytoplasmic effector proteins that are translocated into the host cells. These cytoplasmic effector proteins include crinkler (CRN) and RXLR effectors. RXLR effectors are very abundant in *Phytophthora* pathogens (563 different RXLR effector genes have been predicted from the *P. infestans* genome sequence (Haas et al., 2009)) and have been coined as the major determinants of host specificity (Schulze-Lefert and Panstruga, 2011). Moreover, most Avr proteins characterized up till now belong to this class of effectors. The RXLR proteins contain a common N-terminal motif (the RXLR domain) which functions as a translocation signal for movement into the host cells. This conserved domain also enables computational mining for their encoding genes in genome sequence data. A second, more variable motif, dEER (aspartate, glutamate, glutamate, arginine) is present in many, but not all of the RXLR proteins. Both RXLR and dEER motifs are necessary for entry into the plant cell, but the mechanism of entry mediated by this domain is a matter that is still heavily debated (Petre and Kamoun, 2014).

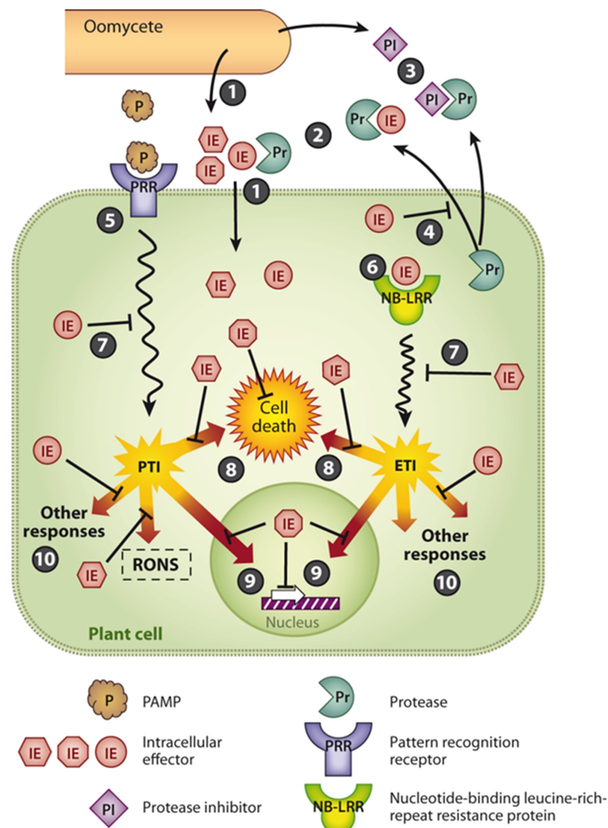
RXLR effectors have several functions in *Phytophthora*-plant interaction and most research up till now has shown that they are mainly involved in the suppression of PTI (Bos et al., 2010; Bozkurt et al., 2011; Gilroy et al., 2011; McLellan et al., 2013; Zheng et al., 2014) and ETI (King et al., 2014; Yin et al., 2013).

The interaction of pathogen effector proteins (Avr) with plant resistance NBS-LRR (R) proteins is almost never a direct one (in *Phytophthora*, no direct Avr-R interaction have been described). Therefore, the gene-for-gene interaction has been expanded to the 'guard' model of plant defense. In this model, R proteins sense the status of so-called effector targets and so they act as guards for the presence of a pathogen (Dangl and Jones, 2001). When an effector interacts with an effector target, the corresponding R gene will trigger plant immunity. In the absence of an R gene, an interaction between effector and effector target will enhance disease progression. This model was later expanded to a 'guard and decoy' model, since some effector targets were found to act as decoys (van der Hoorn and Kamoun, 2008). When an effector binds a decoy effector target, no disease will occur in the absence of an R protein. However, when an R gene is present, plant immunity will be triggered, and so the pathogen is 'tricked' by the plant.

A second large class of cell-entering effectors are the crinkler (CRN) effectors, named for their ability to produce crinkling and necrosis when overexpressed in transient expression assays (Schornack et al., 2010). Just like the RXLR effectors, the crinklers are rapidly evolving, highly diverse and are built in a modular way, with a conserved N-terminal domain essential for host-cell entry, connected to a diverse pool of C-terminal domains. In contrast to the RXLR effectors, crinklers are more widely distributed among oomycetes

pathogens and are probably of ancient origin. Moreover, CRN proteins have been shown to exclusively target the host nucleus (Schornack et al., 2010) where they carry out different functions that mainly lead to host cell death (Stam et al., 2013a,b).

Oomycetes and specifically *Phytophthora* species contain the largest effector repertoires of all plant pathogens sequenced up till now (Raffaele and Kamoun, 2012). Some authors have stated that this could be a unique property of oomycetes, in contrast to plant pathogenic Fungi, in which mainly secondary metabolite toxins play a role in virulence, and families of virulence proteins are less extensively expanded and diversified (Jiang and Tyler, 2012). However, this is a generalization that should be handled with care since recent data on fungal pathogens such as the rusts and powdery mildews shows that also these genera contain large effector repertoires (Spanu et al., 2010; Hulbert and Pumphrey, 2014).
In Figure 2.8, an overview of the possible interactions between plants and (oomycete) pathogens are shown.



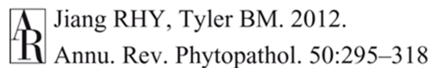
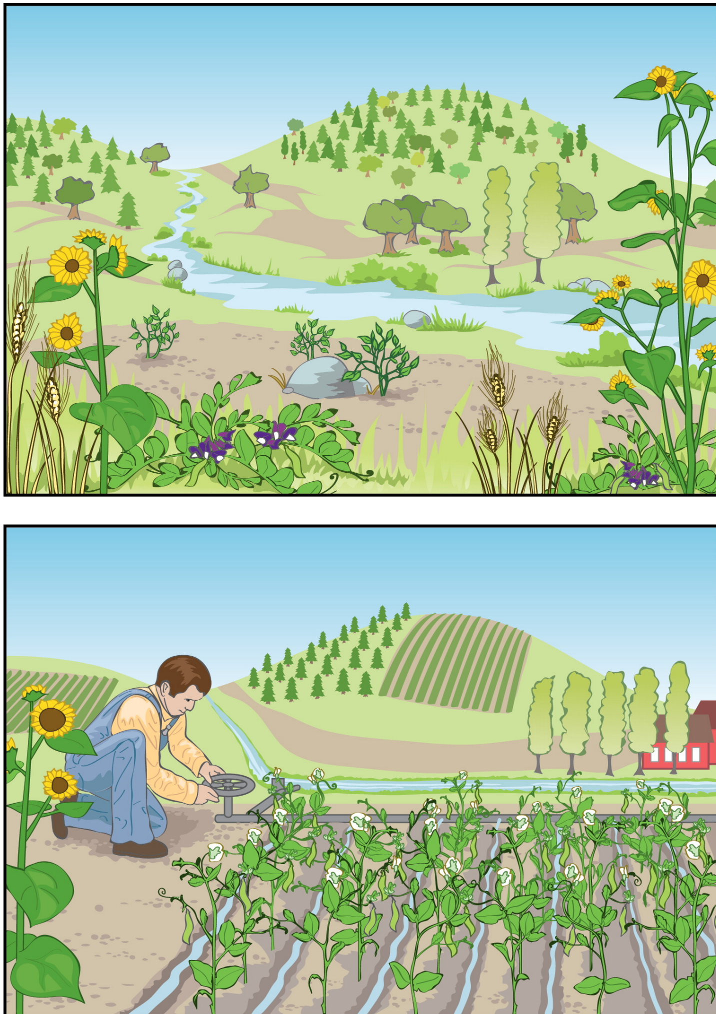


Figure 2.8: Suppression of plant immunity by oomycete effectors. Oomycete pathogens secrete intracellular effectors (IEs) that ① can enter the host cytoplasm. Plants may secrete proteases (Pr) that ② can degrade intracellular or extracellular effectors in the apoplast, but pathogens ③ may secrete protease inhibitors (PIs) that block those proteases, or else produce effectors that block secretion ④. Recognition of pathogen-associated molecular patterns (PAMPs) by pattern-recognition receptors (PRRs) ⑤ produces signaling events that activate PAMP-triggered immune responses (PTI). Recognition of intracellular effectors by nucleotide-binding site leucine-rich repeat receptors (NBS-LRRs) ⑥ leads to effector-triggered immune responses (ETI) ⑦. Both PTI and ETI can induce programmed cell death ⑧, and effectors may inhibit the triggering of cell death or the cell death machinery itself. PTI and ETI both involve transcriptional changes ⑨, and nuclear-targeted effectors may directly interfere with signaling within the nucleus or transcriptional events. PTI and ETI involve numerous other responses ⑩, including the production of reactive oxygen and nitrogen species (RONS), and effectors may also interfere with those responses. Reprinted from Jiang and Tyler (2012) with permission from the publisher.

2.2 Host adaptation in *Phytophthora* plant pathogens

Gaining insight in the molecular mechanisms of host adaptation and specialization is fundamental if we want to create durable crop protection strategies and assess the risks posed by potential future pathogens. We are gaining more and more insight into the molecular basis of plant-pathogen interaction (see section 2.1.4), and it is clear that the complicated defense mechanisms of pathogen attack and plant defense have evolved over very long evolutionary timescales. Compared to those long timescales, agriculture is very young (roughly 10.000 years), so how do new pathogens emerge in such short time scales? In an agricultural system, plants and pathogens are coupled and their interdependence causes evolutionary changes to occur faster compared to those in natural ecosystems (the co-evolutionary arms race). Moreover, the way humans have shaped the agricultural ecosystem provides an especially conducive environment for rapid evolution of plant pathogens (Stukenbrock and McDonald, 2008). In Figure 2.9, the main differences between natural and agricultural ecosystems are shown. The high environmental homogeneity, low species diversity and high host plant density (facilitating pathogen transmission) enables the development of highly virulent, host-specialized pathogens, thereby increasing the risk of epidemics.

The emergence of new plant pathogens is possible through various mechanisms. Host-tracking or co-speciation occurs when a plant pathogen is domesticated together with its host and associated agro-ecosystem. In this scenario, the center of origin will be the same for plant and pathogen and such domestication is a gradual evolutionary change. A pathogen can also emerge by adaptation to a new host following a host shift or host jump. Host shifts/jumps are abrupt evolutionary changes. A host shift involves a new host plant that is taxomically related to the old host plant, while a host jump occurs between taxonomically distant host plants. Host jumps can be recognized by comparing host plant phylogenies with pathogen phylogenies. Co-speciation of plant pathogens, creates largely congruent plant and pathogen phylogenies. When the phylogenies are incongruent, this is indicative of a host jump (Schulze-Lefert and Panstruga, 2011; Slippers et al., 2005; Giraud et al., 2010).



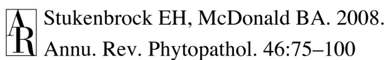


Figure 2.9: An illustration of the main differences between natural and agricultural ecosystems.
Agro-ecosystems (bottom panel) exhibit greater environmental homogeneity, lower species diversity, and higher host density that facilitate pathogen transmission to uninfected neighboring plants. These properties enable the development of epidemics characterized by several cycles of pathogen reproduction every year and drive the emergence of highly virulent, host-specialized plant pathogens. Natural ecosystems (top panel) have greater environmental heterogeneity, higher species diversity, and lower host density that favor less virulent, generalist pathogens that can infect many host species. Reprinted from Stukenbrock and McDonald (2008) with permission from the publisher.

The molecular basis underlying host shifts/jumps is believed to involve changes in effector repertoire (Raffaele and Kamoun, 2012; Schulze-Lefert and Panstruga, 2011). Molecular mechanisms that can cause effector repertoire diversification include (i) Random mutations (mostly SNPs) in effector genes, followed by positive selection, which has recently been shown to have played an important role in the relatively recent adaptation of *Phytophthora mirabilis* to *Mirabilis jalapa* (Dong et al., 2014); (ii) Mobile genetic elements (transposable elements or TEs) are often associated with effector candidates in *Phytophthora* (Haas et al., 2009); (iii) Components related to epigenetic regulation seem to be under accelerated evolution in *Phytophthora* (Raffaele et al., 2010a), epigenetic modification is known to regulate gene expression in eukaryotes, thus they might be involved in the regulation of effector gene expression; (iv) Sexual or meiotic recombination can cause chromosomal rearrangements such as translocations, inversions, gene conversion, etc. and could therefore play an important role in generating effector diversity; (v) Conditionally dispensible chromosomes (CDCs) carrying effector genes have been described in *Fusarium oxysporum* (Ma et al., 2010); (vi) Horizontal gene transfer (HGT), defined as the exchange of specific genes or genomic regions between species that are normally reproductively isolated, is known to be an important mechanism of bacterial adaptation and speciation (Ochman et al., 2000) and until recently, it was thought to be of minor importance in eukaryotes. However, the increasing number of comparative genomics projects of fungal and oomycete plant pathogens indicate that HGT is much more frequent as previously thought (Keeling and Palmer, 2008). In *Phytophthora ramorum*, as much as 7.6% of the secreted proteome has been acquired from fungi by HGT (Richards et al., 2011) (vii) Interspecific hybridization has been described several times in *Phytophthora*, as well as in other plant pathogens , and can cause host range expansion and host jumps (Brasier et al., 1999; Man in 't Veld et al., 2006). Hybridization can be associated with polyploidy, which in plants is known to be a very important driver of speciation and adaptive evolution.

With the increased global trade of (infected) plant material, closely related lineages of pathogens that have been separated geographically, can be brought together, hereby increasing the risk of interspecific hybridization between closely related pathogens. It is known that related species evolving in allopatry (different geographic regions) have lower selection pressure on the formation of reproductive barriers than species evolving in sympatry (existing in the same geographic area) (Stukenbrock and McDonald, 2008; Stukenbrock, 2013).

2.3 Introduction to polyploidy

A large part of this thesis will be devoted to polyploidy in *Phytophthora*. Therefore, a brief introduction about the general concept of polyploidy will be given here. In Table 2.2 some of the most common terminology is defined.
Polyploid species are very common among plants, but also occur in some animal classes like fish and amphibians (Mable, 2003). During the last two decades, many eukaryotic genomes (plants and animals) have been sequenced and a large part of them show signs of ancient polyploidy. In plants, it is now clear that practically all plants have experienced one or more rounds of polyploidization in their evolutionary history (Adams and Wendel, 2005; Cui et al., 2006). This contradicts the notion that polyploidization is an evolutionary 'dead-end', as had been originally proposed Otto (2007). Although polyploidy has already been studied for more than a century, the recent developments in molecular technology (DNA and genome sequencing) and the development of advanced techniques for studying DNA contents of cells (such as flow cytometry, Doležel et al. (2007)), has triggered a renewed interest in polyploidy from the scientific community. Polyploids are generally divided into two classes based on their mode of origin: *allopolyploids* are formed as a consequence of interspecific hybridization combined with a genome duplication event allowing stable meiosis in the absence of *homeologous recombination* (see below). *Autopolyploids* are formed through the doubling of a single species' DNA content, although in the latter case, hybridization between genetically diverse populations of a species can also occur. Therefore, the terminology can be confusing, since the definition of a species is not always straightforward.

It is now largely accepted that polyploids, after going through a bottleneck of instability in the early generations after formation, can be efficient competitors of their diploid counterparts. This instability is mainly a problem in allopolyploids, due to the incompatibilities between the divergent genomes from which they are built (Parisod et al., 2010). Studies have shown that the suppression of homeologous recombination plays an important role in the stabilization and improvement of fertility in allopolyploid plants (Griffiths et al., 2006; Henry et al., 2014). Alternatively, large scale genomic rearrangements can lead to *diploidization*, in which the genomic redundancy is reduced, thereby favoring homologous recombination over homeologous recombination and thereby increasing meiotic stability (Cifuentes et al., 2010). In Figure 2.10, the evolutionary alteration between diploidy and polyploidy is shown. When two closely related species cross, a homoploid F1 zygote is formed which will be meiotically unstable because of divergence in the different parental

Table 2.2: Glossary of common terminology in polyploidy research.

Term	Definition
Allopolyploidy	Polyploidy that arises as a consequence of hybridization between two biological species
Aneuploidy	A type of chromosome abnormality in which there are one or more extra or missing chromosomes
Autopolyploidy	Polyploidy that arises within a single species, although it may involve crossing between genetically differentiated populations
Diploidization	The process of a polyploid going back to the more stable diploid cytogenetic state
Endopolyploidy	The property of cells in certain developmental stages of having more chromatid sets or, less frequently, more chromosome sets than the germ line
Epigenetic change	A mitotically stable change in gene expression that depends not on a change in DNA sequence, but on covalent modifications of DNA or chromatin proteins such as histones
Haploid	Refers to the number of chromosome sets in the germ cells, which is half of the number of chromosome sets in normal cells (symbolized by n)
Heterosis	The increase in performance displayed by hybrids compared with their inbred parents.
Homeolog	A special case of paralogy resulting from polyploidy. Homeologous chromosomes are created through chromosome duplication events (mostly after interspecific hybridization)
Homolog	Chromosomes or genomic regions that share a common ancestry
Homoploid hybrid	A hybrid that has the same ploidy level as the parental species
Mixoploidy	The presence of two nuclei types, one diploid and one polyploid, within an organism
Monoploid	A cell containing only one chromosome set
Neofunctionalization	Acquisition of novel function by a duplicated gene
Neopolyploidy	Polyploidy that has been created by artificially inducing chromosome doubling
Non-disjunction	The failure of chromosome pairs to separate properly during meiosis
Paleopolyploidy	Ancient genome duplication (at least several million years ago)
Paralog	Chromosomes or genomic regions that are homologous due to a gene or genome duplication event
Polyploidy	The presence of more than two sets of chromosomes in a cell
Subfunctionalization	Retention by duplicated genes of different components of the original common function
Synteny	The conservation of blocks of order within two sets of chromosomes that are being compared with each other
Unreduced (2n) gametes	Gametes containing the somatic chromosome number, arising from problems in meiosis

chromosome sets, which causes problems in the correct recognition of homologs in meiosis. A way of restoring fertility is through whole genome duplication, creating again compatible chromosome sets (possibly through the formation of *unreduced (2n) gametes* by the

homoploid hybrid). In this case, heterozygosity within the parental genomes is lost. Allopolyploid hybrids can also be formed in one step through the fusion of unreduced (2n) gametes produced as a consequence of meiotic irregularities in the parental species. In the latter case, heterozygosity in the parental genomes is not always lost. Autopolyploid species are mainly formed through the production of unreduced gametes (De Storme and Geelen, 2013).

Although diploidization is defined as 'the process of a polyploid going back to the diploid state', it is not necessarily associated with a reduction in chromosome number. Processes contributing to diploidization include subfunctionalization and/or neofunctionalization causing differentiation between redundant gene copies; large scale chromosomal rearrangements such as translocations; chromosome loss, etc. (Mayfield-Jones et al., 2013). Polyploidy has long since been linked to increased adaptability, although clear-cut examples are hard to come by (see Chapter 6). Certainly, there are a few important advantages of polyploidy, including heterosis and genetic redundancy (which shields polyploids from the deleterious effects of mutations), but there are also clear disadvantages such as changes in cellular architecture and consequential regulatory implications, problems in meiosis, regulatory changes in gene expression and epigenetic instability (Comai, 2005).

Most of the knowledge about polyploidy gathered up till now comes from plant science. However, polyploidy also appears to be ubiquitous in certain eukaryotic microbes such as Fungi and oomycetes. This has been largely neglected by the scientific community studying polyploidy. The fact that karyotyping in these organisms is extremely difficult because of the small size of the chromosomes and because of difficulties in obtaining clear metaphase preparations played an important role. During the last decade, however, alternative techniques have been developed for studying polyploidy in these taxa, such as flow cytometry for determination of DNA contents (D'hondt et al., 2011) and other alternative karyotyping methods (Mehrabi et al., 2012). A recent review paper on polyploidy in Fungi and oomycetes gives a good overview of past work and future possibilities for polyploidy research in these organisms (Albertin and Marullo, 2012). Polyploidy in Fungi is usually reduced to the well-described whole genome duplication that occurred in the yeast lineage about 100 million years ago (Wolfe and Shields, 1997). There are a few attractive benefits about using a microbial model system for studying polyploidy. One is their faster generation time compared to plants: a few hours for *Saccharomyces* and about four weeks for *Phytophthora porri* compared to six weeks for *Arabidopsis thaliana*, the model plant with the fastest generation time. Another is their small genome size making the use of

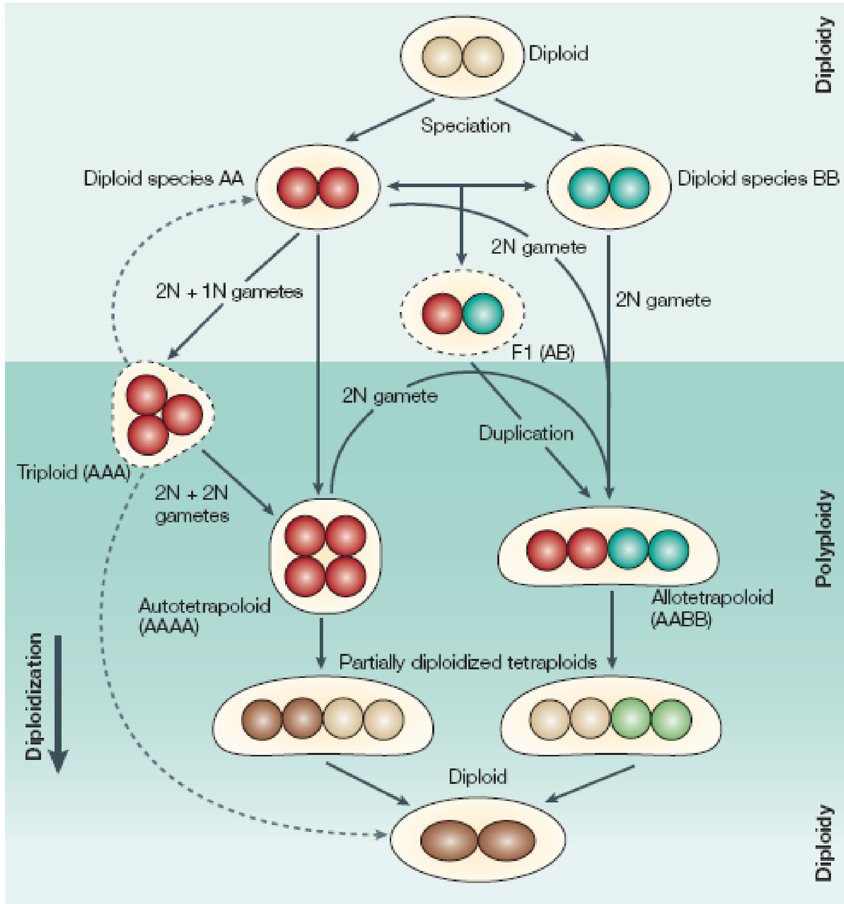


Figure 2.10: Evolutionary alternation between diploidy and polyploidy. The figure shows the possible paths that result in the sudden transition from diploidy to polyploidy and the gradual transition from polyploidy back to diploidy. For simplicity, not all possible paths are shown. Triploids, for example, are shown contributing to autotetraploids but they can also contribute to allopolyploids. For each ploidy form, the haploid genome is represented by a coloured circle or oval inside the beige-filled nuclear shape. Genomes that are illustrated by ovals reflect the increased gene number that results from the retention and subfunctionalization of duplicates during diploidization. Circles or ovals of different colours represent diverged genomes. Highly unstable ploidy forms have dashed nuclear contours. A and B represent genome types and N is the gametic chromosome number. Reprinted from Comai (2005) with permission from the publisher.

next generation sequencing technology relatively straight-forward. Pathogens like *Phytophthora* are also under strong selection pressure from their hosts and are continuously

forced to adapt to new or altered hosts or environments. Polyploidy could deliver the pathogen a means of genomic flexibility to survive this constant arms race with its hosts. Therefore, *Phytophthora* could be an interesting model for studying adaptive evolution in polyploid species (see Chapter 7).

Science may be described as the art of systematic over-simplification.

Karl Popper

3

The expansion of *Phytophthora* clade 8b: three new species associated with winter grown vegetable crops

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Abstract

Despite its association with important agricultural crops, *Phytophthora* clade 8b is a poorly studied group of species. The clade currently consists of three officially described species (*Phytophthora porri*, *Phytophthora brassicae* and *Phytophthora primulae*) that are host-specific pathogens of leek, cabbages and *Primula* spp., respectively. However, over the past few decades, several other clade 8b-like phytophthoras have been found on a variety of different host plants that were all grown at low temperatures in winter seasons. In this study, a collection of 30 of these isolates was subjected to a phylogenetic study using two loci (the rDNA ITS region and the mitochondrial *Cox1* gene). This analysis revealed a clear clustering of isolates according to their host plants. To verify whether these isolates belong to separate species, a detailed morphological study was conducted. On the basis of genetic and morphological differences and host specificity, we now present the official description of three new species in clade 8b: *Phytophthora dauci* sp. nov., *Phytophthora cichorii* sp. nov. and *Phytophthora lactucae* sp. nov. Two other groups of isolates (*Phytophthora* taxon castitis and *Phytophthora* taxon parsley) might also represent new species but the data available at this time are insufficient for an official description. This brings *Phytophthora* clade 8b to a group of six species that are all host-specific, slow-growing and specifically infect herbaceous crops at low temperatures.

3.1 Introduction

Phytophthora is a genus of plant pathogenic, filamentous oomycetes, belonging to the Stramenopiles (Adl et al., 2012). Morphologically, they are very similar to the filamentous fungi (kingdom Fungi), but recent phylogenetic studies have proven them to be of a very different evolutionary origin. Oomycetes differ from Fungi in some important morphological and biochemical aspects and in the fact that they are diploid in their vegetative lifestyle, whereas most Fungi are haploid (Beakes et al., 2012).

Since the official description of *Phytophthora* in 1876, the number of known *Phytophthora* species has increased steadily until a sharp increase in the number of new species descriptions occurred with the introduction of molecular tools in the last decade of the previous century. These new techniques (together with the use of specific selective media and proper isolation techniques) have made rapid and reliable identification of *Phytophthora* species possible. Therefore, large-scale surveys for new species have become more feasible, and many new species have been found in such surveys in recent years. Today, over 100 *Phytophthora* species have been officially described (Kroon et al., 2012), and practically all of them are plant pathogens.

According to the most recent phylogenetic studies (Blair et al., 2008; Cooke et al., 2000; Kroon et al., 2004), the genus *Phytophthora* consists of 10 clades. In this paper, we focus on clade 8b. According to the phylogenetic studies cited above, this group consists of five species: the closely related *P. porri* (Foister, 1931), *P. primulae* (Tomlinson, 1952) and *P. brassicae* (Man in 't Veld et al., 2002) and the more distantly related *P. syringae* (Klebahn, 1909) and *P. austrocedrae* (Greslebin et al., 2007). However, Grünwald et al. (2011) recently described a new species in clade 8 and revised the subclade structure using the same loci that were used in the study by Blair et al. (2008). In this analysis *P. porri*, *P. brassicae* and *P. primulae* are placed in clade 8b, while *P. syringae*, *P. austrocedrae* and the newly described *P. obscura* form a new clade 8d. In this work, we follow the new subclade organization as defined by Grünwald et al. (2011).

Phytophthora porri is known to cause the white tip disease of leek (*Allium porrum*), one of the most important diseases in leek cultivation in autumn and winter seasons in temperate regions (Declercq et al., 2009, 2012). Isolates pathogenic to cabbages (*Brassicaceae*) used to be regarded as a host-specific subspecies of *P. porri*, but were officially described as *Phytophthora brassicae* on the basis of genetic and morphological differences and host specificity by Man in 't Veld et al. (2002). *Phytophthora primulae* has been described as a root pathogen of primrose (*Primula* spp.), an ornamental plant (Tomlinson, 1952).

During the past few decades, isolates morphologically similar to the species described

above have been reported to cause diseases in a range of other hosts. Firstly, isolates similar to *Phytophthora porri* have been isolated from lettuce (*Lactuca sativa*). One report describes the occurrence of stem rot of lettuce in South Australia, resulting in complete wilting of the heads (Sitepu and Bumbieris, 1981). Another report describes the same symptoms in Greece (Elena et al., 2006). Secondly, a disease named 'rubbery brown rot' occurred in Canada in stored carrots (*Daucus carota*; Stelfox and Henry (1978)). Furthermore, in some regions in the north of France, a *P. porri*-like organism is associated with a carrot disease called 'ring rot disease' (Danielle Breton, pers. comm.). Thirdly, isolates similar to *Phytophthora primulae* were derived from parsley plants (*Petroselinum crispum*) in Greece. They caused stem base rot and consequently wilting of entire plants in consecutive years starting from 2002, resulting in significant yield losses (Elena et al., 2008). Fourthly, isolates belonging to a clade 8b-like species have been causing rotting in chicory roots (*Cichorium intybus* var. foliosum) in the UK (Kim Green & John Scrace, ADAS, pers. comm.) and in the Netherlands during the past decade. Fifthly, a *P. porri*-like organism provoking crown rot of strawberry (*Fragaria* x *anannassa*) was collected in Sweden. Last of all, a Japanese report from the 1960s describes leaf blight and bulb rot in scallion (*Allium bakeri*), caused by a *P. porri*-like species, as one of the most serious problems in scallion cultivation in some regions in Japan (Katsura et al., 1969). The same disease was described in Great Britain (Griffin and Jones, 1977) and in South Africa (von Maltitz and von Broembsen, 1984). These isolates may possibly represent an incipient species arising from interspecific hybridization between *P. porri* and a closely related species (Declercq (2009) and Chapter 4). Because of their probable hybrid nature, these isolates will not be discussed in this chapter. In all cases mentioned above, the disease occurred at low temperatures during winter seasons.

Despite its association with these important agricultural crops, up until now clade 8b has been an understudied group of species. A reason for this might be the recalcitrance of the clade 8b species: they are all very slow growing on culture media, and therefore extremely hard to detect and isolate from infected plants. In an attempt to fill this gap, we performed a multi-locus phylogenetic study on a collection of clade 8b isolates from the hosts mentioned above. From this phylogenetic study, it became clear that some groups of isolates showed considerable amounts of genetic variation from the known clade 8b species. To verify whether these groups of isolates belong to separate species, the isolates were subjected to a detailed morphological study. On the basis of genetic and morphological differences and host specificity, we present the official description of three new species in clade 8b, namely *Phytophthora dauci* sp. nov., *Phytophthora cichorii* sp. nov. and *Phytophthora lactucae* sp. nov. Next to this, we confirm the existence of two

possible new taxa, *Phytophthora* taxon parsley and *Phytophthora* taxon castitis.

3.2 Materials and methods

3.2.1 Isolate collection and maintenance

All 31 isolates used in this study are listed in Table 3.1. They were either freshly isolated from diseased plants or obtained from culture collections or from other researchers via personal contacts. The cultures were routinely maintained on V8 agar (V8A, 200 mL V8 juice (Campbell); 3 g $CaCO_3$; 15 g agar and 800 mL of sterile water) or Corn Meal Agar (CMA-BD, Beckton Dickinson) or CBS cornmeal agar (CMA-CBS, (Crous et al., 2009)) and kept as V8A plugs in 10 % glycerol at -80°C for long term storage. All cultures are available at the CBS Fungal Diversity Center in the Netherlands (CBS) or at the Benaki Phytopathological Institute in Greece.

3.2.2 DNA-extraction, PCR and sequencing

Isolates were grown in clarified V8 broth (100 mL of clarified V8 juice, 3 g $CaCO_3$ and 900 mL of sterile water) for 7-10 d at 15°C in the dark. The mycelial mats were harvested by filtration, blotted dry, frozen in liquid nitrogen and pulverized using mortar and pestle. DNA was extracted using Qiagen's DNeasy Plant Mini Kit (Hilden, Germany). Amplification and sequencing of the ITS region was performed using primers ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') (White et al., 1990). PCR reactions were performed in a 25 µl mix containing 2.5 µl PCR buffer (10X, Qiagen), 0.5 µl dNTPs (10 mM, Qiagen), 1 µl of each primer (10 µM), 0.15 µl Taq polymerase (5 units per µl; Fermentas), 17.85 µl milli-Q water and 2 µl of DNA template (25 ng μl^{-1}). The amplifications were done in a Flexcycler PCR Thermal Cycler (Analytikjena) programmed as follows: initial denaturation for 10 min at 94°C; 35 cycles of denaturation for 1 min at 94°C; annealing for 1 min at 60°C; extension for 1 min at 72°C; final extension for 10 min at 72°C. For the *Cox1* gene, the degenerate primers Oom-CoI-Lev-up (5'-TCA WCW MGA TGG CTT TTT TCA AC-3') and FM-85-mod (5'-RRH WAC KTG ACT DAT RAT ACC AAA-3') were used as described by Bala et al. (2010).

Table 3.1: List of the 31 *Phytophthora* isolates used in this study.[a]Genbank accession numbers

Code	Alternative collections	Host	Origin	Year of isolation	(Proposed) Species name	*Cox1* [a]	ITS [a]
CBS 802.95	PD 92/214	*Allium porrum*	Netherlands	1992	*Phytophthora porri*	KC478717	KC478747
CBS 114100	-	*Allium porrum*	Denmark	1992	*Phytophthora porri*	KC478718	KC478748
CBS 116662	Smilde GG	*Allium porrum*	UK	1994	*Phytophthora porri*	KC478719	KC478749
CBS 127099	K06006(2)	*Allium porrum*	Belgium	2006	*Phytophthora porri*	KC478720	KC478750
CBS 127101	S05029(1)	*Allium porrum*	Belgium	2005	*Phytophthora porri*	KC478721	KC478751
CBS 110167	BBA 71108	*Primula eliator*	Germany	1999	*Phytophthora primulae*	KC478722	KC478752
CBS 116663	PD 99/2429	*Primula* sp.	Netherlands	1999	*Phytophthora primulae*	KC478723	KC478753
CBS 114346	LYN 916-A	*Primula polyantha*	New-Zealand	2003	*Phytophthora primulae*	KC478724	KC478754
CBS 110162	BBA 70403	*Primula* sp.	Germany	1997	*Phytophthora primulae*	KC478725	KC478755
CBS 620.97	PD 97/875	*Primula acaulis*	Germany	1997	*Phytophthora primulae*	KC478726	KC478756
BPIC 2584	-	*Petroselinum crispum*	Greece	2006	*Phytophthora* taxon parsley	KC478727	KC478757
CBS 114156	-	*Petroselinum crispum*	Australia	2003	*Phytophthora* taxon parsley	KC478728	KC478758
CBS 688.79	-	*Daucus carota*	Canada	1978	*Phytophthora* taxon castitis	KC478729	KC478759
CBS 131246	CH112	*Fragaria times ananassa*	Sweden	1995	*Phytophthora* taxon castitis	KC478730	KC478760
CBS 127102	BorfSP370	*Daucus carota*	France	2009	*Phytophthora dauci* sp. nov.	KC478731	KC478761
CBS 114039	-	*Daucus carota*	Australia	2003	*Phytophthora dauci* sp. nov.	KC478732	KC478762
CBS 782.97	Smilde HH	*Brassica chinensis*	Netherlands	1994	*Phytophthora brassicae*	KC478733	KC478763
CBS 212.82	P3273	*Brassica oleraceae*	Netherlands	1982	*Phytophthora brassicae*	KC478734	KC478764
CBS 113350	PD 94/166	*Brassica oleraceae*	Netherlands	1994	*Phytophthora brassicae*	KC478735	KC478765
CBS 112277	ICMP 14271	*Brassica oleraceae*	New-Zealand	2001	*Phytophthora brassicae*	KC478736	KC478766
CBS 127274	B10001	*Brassica oleraceae*	Belgium	2010	*Phytophthora brassicae*	KC478737	KC478767
BPIC 1985	-	*Lactuca sativa*	Greece	2001	*Phytophthora lactucae* sp. nov.	KC478738	KC478768
BPIC 1986	-	*Lactuca sativa*	Greece	2001	*Phytophthora lactucae* sp. nov.	KC478739	KC478769
BPIC 1987	-	*Lactuca sativa*	Greece	2002	*Phytophthora lactucae* sp. nov.	-	-
BPIC 1988	-	*Lactuca sativa*	Greece	2002	*Phytophthora lactucae* sp. nov.	KC478740	KC478770
BPIC 1991	-	*Lactuca sativa*	Greece	2003	*Phytophthora lactucae* sp. nov.	KC478741	KC478771
BPIC 1992	-	*Lactuca sativa*	Greece	2003	*Phytophthora lactucae* sp. nov.	KC478742	KC478772
CBS 115029	-	*Cichorium intybus*	Netherlands	2004	*Phytophthora cichorii* sp. nov.	KC478743	KC478773
CBS 114345	-	*Cichorium intybus*	Netherlands	2003	*Phytophthora cichorii* sp. nov.	KC478744	KC478774
CBS 115030	-	*Cichorium intybus*	Netherlands	2004	*Phytophthora cichorii* sp. nov.	KC478745	KC478775
CBS 133815	SCRACE5388	*Cichorium intybus*	UK	1999	*Phytophthora cichorii* sp. nov.	KC478746	KC478776

3.2.3 Phylogenetic analysis

Sequence alignments of 44 isolates were made for the two loci (ITS and *Cox1*) using ClustalW in Bio-Edit and manually edited afterwards. Of these 44 isolates, 30 isolates represented the clade 8b strains that were sequenced in this study; the other 14 isolates represent species of the other clade 8 subclades (8a, 8c and 8d) and their sequences were derived from Q-bank (`http://www.q-bank.eu`) or from GenBank (`http://www.ncbi.nlm.nih.gov`). For each locus, two different phylogenetic analyses were performed, one using Maximum Likelihood analysis as implemented in the MEGA5 software (Tamura et al., 2011) and the other using Bayesian Inference of Phylogeny (MrBayes v. 3.1.2; Huelsenbeck and Ronquist (2001); Ronquist and Huelsenbeck (2003)). Model selection was done by jModeltest (Guindon and Gascuel, 2003; Posada, 2008) with the Akaike Information Criterion (AIC). The model that best fitted the data and that was available in MEGA5 and MrBayes was the General Time Reversible model with gamma distributed rate variation (GTR + G). For the Maximum Likelihood phylogenetic analysis, the data were described as coding (*Cox1*) or non-coding (ITS). A bootstrap consensus tree inferred from 1000 replicates was built. For the Bayesian analysis, two analyses were run simultaneously for 1 000 000 generations with three heated chains and one cold chain. The majority-rule consensus tree was calculated after discarding the first 250 000 generations (25%) from each run as burn-in. Both alignments, trees, as well as the phylogenetic source files are available on TreeBASE (`http://treebase.org/treebase-web/home.html`; study number 13805).

3.2.4 Determination of growth curves

For the study of temperature growth relationships, CMA-Oxoid medium was used (Oxoid, Basingstoke, Hampshire, England). This medium differs from the other CMA agars used in this study in that it is a clear medium that allows better judgement of colony diameter. Inoculum plugs were taken from the margin of an actively growing, young colony. The plugs were transferred to the centre of a series of 13 Petri dishes that were incubated in darkness at 18°C. In most cases, cultures showed some growth after one day. If not, the cultures were incubated one or several days longer. After this initial incubation, Petri dishes were transferred to a series of incubators. Thirteen incubators ranging in temperature from 0-36°C with increments of 3°C were used. One additional incubator was set at 40°C. After an hour, two perpendicular lines were drawn on the back of the Petri dish, intersecting beneath the inoculum plug. Radial growth was determined after 24 h, 48 h and 1 wk by marking the margin of the colony along these lines in all four directions.

3.2.5 Colony Morphology

Colony morphology of isolates was studied on V8-CBS, PDA-CBS (Crous et al., 2009) and CMA-Oxoid. Round inoculum plugs with a diameter of 5 mm were taken from the margin of young, actively growing colonies. The mycelium plugs were placed in the center of Petri dishes with the aforementioned media and were placed at 18°C and 24°C in darkness. Photographs were taken after one week of incubation.

3.2.6 Morphology

Sporangium production was studied on colonized hemp and bell pepper seeds in sterile pond water. Autoclaved bell pepper and hemp seeds were placed on the edge of young *Phytophthora* colonies, and incubated for one or several days at 18°C in the dark until the seeds were colonized by the oomycete. The colonized seeds were transferred to new Petri dishes with sterile filtered pond water, and were incubated at 18°C. Isolates that sporulated poorly were also incubated at other temperatures and light conditions. However, this did not lead to better results. CMA-CBS agar was used for the production of gametangia. Homothallic isolates produced oogonia after incubation in darkness at 18°C for one or several weeks. Isolates that failed to produce oogonia in single culture were mated with heterothallic isolates of known mating type to test if they were heterothallic. Gametangia used for the morphological description of heterothallic isolates were produced by the following procedure. A plug of mycelium approximately a centimeter in diameter was transferred to an empty Petri dish and covered with a polycarbonate filter. A mycelium plug of a culture of the opposing mating type was put on top and the Petri dish was sealed with parafilm, and incubated in the dark for 1-2 wk. Experiments with plugs without mycelium on one side were used as a control to confirm that the membrane was impermeable to hyphae. When two-celled antheridia were produced, the entire globose structure was measured when determining antheridial size, even though by biological function, only the top cell is the antheridium. Production of hyphal swellings and chlamydospores occurred both in the water cultures used for sporangium production and on CMA-CBS agar. Nomenclature and descriptions linked to taxonomic novelties were deposited in MycoBank (`www.MycoBank.org`; Crous et al. (2012)).

3.3 Results

3.3.1 Phylogenetic analysis

The number of variable sites among the 30 clade 8b isolates in the alignments differed between the two loci. The *Cox1* gene showed the highest variability with 79/716 variable characters. The ITS region had a lower variability with 39/855 variable characters. However, there was enough phylogenetic signal to resolve reliable clustering with Bayesian and Maximum Likelihood analysis in both ITS and *Cox1* phylogenies. In Figures 3.1 and 3.2, the Maximum Likelihood bootstrap consensus trees derived from the ITS and *Cox1* alignments, respectively, are shown. The majority consensus rule trees derived from the Bayesian analysis showed a nearly identical clustering for both loci. Bayesian Posterior Probability values of clusters are shown only when higher than 0.90. Maximum likelihood bootstrap measures are shown only when higher than 70. From the phylogenetic analyses, it is clear that all isolates examined (Table 3.1) belong to clade 8b and are closely related to the three described clade 8b species *P. porri*, *P. brassicae* and *P. primulae*. However, there is a clear phylogenetic difference between groups of isolates derived from different host plants (Figures 3.1 and 3.2). The *P. porri* isolates from leek form a distinct group in both phylogenies with high support from both analyses. Their closest relatives are the *P. primulae* isolates and the isolates from parsley. The two isolates from parsley only form a separate cluster from the primrose isolates in the *Cox1* phylogeny. In more detail, the ITS sequences of the parsley isolates are 100% identical to those of *P. primulae*; in the *Cox1* gene however, there are two point mutations compared to *P. primulae*. The *P. brassicae* isolates form a cluster in both phylogenies with high support from both analyses, supporting its reclassification as a species separate from *P. porri*. The four isolates from chicory form a separate cluster in both phylogenies receiving high support from both analyses. The six isolates from lettuce also form a separate cluster in both phylogenies with high support from both analyses. Isolates from carrot fell into two distinct groups. The first group contains two isolates from carrot (CBS 127102 and CBS 114039) and forms a distinct cluster with high support in both phylogenies. The third isolate from carrot (CBS 688.79) seems to be genetically different and clusters together with another isolate that was derived from strawberry (CBS 131246). To avoid confusion, isolates from the first group will be referred to as *P. dauci*, while isolates from the clade containing the strawberry and carrot isolate will be referred to as *P.* taxon castitis. Similarly, isolates from chicory will be referred to as *P. cichorii*, isolates from lettuce will be referred to as *P. lactucae* and the isolates from parsley as *P.* taxon parsley. Our phylogenetic data also supports the revised clade 8 structure as proposed by Grünwald et al. (2011), in which

P. syringae, *P. austrocedrae* and the newly described *P. obscura* form a distinct clade 8d. In our study, the *Cox1* gene proved more useful for distinguishing the separate taxa than the ITS region, while the latter provided a better resolution at a higher taxonomical level, namely that of the subclades.

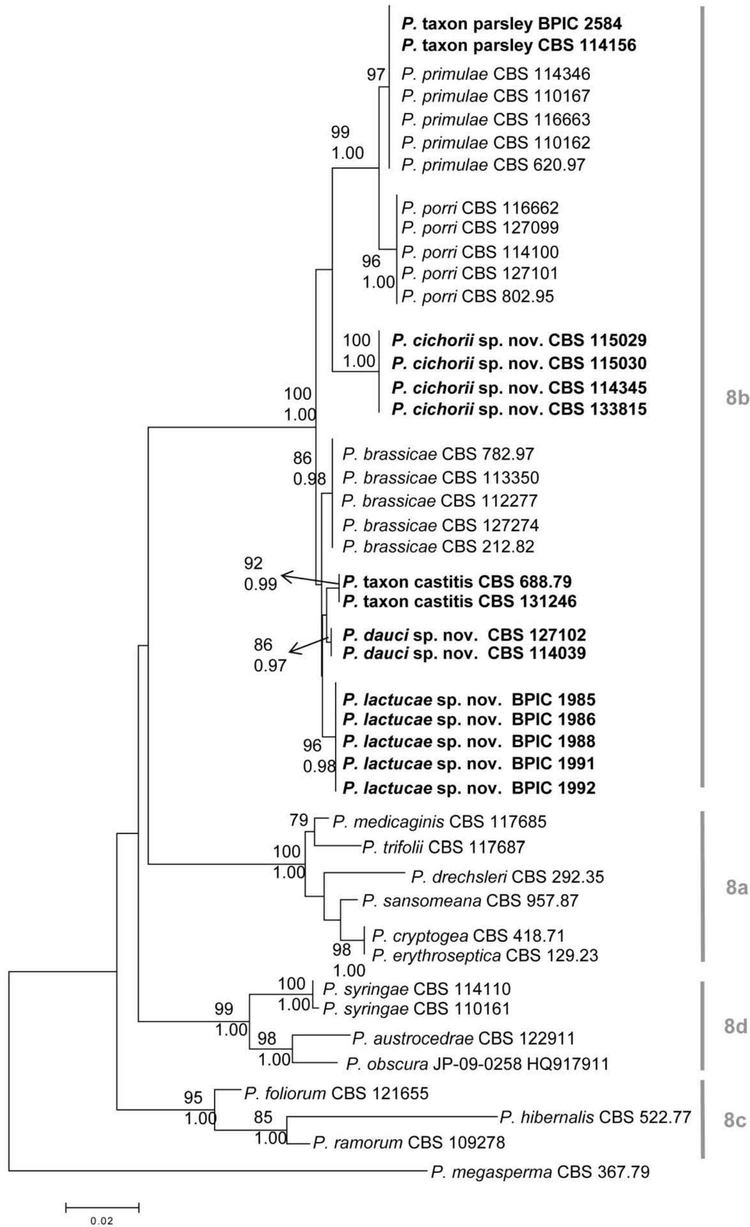

Figure 3.1: ITS phylogeny of *Phytophthora* clade 8. Phylogenetic tree derived from ITS sequence data. The bootstrap consensus tree from the Maximum Likelihood analysis, with its according branch lengths, is presented. The Maximum Likelihood bootstrap support values are shown only for those branches with a bootstrap support higher than 70 (top). Bayesian Posterior Probability values are shown only for those branches having support values higher than 0.90 (bottom). The tree is rooted with *P. megasperma* isolate CBS 367.67 (clade 6). Sequences from species from subclades 8a, 8c and 8d are derived from Q-bank (`http://www.q-bank.eu`; CBS number is shown) or from GenBank (GenBank accession number is shown).

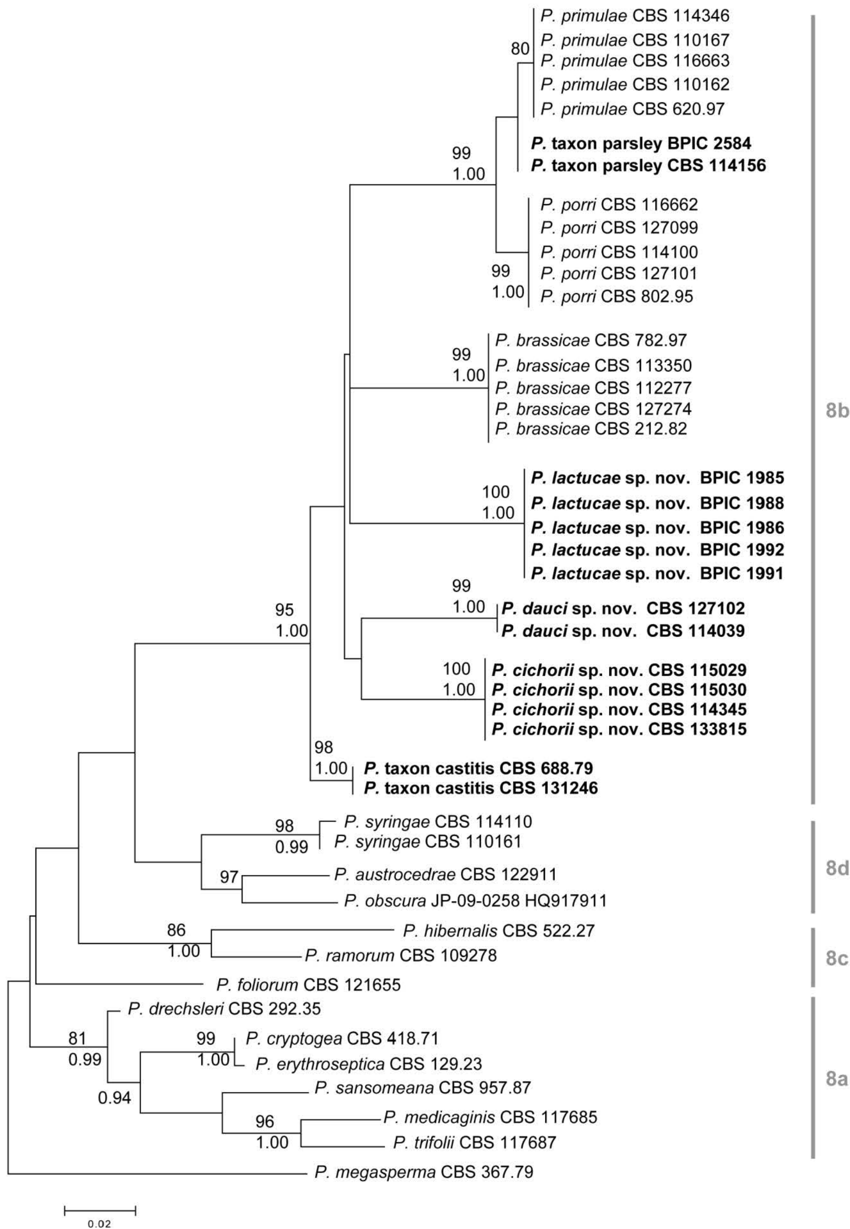

Figure 3.2: *Cox1* phylogeny of *Phytophthora* clade 8. Phylogenetic tree derived from *Cox1* sequence data. The bootstrap consensus tree from the Maximum Likelihood analysis, with its according branch lengths, is presented. The Maximum Likelihood bootstrap support values are shown only for those branches with a bootstrap support higher than 70 (top). Bayesian Posterior Probability values are shown only for those branches having support values higher than 0.90 (bottom). The tree is rooted with *P. megasperma* isolate CBS 367.67 (clade 6). Sequences from species from subclades 8a, 8c and 8d are derived from Q-bank (`http://www.q-bank.eu`; CBS number is shown) or from GenBank (GenBank accession number is shown).

3.3.2 Morphological and physiological characters

In the phylogenetic analysis, several highly supported clades were found. The morphology and physiology of these isolates was studied to determine whether there was support for these clades representing new species. Morphological measurements and observations are summarized in Table 3.2. For comparison with the other clade 8b species, we refer to Q-bank (`http://www.q-bank.eu`). Temperature growth relationships for *P. cichorii*, *P. lactucae*, *P. dauci*, *P.* taxon castitis and *P.* taxon parsley are shown in Figure 3.3. Morphology for each of the new species and for *P.* taxon castitis and *P.* taxon parsley is shown in Figures 3.4 to 3.8. Colony morphology photographs are shown in Figure 3.9. Concerning the production of sexual structures, the following findings should be mentioned. Isolates belonging to *P.* taxon castitis failed to produce gametangia in our experiments in both single culture and in mating tests. The *P. cichorii* isolates tested were heterothallic: solates CBS 115029 and CBS 114345 produced oogonia in a polycarbonate filter separated mating setup with *P. capsici* CBS 128.23, a strain of A2 mating type. Isolate CBS 115030 failed to produce oogonia, but did induce oogonium formation in an A2 strain of *P. capsici* CBS 128.23 indicating that it is silent heterothallic under the conditions tested. Strain CBS 114345 produced only aborted oospores. The mating system of CBS 133815 was not determined. All isolates belonging to *P. dauci* and *P. lactucae* were homothallic. For *P. lactucae*, two isolates produced almost exclusively aborted oospores (BPIC 1988, BPIC 1992). Only a limited number of gametangia could be measured for these isolates but for those measured, oogonium sizes fell within the size ranges reported for other *P. lactucae* isolates. Not all isolates produced sporangia in our experiments; two of the six *P. lactucae* isolates failed to sporulate or produced only a few sporangia (BPIC 1987, BPIC 1991). Isolate CBS 688.79 (*P.* taxon castitis) sporulated very poorly.

Table 3.2: Overview of morphological data of the new *Phytophthora* clade 8b species.[a]Oogonia not produced under normal circumstances or in mating test, oogonia were induced by Ho (1983) after exposing a culture to X-rays

	Phytophthora cichorii	***Phytophthora dauci***	***Phytophthora lactucae***	**Phytophthora** taxon castitis	**Phytophthora** taxon parsley
Number of isolates examined	3	2	6	2	2
Cardinal growth temperature					
minimum	0 °C	0 °C	0 °C	0 °C	0 °C
maximum	24 °C	21 °C	24 °C	27 °C	24 °C
optimum	15-21 °C	9-18 °C	15-21 °C	15-24 °C	15-21 °C
Growth rate at 21 °C (mm/day)	0.6-1.1	0.8-1.0	0.7-2.1	2.0-2.6	1.5-2.0
Sporangia					
length × width:					
range	30.6-142.9 × 20.7-63.1	27.4-89.9 × 24-56.2	27.6-101.3 × 18.3-53.5	23.6-97.9 × 14.9-49.5	25.2-107,7 × 17.8-46.5
isolate means	69.0-74.9 × 37.5-42	52.6-58.8 × 37.2-40.1	55.3-61.1 × 35.5-38.5	50.8-55.4 × 32.6-35.6	52.8-54.3 × 29.4-30.6
length/width ratio:					
Range	1.2-3.2	1.1-2.0	1.2-2.4	1.2-2.6	1.2-3.5
isolate means	1.7-1.8	1.4-1.5	1.5-1.6	1.4-1.7	1.7-1.9
discharge pore width:					
Range	6.0-16.9	6.5-11.7	6.0-15.5	-	5.2-10.7
isolate means	8.9-11.0	9.1-9.5	9.5-10.3	-	7.9-8.0
Mating system	Heterothallic	Homothallic	Homothallic	sterile[a]	Homothallic
Antheridia					
Type	Amphigynous	Paragynous	Paragynous, some amphigynous	-	Both amphigynous and paragynous
length × width:					
Paragynous range	-	12.1-21.9 × 8.5-16.8	10.7-25.2 × 7.5-16.5	-	11.8-30.7 × 8.5-18.3
Paragynous isolate means	-	16.0-17.1 × 11.6-12.5	16.1-17.5 × 9.8-11.9	-	18.1-19.3 × 13.2-14.0
Amphigynous range	16.4-37.7 × 13.2-26.4	-	Rare	-	10.2-21.3 × 10-16.9
Amphigynous isolate means	23.4-27.4 × 19.8-19.8	-	Rare	-	15.8-16.5 × 13.6-14.2
Oogonia					
length × width:					
range	23.7-49.5 × 19.2-45.9	25.3-37.2 × 25-37.2	22-44.1 × 21-44.1	-	24.3-44.7 × 23.4-42.6
isolate means	34.5-37 × 30.3-35.4	31.9-32.3 × 31.2-31.6	33.5-36.4 × 33.0-36.4	-	34.4-37.6 × 33.9-37.3
oogonium wall (isolate means)	1.0-1.2	1.0-1.1	1.3-1.7	-	1.1-1.4
oospore width:					
range	21.6-42.0	20.6-30.6	19.0-38.7	-	19.4-37.3
isolate means	27.5	26.3-27.6	27.2-31.0	-	28.9-30.9
oospore wall (isolate means)	1.2	1.1-1.3	1.1-1.6	-	0.9-1.4
Hyphal swellings					
length × width:					
range	16.0-56.5 × 13.5-42.3	17.1-59.9 × 14.3-41.4	14.3-49.8 × 10.4-39.3	14.6-61.8 × 12.6-52.1	11.3-61.5 × 10.3-38.7
isolate means	25.1-32.2 × 19.1-26.2	30.3-30.8 × 22.6-25.1	26.0-31.6 × 21.8-26.4	32.5 × 28.8	24.1-28.1 × 19.8-20.7
Chlamydospores					
length × width:					
range	-	23.7-57.4 × 20.9-56.0	22.0-51.4 × 20.4-52.4	29.1-73.2 × 29.1-73.2	-
average	-	31.9-38.3 × 30.8-37.2	31.7-37.2 × 29.7-35.3	36.4-51.4 × 36.4-51.4	-

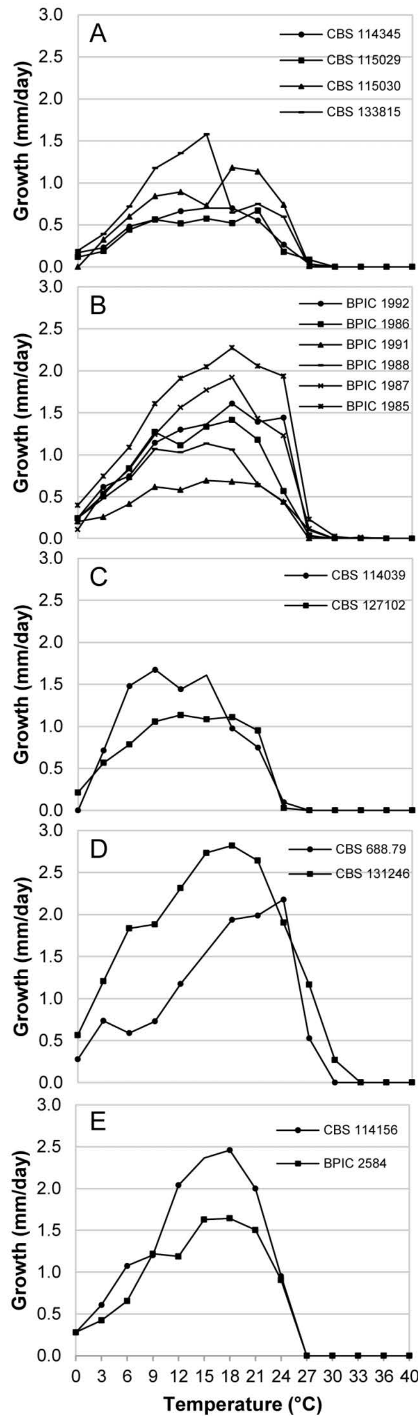

Figure 3.3: Temperature growth relationships of the *P. cichorii* (A), *P. lactucae* (B), *P. dauci* (C), *P.* taxon castitis (D) and *P.* taxon parsley (E) isolates.

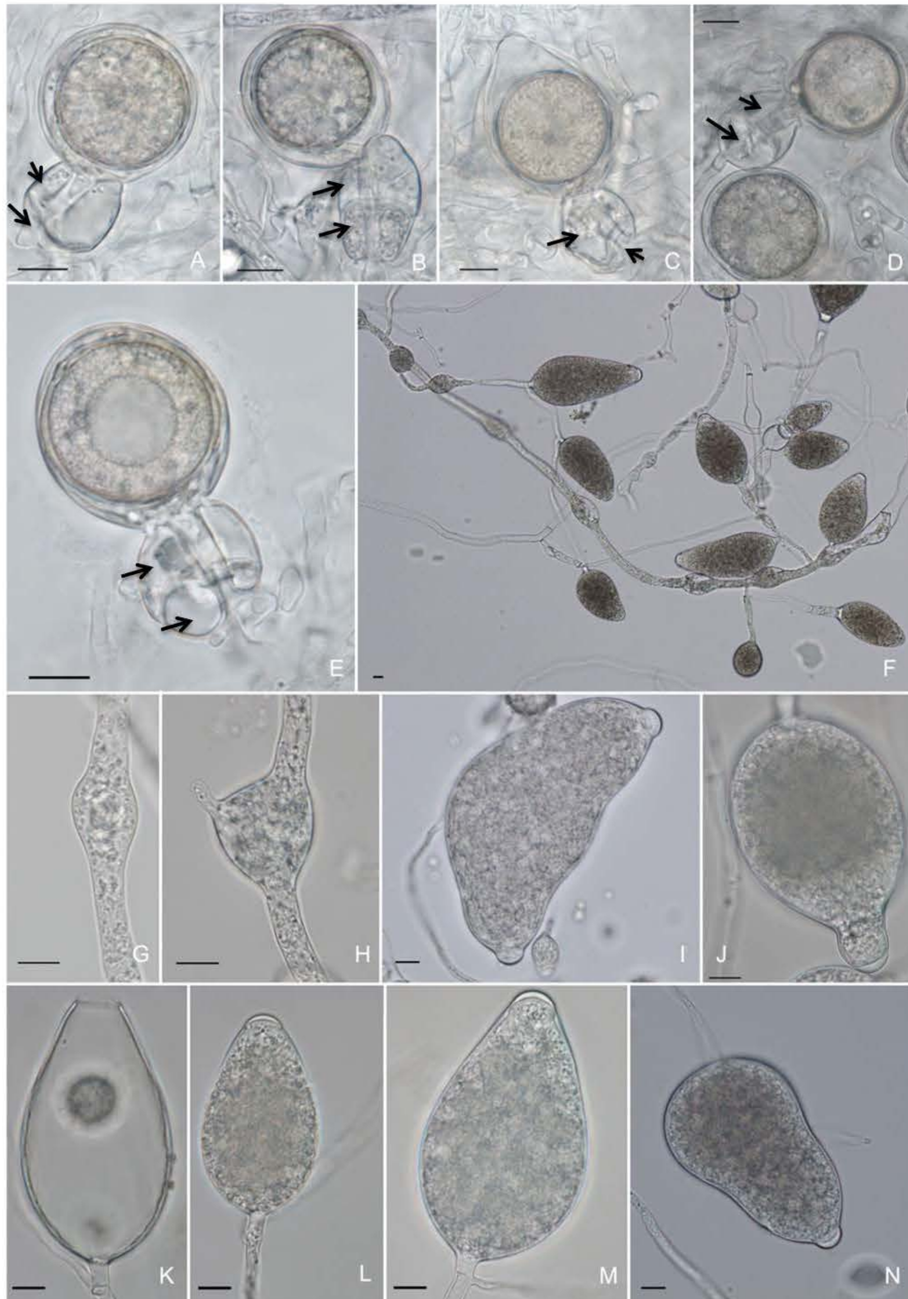

Figure 3.4: ***Phytophthora cichorii*** **morphology.**
All scale bars are 10 µm. A-E, Oogonia with '2-celled' amphigynous antheridia; F, sporangia and hyphal swellings at low magnification; G-H, intercalary hyphal swellings; I-N, sporangia; I, bipapillate sporangium; J, sporangium with elongated neck; K, empty sporangium with wide discharge pore.

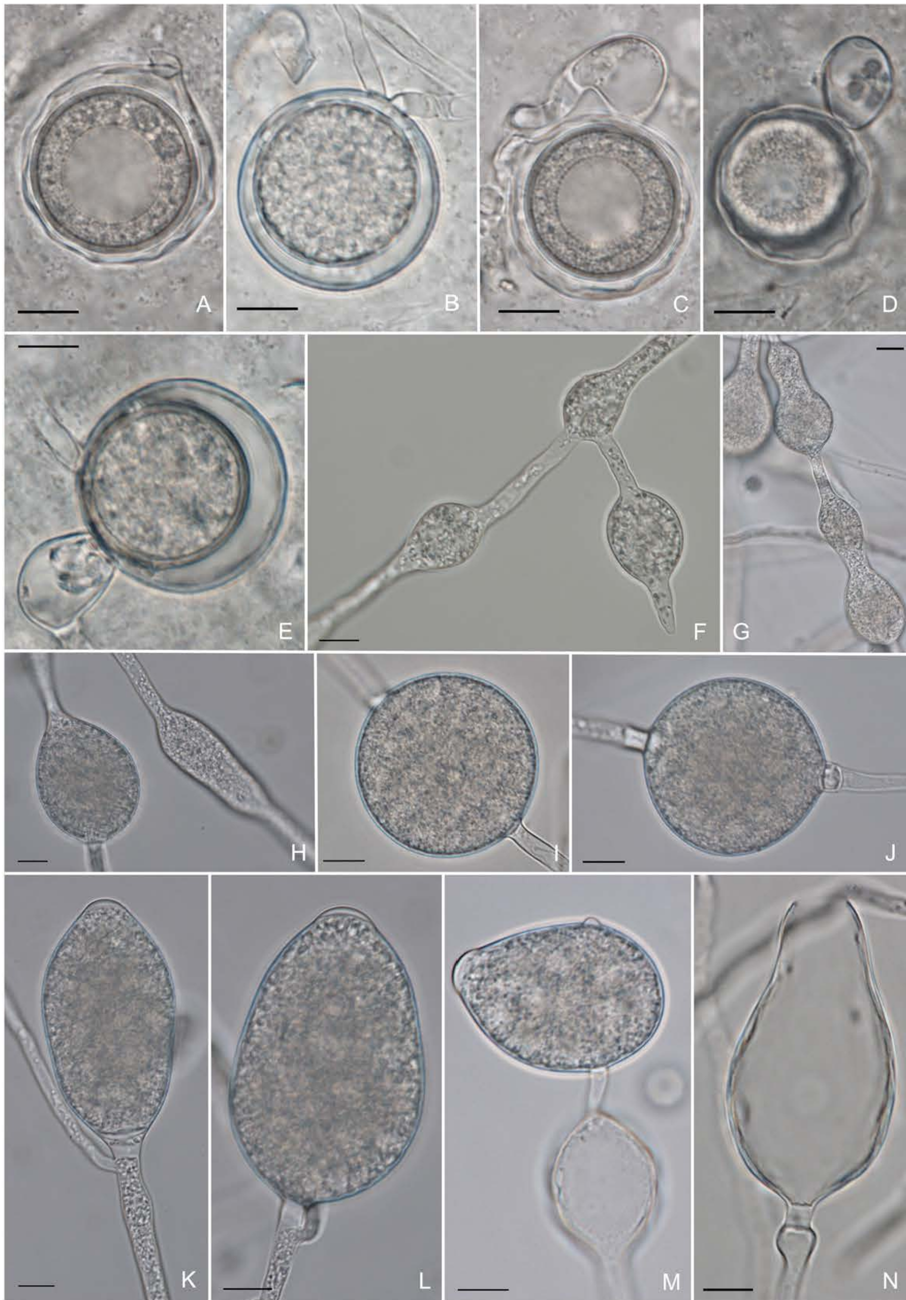

Figure 3.5: ***Phytophthora dauci*** **morphology.**
All scale bars are 10 µm. A-E, Oogonia; B, intercalary oogonium; A, C, D, oogonia with 'wavy' oogonium walls; C-E, paragynous antheridia; F-H, hyphal swellings; I-J, chlamydospores; K-N, sporangia; N, empty sporangium with wide discharge pore; M, sporangium with lateral attachment, with a hyphal swelling in the subtending hypha.

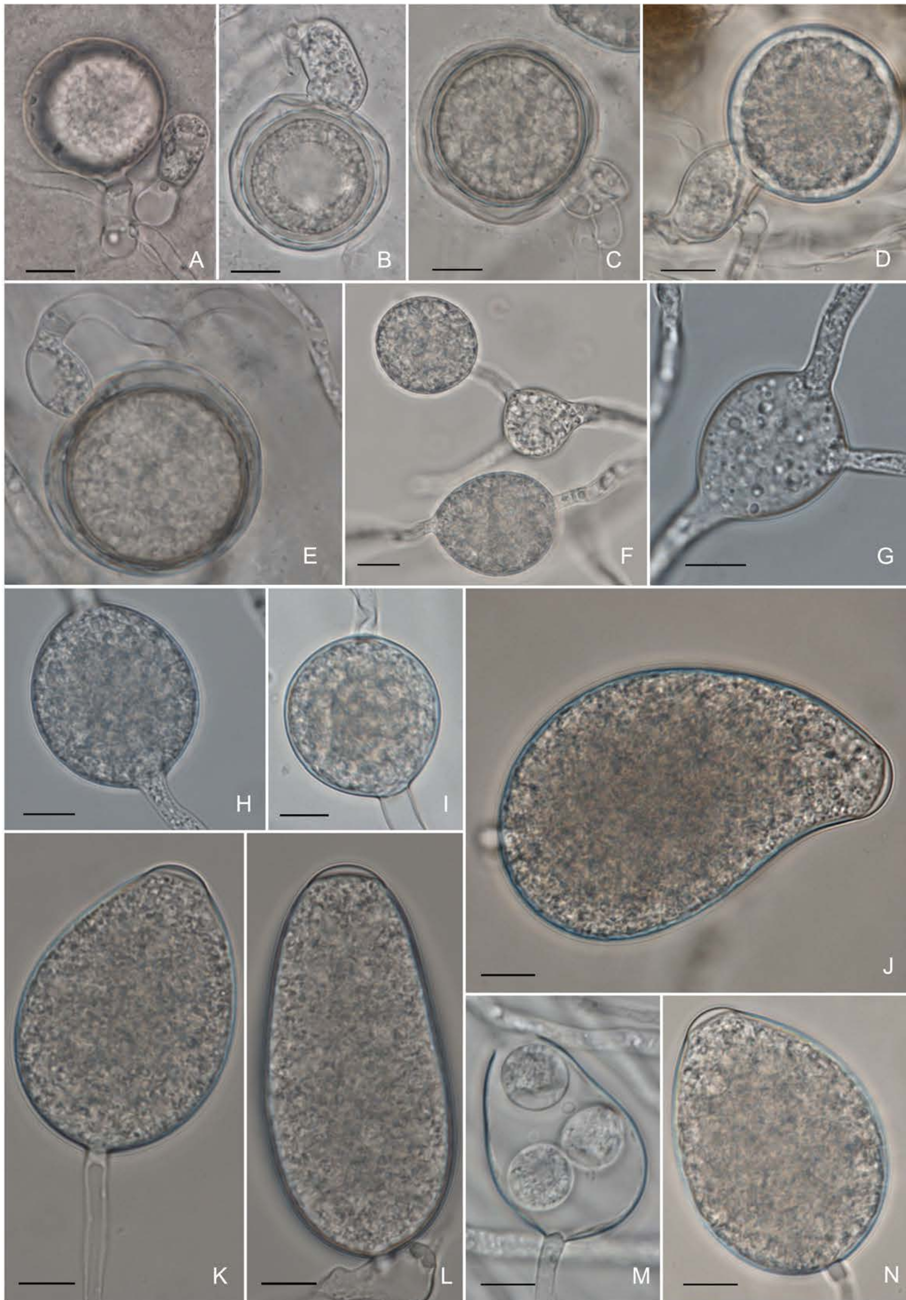

Figure 3.6: *Phytophthora lactucae* morphology.
All scale bars are 10 µm. A-E, Oogonia; A, B and E, paragynous antheridia; C-D, amphigynous anteridia; F and H, hyphal swellings; I, chlamydospore; J-N, sporangia; M, discharged sporangium, several zoospores have encysted within the sporangium.

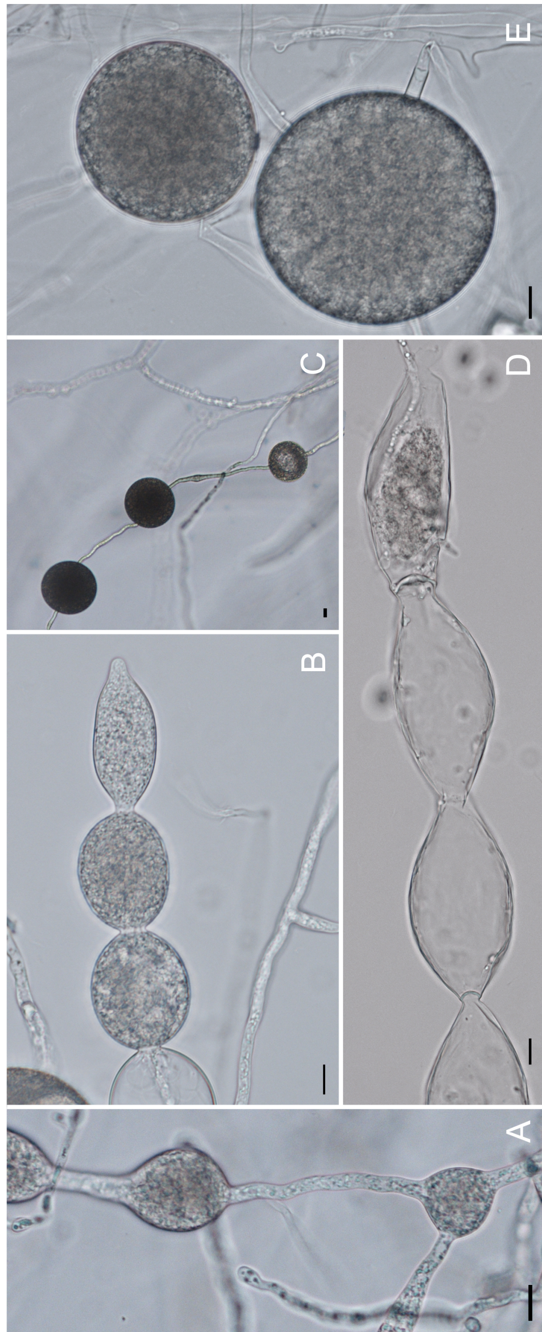

Figure 3.7: ***Phytophthora* taxon castitis morphology.**
All scale bars are 10 µm. A, hyphal swellings; B, catenulate hyphal swellings with a terminal sporangium; D, empty swellings with a terminal empty sporangium; C and E, chlamydospores.

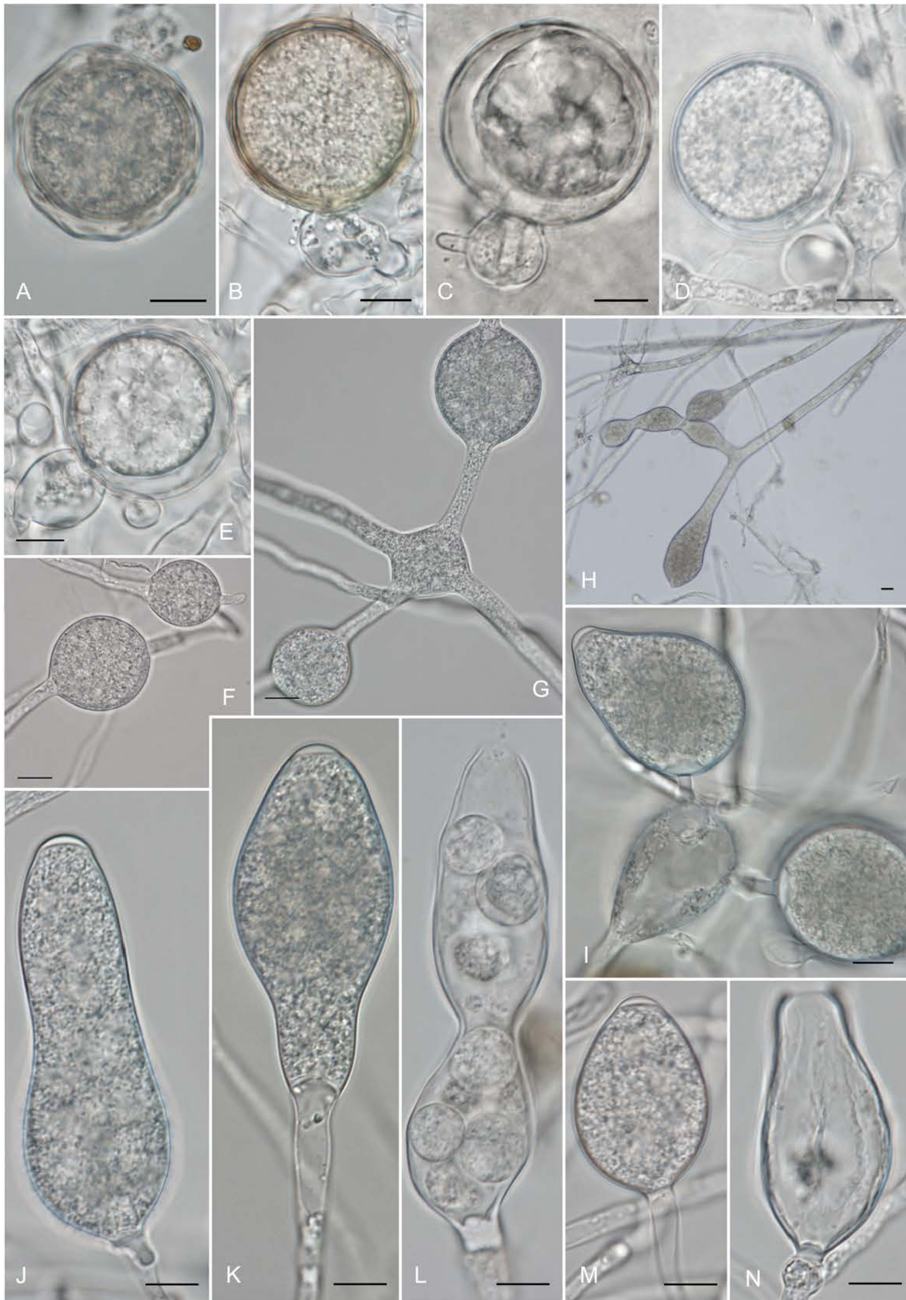

Figure 3.8: *Phytophthora* taxon parsley morphology.
All scale bars are 10 µm. A, Oogonium with 'wavy' wall; B and D-E, oogonia with paragynous antheridia; C, oogonium with amphigynous antheridium; D, oogonium with two antheridia; F-H, hyphal swellings; I-N, sporangia; J, elongated sporangium; K, sporangium with a tapering base; L, constricted sporangium that failed to discharge all its zoospores.

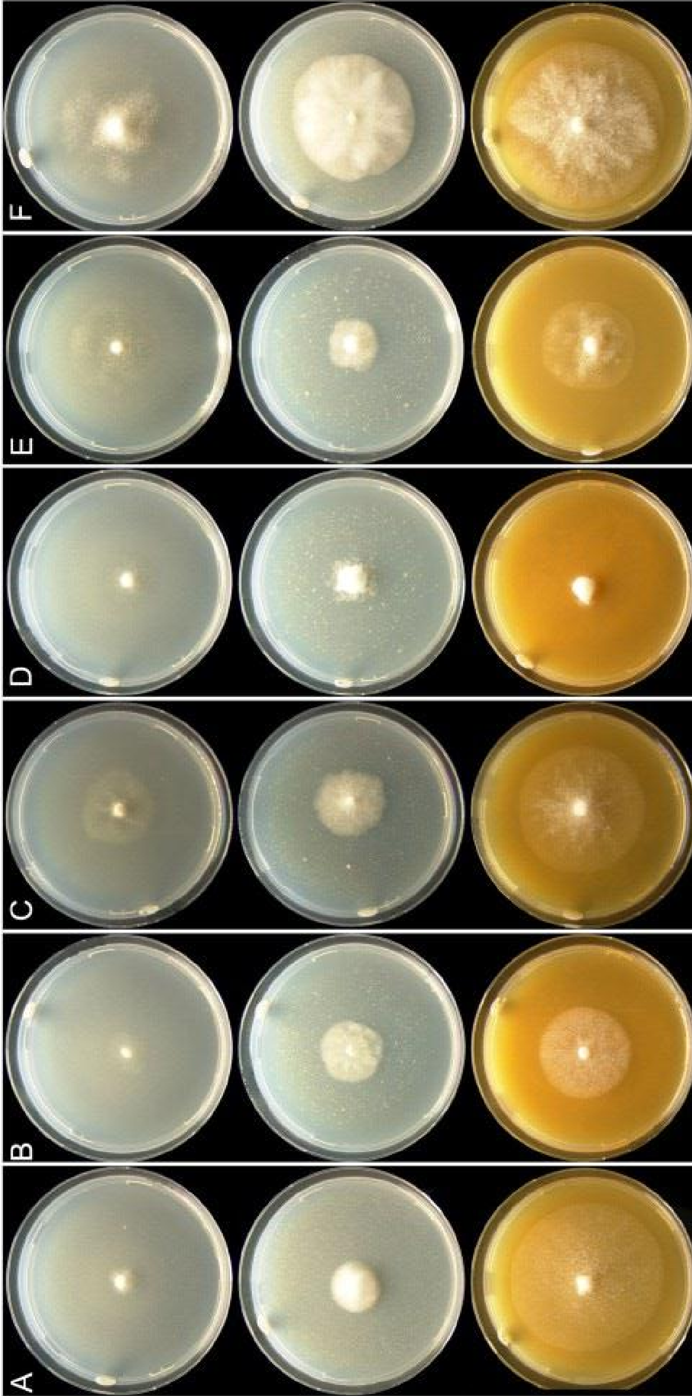

Figure 3.9: Colony morphology of the new species *P. cichorii*, *P. dauci* and *P. lactucae* compared to that of *P. porri*. Colony morphology on CMA-Oxoid (top row), PDA (middle row) and V8 agar (bottom row) after 1 wk of incubation at 18 °C; A, *P. cichorii* (CBS 115029); B, *P. dauci* (CBS 127102); C, *P. lactucae* (BPIC 1985); D, *P.* taxon castitis (CBS 688.79); E, *P.* taxon parsley (CBS 114156); F, *P. porri* (CBS 114100).

3.4 Taxonomy

In the following species descriptions, all averages are given with standard deviations (average ± SD).

Phytophthora cichorii Bertier, Brouwer, de Cock & D.E.L. Cooke, sp. nov.
MycoBank MB803102; Figure 3.4

Etymology
Named after the host plant, *Cichorium intybus* (chicory).

Description
This species is heterothallic with amphigynous, often two-celled antheridia. Antheridia measure 23.4 ± 3.5 × 19.8 ± 1.7 µm in size. Oogonia are smooth walled and 34.5 ± 4.7 µm long by 30.3 ± 2.7 µm wide; oospores are aplerotic to almost plerotic and on average 27.5 ± 2.4 µm wide. The semi-papillate sporangia are 70.3 ± 19.1 × 42.0 ± 9.4 µm in size. Hyphal swellings measure on average 25.1 ± 5.9 × 19.1 ± 3.5 µm. Chlamydospores were not observed. The minimum temperature for growth is 0 ˚C, maximum 24 ˚C and optimum temperature 18-21 ˚C. Average growth rate at 21 ˚C on CMA-Oxoid is 0.8 mm/day. No colony pattern was observed on all media examined (V8, PDA and CMA-Oxoid).

Observations on other isolates examined
Colonies on V8, CMA-Oxoid and PDA without a discernible colony pattern. The species grows very poorly on CMA-Oxoid, with diffuse colony edges. On both V8 and PDA medium, colonies have a well-defined edge. Growth on V8 was submerged, with very sparse aerial mycelium. On PDA agar colonies form a thick, cottony, aerial mycelium (Figure 3.9 A). Growth occurred from 0-24 ˚C. No growth occurred at 27 ˚C and higher. Growth rate at 21 ˚C was 0.6 to 1.1 mm/d (range of isolate means).
Phytophthora cichorii is heterothallic, antheridia are amphigynous, most antheridia are '2-celled' (Figure 3.4 A-E). Size ranges for antheridia are 16.4-37.7 × 13.2-26.4 µm, with isolate means of 23.4-27.4 × 19.8-19.8 µm. Sizes of oogonia range from 23.7-49.5 × 19.2-45.9 µm, with a range of isolate means of 34.5-37 × 30.3-35.4 µm. Oogonial wall thickness ranges from 0.6-1.8 µm with a range of isolate means of 1.0-1.2 µm. Oogonia are smooth

walled and lack ornamentation (Figure 3.4 A-E). Oospores measure 21.6-42.0 µm diam (av. 27.5 µm). The size range for the semipapillate sporangia is 30.6-142.9 × 20.7-63.1 µm, with a range of isolate means of 69.0-74.9 × 37.5-42.0 µm. Discharge pore width is 8.9-11.0 µm (range of isolate means). Larger sporangia with distorted shapes were seen but are rare. Sporangia often had thick basal plugs (Figure 3.4 M). Sporangia are usually persistent on the hyphae, but a few detached sporangia were seen. Sporangia are ovoid to obpyriform, sometimes with distorted shapes or constrictions. Sporangia with elongated necks (Figure 3.4 J) and bipapillate sporangia (Figure 3.4 I) occur occasionally. Internal proliferation was not observed. Hyphal swellings occur in water and in rare cases in agar. The size range for hyphal swellings is 16.0-56.5 × 13.5-42.3 µm with a range of isolate means of 25.1-32.2 × 19.1-26.2 µm.

Notes

Phytophthora cichorii can be distinguished from related species by ITS and *Cox1* sequence data, and morphologically by its heterothallic mating behaviour, and the occurrence of two-celled antheridia.

Specimens examined

CBS 133815, CBS 115029 (holotype, Herb. CBS H-21127 (dried culture)), CBS 114345, CBS 115030 (see Table 3.1)

Phytophthora dauci Bertier, Brouwer & de Cock, sp. nov.
MycoBank MB803103; Figure 3.5

Etymology

Named after the host plant, *Daucus carota* (carrot).

Description

The species is homothallic with paragynous antheridia measuring 16.0 ± 1.7 × 11.6 ± 1.3 µm. Oogonia are subglobose, often intercalary and 32.3 ± 2.2 × 31.6 ± 2.3 µm in size. Oospores measure on average 27.6 ± 2.2 µm; oogonium walls are smooth or wavy. Sporangia are semipapillate and 58.8 ± 11.6 × 40.1 ± 7.2 µm in size. Hyphal swellings present, on average, 30.8 ± 6.8 × 22.6 ± 5.5 µm. Chlamydospores are found on CMA-CBS agar in low numbers and are thin-walled, often slightly subglobose and measure 31.9 ± 4.1 × 30.8 ± 4.7 µm. Minimum growth temperature 0°C; maximum 21°C; optimum 12-21°C. Average growth rate at 21°C on CMA-Oxoid is 1.2 mm d^{-1}. Colonies on V8, CMA-Oxoid and PDA are without a discernible colony pattern.

Observations on other isolates examined

Colonies on V8, CMA-Oxoid and PDA are without a discernible colony pattern. The species grows very poorly on CMA-Oxoid, with diffuse colony edges. On both V8 and PDA medium, colonies have a well-defined edge. Growth on V8 was submerged, with very sparse aerial mycelium. On PDA agar colonies form a thick, cottony, aerial mycelium (Figure 3.9 B). Growth occurred from 0-21°C. No growth occurred at 24°C and higher. Growth rate at 21°C was 0.8 to 1.0 mm/d (range of isolate means). Antheridia are paragynous and mostly diclinous; size ranges are 12.1-21.9 × 8.5-16.8 µm, with a range of isolate means of 16.0-17.1 × 11.6-12.5 µm. Intercalary antheridia are quite common. *Phytophthora dauci* is homothallic. Compared to other clade 8b species, oogonium production is very low. Oogonia were produced in CMA-CBS agar and were sparsely and evenly distributed through the medium. Oogonium production did not increase when paired with A1 or A2 strains. Oogonia often had wavy walls, but smooth walls also occurred (Figure 3.5 A-E). The size range for oogonia was 25.3-37.2 × 25-37.2 µm, with a range of means of 31.9-32.3 × 31.2-31.6 µm. Oogonia are often found in intercalary positions in

the hyphae, which in many cases could be easily mistaken for laterally attached oogonia, as the subtending and outgrowing hyphae are usually close together on the same side of the oogonium (Figure 3.5 B). Oogonium wall thickness ranges from 0.6-1.8 µm with a range of isolate means of 1.0-1.1 µm. Oospore diameter ranges from 20.6-30.6 µm, with average diameters of 26.3-27.6 µm for the tested isolates. Oospore wall thickness ranges from 0.6-1.9 µm, with a range of isolate means of 1.1-1.3 µm. Sporangium size ranges from 27.4-89.9 × 24-56.2 µm with a range of isolate means of 52.6-58.8 × 37.2-40.1 µm. Discharge pore width ranges from 6.5-11.7 µm (av. 9.1 and 9.5 µm for the two isolates). Hyphal swellings in agar cultures range in size from 17.1-59.9 × 14.3-41.4 µm, with a range of isolate means of 30.3-30.8 × 22.6-25.1 µm. Chlamydospores are quite rare, they were occasionally found in low numbers in CMA-CBS agar cultures. The size range of chlamydospores is 23.7-57.4 × 20.9-56.0 µm(range of isolate means 31.9-38.3 × 30.8-37.2 µm).

Notes

Phytophthora dauci can be distinguished from other related species by its low maximum temperature for growth, and ITS and *Cox1* sequence data.

Specimens examined

CBS 114039, CBS 127102 (holotype, Herb. CBS H-21128 (dried culture)) (see Table 3.1).

Phytophthora lactucae Bertier, Brouwer & de Cock, sp. nov.
MycoBank MB803104; Figure 3.6

Etymology

Named after the host plant, *Lactuca sativa* (lettuce).

Description

Phytophthora lactucae is homothallic, antheridia are mostly paragynous, but amphigynous antheridia occur as well. Paragynous antheridia are 16.1 ± 2.1 × 10.5 ± 1.1 µm in size. Antheridia were mostly diclinous or distantly monoclinous. The subglobose oogonia averaged 33.5 ± 4.0 × 33.0 ± 4.0 µm in size. Oospores measure on average 28.2 ± 3.8 µm. Sporangia are semipapillate and ovoid to ellipsoid or irregular in shape and on average 55.5 ± 12.3 × 35.5 ± 7.1 µm in size. Hyphal swellings averaged 27.6 ± 5.4 × 23.8 ± 5.7 µm. The minimum temperature for growth on CMA-Oxoid is 0°C, and the maximum 24°C. Optimum temperature is 21°C. Average growth rate at 21°C on CMA-Oxoid is 3.1 mm/d. Colonies on V8 had a faint chrysanthemum pattern; no discernible pattern on V8 or CMA-Oxoid.

Observations on other isolates examined

Colony morphology is somewhat variable, colonies on CMA-Oxoid have no discernible pattern (Figure 3.9). Colonies have sparse aerial or submerged mycelium. Colony patterns on PDA are more variable and range from no discernible pattern to chrysanthemum with dense, velvety mycelium. Colony edges were either sharp or vague. Colonies on V8 have no discernible pattern or a very faint chrysanthemum pattern. Aerial mycelium is sparse and fluffy to velvety (Figure 3.6 C). Growth occurred from 0-24°C. No growth occurred at 27°C and higher. Growth rate at 21°C was 0.7 to 2.1 mm/d (range of isolate means). *Phytophthora lactucae* is homothallic. Antheridia are predominantly paragynous, but some amphigynous antheridia occur in all isolates tested. Paragynous antheridia are 10.7-25.2 × 7.5-16.5 µm in size with isolate means of 16.1-17.5 × 9.8-11.9 µm. Oogonia are subglobose, measuring 22-44.1 × 21-44.1 µm, with a range of isolate means of 33.5-36.4 × 33.0-36.4 µm. Oogonium wall thickness varies from 0.6-2.7 µm with a range of isolate means of 1.3-1.7 µm. Oospore diameter is 19.0-38.7 µm, with isolate means of 27.1-31 µm. Oospore wall thickness varies from 0.5-2.5 µm, with isolate means of 1.1-1.6 µm.

The semipapillate sporangia are mostly ovoid in shape, but ellipsoid, slightly obpyriform and asymmetrical shapes are also found (Figure 3.6 J-N). Tapered bases are rare. Sizes ranged from 27.6-101.3 × 18.3-53.5 µm, with isolate means of 55.3-61.1 × 35.5-38.5 µm. Discharge pore width ranged from 6-15.5 µm with isolate means of 9.5-10.3 µm. Hyphal swellings are produced in agar and in water cultures, occur in sparse clusters and are globose to somewhat angular or irregular in shape (Figure 3.6 F-H). Hyphal swellings are 14.3-49.8 × 10.4-39.3 µm in size with isolate means of 26-31.6 × 21.8-26.4 µm. Chlamydospores are rare and measure 22.0-51.4 × 20.4-52.4 µm, with isolate means of 31.7-37.2 × 29.7-35.3 µm. Only isolates BPIC 1987 and BPIC 1988 produced chlamydospores occasionally in larger numbers, for other isolates only a few chlamydospores were seen. Chlamydospores were thin-walled.

Notes

Phytophthora lactucae is morphologically similar to other homothallic species from clade 8b; it can be distinguished from *P. brassicae* by antheridial type, with *P. brassicae* having predominantly amphigynous antheridia instead of paragynous antheridia; from *P. dauci* by the lower maximum temperature for growth of the latter species; from *P. primulae* by the higher growth rate and maximum temperature for growth of that species. *P. lactucae* can also be distinguished from all other clade 8b isolates based on ITS and *Cox1* sequence data.

Specimens examined

BPIC 1985 (holotype, Herb. CBS H-21129 (dried culture)),BPIC 1986, BPIC 1987, BPIC 1988, BPIC 1991, BPIC 1992 (see Table 3.1).

***Phytophthora* taxon castitis**

Figure 3.7

Etymology

From the Latin 'castita' meaning chastity; referring to the sexual dormancy of the isolates.

Description

Two isolates are available for this potential new species, one from strawberry and one from carrot (see Table 3.1). For both isolates, the minimum temperature for growth was 0°C; the optimum growth temperature was 18-24°C. No growth occurred at 30°C or higher. Isolate CBS 688.79 from carrot is characterized by very abundant production of chlamydospores, both in agar and in water cultures (Figure 3.7 C,E). Chlamydospores are globose and very large, ranging from 29.1-73.2 µm diam with an average of 51.4 µm. Isolate CBS 131246 produced smaller chlamydospores, with an average of 36.4 µm. They can be intercalary or terminal. Hyphal swellings ranged in size from 14.6-61.8 × 12.6-52.1 µm (av. 32.5 × 28.8 µm). Sporangia were only sporadically produced and were 23.6-97.9 × 14.9-49.5 µm in size (av. 50.8-55.4 × 32.6-35.6 µm). In case of CBS 688.79, sporangia were often found at the end of long chains of hyphal swellings, or swollen, constricted hyphae. In many cases, only the very terminal tip of these chains of swellings would contain cytoplasm. During development of these catenulate swellings, in some cases septa are formed, delineating the subtending, empty part of the hypha from the living part as the cytoplasm moves along with the growing tip. This leaves behind a long string of empty hyphal swellings (Figure 3.7 B,D). These chains of swellings have also been described for *P. primulae*, and are quite common in that species (Tomlinson, 1952). Neither isolate produced oogonia in single culture or when mated with other strains in our study.

***Phytopthhora* taxon parsley**

Figure 3.8

Description

Two *P. primulae*-like isolates were available from parsley. Minimum growth was 0°C. Optimum growth was between 15-21°C. The maximum temperature for growth was 24°C. The morphology of these isolates was mostly similar to the *P. primulae* isolates. A notable difference was the ratio of paragynous to amphigynous anteridia. These were produced in roughly equal amounts in the isolates from parsley, while *P. primulae* was originally described as having only paragynous antheridia (Tomlinson, 1952). Both examined isolates from primrose (CBS 116663 and CBS 114346) did indeed produce mostly paragynous antheridia, but a few amphigynous antheridia were found in both isolates. Other morphological characters did not differ much between the parsley and *P. primulae* isolates. Sporangial shapes for the parsley isolates varied from ovoid and ellipsoid to elongated with distorted shapes, often with constrictions (Figure 3.8 I-N). Sporangia can, but do not always, have a tapered base. The average size for sporangia was 52.8-54.3 × 29.4-30.6 µm with a size range of 25.2-107.7 × 17.8-46.5 µm. Discharge pores of empty sporangia ranged in diameter from 5.2-10.7 µm; averages for the two isolates were 7.9-8.0 µm. Larger sporangia may occur as larger, already discharged sporangia were found that could no longer be measured accurately. One of the typical features of *P. primulae* is chains of constricted hyphal swellings, like those that are described above for *P.* taxon castitis (Figure 3.8 F-H). These were rare but were seen on a few occasions and were also observed in one of the parsley isolates by Elena et al. (2008). Hyphal swellings size ranges were 11.3-61.5 × 10.3-38.7 µm. The average size for CBS 114156 was 24.1 × 19.8 µm and for BPIC 2584, 28.1 × 20.7 µm. Chlamydospores were not observed. Oogonia measured 24.3-44.7 × 23.4-42.6 µm with a range of isolate means of 34.4-37.6 × 33.9-37.3 µm. Oospores were 19.4-37.3 µm in diameter with isolate means of 28.9 µm and 30.9 µm for the two isolates. Both isolates produced amphigynous and paragynous antheridia in roughly equal amounts. Paragynous antheridia were 11.8-30.7 × 8.5-18.3 µm in size. Amphigynous antheridia ranged from 10.2-21.3 × 10.0-16.9 µm. The isolate means were 18.1-19.3 × 13.2-14 µm and 15.8-16.5 × 13.6-14.2 µm, respectively for the two aforementioned antheridia types.

3.5 Discussion

Our publication provides further evidence for the revised structure of *Phytophthora* clade 8 as proposed by Grünwald et al. (2011). All isolates discussed in this paper are closely related to the three known clade 8b species *P. porri*, *P. brassicae* and *P. primulae* and together they form a distinct clade from the newly raised clade 8d. Moreover, a difference in host-plant preference is clear. The species of clade 8d are all pathogens of woody perennials, while the species in clade 8b are pathogens of herbaceous plants. The description of *P. cichorii*, *P. dauci* and *P. lactucae* brings the total number of clade 8b species to six. The *P.* taxon castitis and the *P.* taxon parsley isolates may represent additional new species, but formal description of these taxa would be premature. In the case of the *P.* taxon parsley isolates, difference in growth rate, maximum temperature for growth and ratio of amphigynous and paragynous antheridia were found when compared to *P. primulae* isolates. Elena et al. (2008) also found evidence for differences in host range. The genetic differences between the parsley isolates and isolates from primrose, however, are small. A study involving more isolates, or a more in-depth study of gene flow between the two groups of isolates may offer more insight into the taxonomic relationship between the *P.* taxon parsley and *P. primulae* isolates. In the case of *P.* taxon castitis, there is also genetic and morphological evidence that these isolates could be considered a separate species. Sporulation of this species, however, was very poor. Only a few sporangia were produced, and gametangia were not observed. More data, if possible using fresh isolates from the field, are needed if the species is to be properly described.

Phytophthora cichorii is the only clade 8b species known to have a heterothallic mating system. Heterothallic species produce oospores when isolates of compatible mating type of the same or different species are paired in cultures. Two mating types are known, A1 and A2. Heterothallic species do not require exchange of genetic material with the mating partner for successful formation of oospores, after induction of gametangium formation by mating hormone from the mating partner all oospores may be formed by selfing without further interaction with the other isolate (Ko, 1978; Judelson, 2007). *Phytophthora cichorii* gametangia in our study were formed by pairing isolates with compatible isolates

of *P. capsici* using the polycarbonate filter technique developed by Ko (1978). *Phytophthora capsici* belongs to clade 2, and is unrelated to the clade 8b species. The reason for our use of strain *P. capsici* CBS 128.23 is that it was found to be a good mating partner for a wide variety of A1 strains from different phylogenetic clades (Brouwer *et al.*, unpublished data).

Oomycete strains may degenerate when kept under artificial conditions for a prolonged period of time. Strains of clade 8b seem to be especially likely to develop such problems. During this study, several *P. brassicae* and *P. porri* strains studied by De Cock et al. (1992); Man in 't Veld et al. (2002) were considered. Some of these no longer produced oogonia, or only produced aborted oogonia, often with distorted shapes. The failure of several isolates belonging to the new species presented in this paper to produce oogonia is likely to be caused by this phenomenon and should not be taken as evidence of (partial) sterility of these species, as has been reported for other *Phytophthora* spp. (Jung et al., 2011). The lack of gametangium formation by some isolates may also depend on other poorly understood factors. One of the *P.* taxon castitis isolates (CBS 688.79) has been studied quite intensively in the past. This isolate failed to produce gametangia when it was originally isolated (Stelfox and Henry, 1978), and in an earlier study by De Cock et al. (1992) and as mentioned above, also failed to produce oogonia in our study, both in single culture and in mating tests. Ho (1983), however, could induce oogonium formation by irradiating a young culture with x-rays. The irradiated culture produced oogonia just before the culture completely dried out, after four months of storage.

There are more cases where *Phytophthora* isolates that appear sterile may produce oogonia under specific conditions. *Phytopthhora thermophila* isolates failed to produce oogonia in single culture or mating test, but one isolate produced oogonia in single culture when flooded with non-sterile soil filtrate (Jung et al., 2011). These cases provide evidence that isolates or species that appear sterile can actually have a functioning, but dormant sexual system. Gametangium formation in such species may be triggered by internal or external conditions that are poorly understood. The same could be true for other species that are, up to now, considered to be sexually sterile. Elucidation of pathways and genes involved in gametangium formation combined with information from genome sequencing projects may provide another way of determining the sexual status of *Phytophthora* species.

With the discovery of these new species, some interesting common ecological features of this clade are becoming clear. One is their ability to persist at very low temperatures. All isolates studied have a minimum growth temperature of $\leq$ 3°C, most isolates even show some growth at 0°C. Moreover, optimum and maximum growth temperatures are among the lowest observed in the whole genus (`http://www.q-bank.eu`), making them well adapted to infect winter-grown plants.
A second feature is their slow growth. The new species characterized in this study have maximum growth rates between 0.5 and 2.8 $\mathrm{mm\,d^{-1}}$. This slow growth rate makes it very hard to isolate these species from diseased plants, since they are easily overgrown by secondary pathogens. These secondary pathogens can cause a lot of problems, but are not the primary cause. Moreover, secondary pathogens can be mistakenly treated as being the primary cause and as a result, the disease will not be effectively managed. Because these species are not easy to isolate, it is likely that the presence these *Phytophthoras* in the field is underestimated. Another interesting feature is host specificity. The new species described here have so far only been isolated from their respective host plants, lettuce, chicory and carrot, indicating host plant driven speciation. For *P. lactucae*, pathogenicity tests confirmed this host specificity (Elena et al., 2006). Speciation influenced by geographic isolation is less likely since for all species in clade 8b, isolates have been found in different countries and in most cases even in different continents. An exception is *P. lactucae*, for which only isolates from Greece are available. Another culture isolated from lettuce in Australia existed (BRIP 15683; Sitepu and Bumbieris (1981)) but this isolate has been lost.

Combining all this information, we can conclude that *Phytophthora* clade 8b is a clade containing cold-loving species, representing a group of pathogens specifically adapted to cause disease at low temperatures on a range of important agricultural crops, mostly vegetables.

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Author contributions

Lien Bertier performed DNA extractions, PCR, sequencing, phylogenetic analysis and part of the growth/temperature experiments. Henk Brouwer performed all microscopy, morphological measurements and most of the growth/temperature experiments.

Everything should be made as simple as possible, but not simpler.

Albert Einstein

4

Host adaptation and speciation through hybridization and polyploidy in *Phytophthora*

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Abstract

It is becoming increasingly evident that interspecific hybridization is a common event in *Phytophthora* evolution. Yet, the fundamental processes underlying interspecific hybridization and the consequences for its ecological fitness and distribution are not well understood. We studied hybridization events in *Phytophthora* clade 8b. This is a cold-tolerant group of plant pathogenic oomycetes in which six host-specific species have been described that mostly attack winter-grown vegetables. Hybrid characterization was done by sequencing and cloning of two nuclear (ITS and *Ypt1*) and two mitochondrial loci (*Cox1* and *Nadh1*) combined with DNA content estimation using flow cytometry. Three different mtDNA haplotypes were recovered among the presumed hybrid isolates, dividing the hybrids into three types, with different parental species involved. In the nuclear genes, additivity, i.e. the presence of two alleles coming from different parents, was detected. Hybrid isolates showed large variations in DNA content, which was positively correlated with the additivity in nuclear loci, indicating allopolyploid hybridization followed by a process of diploidization. Moreover, indications of homeologous recombination were found in the hybrids by cloning ITS products. The hybrid isolates have been isolated from a range of hosts that have not been reported previously for clade 8b species, indicating that they have novel pathogenic potential. Next to this, DNA content measurements of the non-hybrid clade 8b species suggest that polyploidy is a common feature of this clade. We hypothesize that interspecific hybridization and polyploidy are two linked phenomena in *Phytophthora*, and that these processes might play an important and ongoing role in the evolution of this genus.

4.1 Introduction

Phytophthora is a genus of plant pathogenic filamentous oomycetes containing more than one hundred species. Virtually all of them are plant pathogens causing many important plant diseases worldwide, such as potato late blight, sudden oak death and forest dieback caused by *Phytophthora infestans*, *Phytophthora ramorum* and *Phytophthora cinnamomi*, respectively. Morphologically, oomycetes are very similar to the filamentous Fungi. Therefore, they used to be classified as Fungi. Only during the last few decades, DNA and whole genome sequencing revealed that they have a completely different evolutionary origin but have adopted similar morphology and infection strategies through convergent evolution and horizontal gene transfer. Oomycetes differ from Fungi in some important morphological and biochemical aspects and in the fact that they are diploid in their vegetative lifestyle, whereas most Fungi are monoploid (see Table 2.1 and Beakes et al. (2012)). *Phytophthora* clade 8b contains a group of pathogens specifically adapted to cause disease at low temperatures in a range of important agricultural crops, mostly winter grown vegetables. A previous genetic diversity study of *Phytophthora* clade 8b isolates from around the world resulted in the official description of three new clade 8b species (Chapter 3). This was based on differences in the nuclear rDNA internal transcribed spacer (ITS) and mtDNA cytochrome oxidase I (*Cox1*) barcoding sequences (Robideau et al., 2011), morphological and physiological differences, and host preference. The clade now consists of six host-specific species, namely *P. porri*, *P. primulae* and *P. brassicae* and the newly described *P. dauci*, *P. cichorii* and *P. lactucae*, respectively causing disease in leek, primrose, cabbages, carrot, chicory and lettuce. Two additional taxa were described, which have been tentatively named *P.* taxon castitis (on carrot and strawberry) and *P.* taxon parsley (on parsley), as insufficient data could be collected for an official species description.

During this previous study, we detected intra-isolate sequence polymorphisms in the ITS region in 12 isolates, which were mainly isolated from *Allium* species. These sequence polymorphisms point to additivity, which is a distinctive feature of interspecific hybridization. Therefore, we decided to study the possible hybridity of these isolates using different techniques, which laid the foundation of this work. Natural interspecific hybridization has already been reported several times in the genus *Phytophthora* (Bonants et al., 2000; Brasier et al., 2004, 1999; Goss et al., 2011; Hurtado-Gonzales et al., 2009; Man In 't Veld

et al., 1998; Nagel et al., 2013; Nirenberg et al., 2009). Next to this, synthetic hybrids have repeatedly been created in the lab (Donahoo et al., 2008; Ersek et al., 1995; Goodwin and Fry, 1994). In *Phytophthora*, the fundamental processes underlying interspecific hybridization and the consequences for its ecological fitness and distribution are not well understood. In plants, however, it is known that hybridization and polyploidy are important evolutionary processes contributing to adaptation and speciation and form the basis of the complete (angiosperm) biodiversity known today (Soltis and Soltis, 2009).

One of the main pathways of (angiosperm) speciation is believed to involve a polyploid phase, mostly occurring as a consequence of hybridization. In a hybridization event, (slightly) diverged chromosome sets are brought together in one nucleus. This causes unstable meiosis and in most cases abortion of the zygote. Fertility can be restored by a whole genome duplication event, creating again compatible chromosome sets. The resulting allopolyploid will then harbor the complete genomes of both parents. Although allopolyploids can be stable over long periods of time, it is well documented that most (allo)polyploids evolve back to a diploid state through the process of diploidization (Comai, 2005). Diploidization can be defined as the transitioning of a polyploid organism back to the more stable diploid state. This can be accompanied by chromosome number reduction, although this is not necessarily the case. Processes that contribute to diploidization include subfunctionalization and/or neofunctionalization causing differentiation between redundant gene copies; large scale chromosomal rearrangements such as translocations; chromosome loss, etc. In allopolyploids, an important process that can create new, advantageous gene combinations and phenotypes is homeologous recombination, i.e. recombination between chromosomes of different parental origin (Gaeta and Pires, 2010).

Polyploidy has already been shown in several *Phytophthora* species (Sansome et al., 1991; Sansome and Brasier, 1974; Sansome, 1977). In *P. infestans*, Sansome (1977) showed that British isolates contained approximately twice the number of chromosomes compared to isolates from Mexico, the species' center of origin. She hypothesized that *P. infestans* might exist in the tetraploid condition in temperate regions, and that the higher ploidy levels might enable the pathogen to adapt to cooler environments. This initiated a DNA content screening of *P. infestans* populations in many countries, using cytophotometric methods. Indeed, isolates from Mexico were found to contain much lower DNA contents

compared to isolates from other regions (Tooley and Therrien, 1989), supporting Sansome's hypothesis.
With the advent of the genomic era around the year 2000, research efforts aimed at understanding polyploidy in *Phytophthora* diminished. However, in 2010, a new study with recent *P. infestans* field isolates analyzed using flow cytometry showed large DNA content variation and heterokaryosis (Catal et al., 2010). Moreover, by analysis of the genomes of *P. infestans*, *P. ramorum* and *P. sojae* using bio-informatics, remnants of an ancient polyploidization event were detected. Most likely, a common ancestor of these species has undergone a whole genome duplication that might have played a role in the evolution and pathogenic success of *Phytophthora* pathogens (Martens and Van de Peer, 2010).

In this chapter, we describe three different types of interspecific hybrids in *Phytophthora* clade 8b, as well as the occurrence of polyploidy as a common feature of the clade. We discuss a potential link between polyploidy and past hybridization events and the role that both events could play in host adaptation and speciation of *Phytophthora* pathogens. The implications of these phenomena for *Phytophthora* research are discussed.

4.2 Materials and methods

4.2.1 Isolate collection and maintenance

All isolates used in this study are listed in Table 4.1. The isolates were freshly isolated from diseased plants or obtained from different culture collections around the world. Thirty-one of these isolates have been used previously in a genetic diversity study of *Phytophthora* clade 8b (see Chapter 3). The isolates were maintained routinely on V8 agar (see section 3.2.1) or on Corn Meal Agar (Beckton Dickinson). For long term storage, isolates were kept on V8 plugs at -80°C in 10% glycerol.

Table 4.1: Isolates used in this study

Species	Code	Alternative codes	Host	Origin	Year of isolation	*CoxI*	*Nadh1*	ITS	Ypt1
Phytophthora porri	CBS 802.95	PD 92/214	*Allium porrum*	Netherlands	1992	KC478717	-	KC478747	-
	CBS 114100	-	*Allium porrum*	Denmark	1992	KC478718	KF882685	KC478748	KF882633
	CBS 116662	Smilde GG	*Allium porrum*	UK	1994	KC478719	-	KC478749	-
	CBS 127099	K06006(2)	*Allium porrum*	Belgium	2006	KC478720	KF882686	KC478750	KF882634
	CBS 127101	S05029(1)	*Allium porrum*	Belgium	2005	KC478721	KF882687	KC478751	KF882635
	CBS 181.87	-	*Allium porrum*	Netherlands	1987	-	-	FJ643565	-
	CBS 127098	B06005(1)	*Allium porrum*	Belgium	2006	KF882609	KF882688	FJ643553	KF882636
	S05014(1)	-	*Allium porrum*	Belgium	2005	KF882610	KF882689	FJ643551	KF882637
	B06011	-	*Allium porrum*	Belgium	2006	KF882611	KF882690	-	KF882638
	S05007(2)	-	*Allium porrum*	Belgium	2005	KF882612	KF882691	-	KF882639
	S05017(2)	-	*Allium porrum*	Belgium	2005	KF882613	KF882692	-	KF882640
	K05020(2)	-	*Allium porrum*	Belgium	2005	KF882614	KF882693	-	KF882641
	K05025(1)	-	*Allium porrum*	Belgium	2005	-	-	-	-
	B06008	-	*Allium porrum*	Belgium	2006	-	-	-	-
	S05009	-	*Allium porrum*	Belgium	2005	-	-	-	-
	K07015(4)	-	*Allium porrum*	Belgium	2007	-	-	-	-
	S05012(2)	-	*Allium porrum*	Belgium	2005	-	-	-	-
	K07015(2)	-	*Allium porrum*	Belgium	2007	-	-	-	-
	S12001	-	*Allium porrum*	Belgium	2012	-	-	-	-
Phytophthora primulae	CBS 110167	BBA 71108	*Primula eliator*	Germany	1999	KC478722	KF882694	KC478752	KF882642
	CBS 116663	PD 99/2429	*Primula sp.*	Netherlands	1999	KC478723	KF882695	KC478753	KF882643
	CBS 114346	LYN 916-A	*Primula polyantha*	New Zealand	2003	KC478724	KF882696	KC478754	KF882644
	CBS 110162	BBA 70403	*Primula sp.*	Germany	1997	KC478725	KF882697	KC478755	KF882645
	CBS 620.97	PD 97/875	*Primula acaulis*	Germany	1997	KC478726	KF882698	KC478756	KF882646
	CBS 110165	-	*Primula sp.*	Germany	1998	KF882615	KF882699	KF882684	KF882647
Phytophthora taxon parsley	BPIC 2584	-	*Petroselinum crispum*	Greece	2006	KC478727	KF882700	KC478757	KF882648
	CBS 114156	-	*Petroselinum crispum*	Australia	2003	KC478728	KF882701	KC478758	KF882649
Phytophthora taxon castitis	CBS 688.79	P3827	*Daucus carota*	Canada	1978	KC478729	KF882702	KC478759	KF882650
	CBS 131246	CH112	*Fragaria x ananassa*	Sweden	1995	KC478730	KF882703	KC478760	KF882651
Phytophthora dauci	CBS 127102	BorfSP370	*Daucus carota*	France	2009	KC478731	KF882704	KC478761	KF882652
	CBS 114039	-	*Daucus carota*	Australia	2003	KC478732	KF882705	KC478762	KF882653

Phytophthora brassicae	CBS 782.97	Smilde HH	*Brassica chinensis*	Netherlands	1994	KC478733	KF882706	KC478763	KF882654
	CBS 212.82	P3273	*Brassica oleraceae*	Netherlands	1982	KC478734	KF882707	KC478764	KF882655
	CBS 113350	PD 94/166	*Brassica oleraceae*	Netherlands	1994	KC478735	KF882708	KC478765	KF882656
	CBS 112277	ICMP 14271	*Brassica oleraceae*	New Zealand	2001	KC478736	KF882709	KC478766	KF882657
	CBS 127274	B10001	*Brassica oleraceae*	Belgium	2010	KC478737	KF882710	KC478767	KF882658
	K13001	-	*Brassica oleraceae*	Belgium	2013	KF882616	KF882711	-	KF882659
	CBS 179.87	-	*Brassica oleraceae*	Netherlands	1987	KF882617	AY564025	AF380148	KF882660
	CBS 112967	-	*Brassica oleraceae*	UK	-	-	-	KF882682	-
	CBS 686.95	-	*Brassica oleraceae*	Netherlands	1995	KF882618	KF882712	AF380149	KF882661
	CBS 113352	-	*Brassica oleraceae*	Netherlands	1995	KF882619	KF882713	-	KF882662
Phytophthora lactucae	BPIC 1985	-	*Lactuca sativa*	Greece	2001	KC478738	KF882714	KC478768	KF882663
	BPIC 1988	-	*Lactuca sativa*	Greece	2002	KC478740	KF882715	KC478770	KF882664
	BPIC 1992	-	*Lactuca sativa*	Greece	2003	KC478742	KF882716	KC478772	KF882665
Phytophthora cichorii	CBS 115029	-	*Cichorium intybus*	Netherlands	2004	KC478743	KF882717	KC478773	KF882666
	CBS 114345	-	*Cichorium intybus*	Netherlands	2003	KC478744	KF882718	KC478774	KF882667
	CBS 115030	-	*Cichorium intybus*	Netherlands	2004	KC478745	KF882719	KC478775	KF882668
	CBS 133815	SCRACE5388	*Cichorium intybus*	UK	1999	KC478746	KF882720	KC478776	KF882669
Hybrid (type 1)	ICMP14653	-	*Allium cepa*	New Zealand	2002	KF882620	KF882721	KF882683	KF882670, KF882671
	CBS 114040	-	*Pastinaca sativa*	Australia	-	KF882621	KF882722	Yes	KF882672
	CBS 139.87	NBRC 30417, P6500	*Allium grayi*	Japan	1977	KF882622	KF882723	Yes	Yes
	CBS 112966	-	*Chrysanthemum sp.*	UK	-	KF882623	KF882724	Yes	Yes
	P6817	ADC 10.015	*Allium cepa*	Australia	1980	KF882624	KF882725	Yes	Yes
	CBS 140.87	NBRC 30418, P7516	*Allium cepa*	Japan	1977	KF882625	KF882726	Yes	Yes
	CBS 114101	P6815	*Parthenium argentatum*	Australia	1983	KF882626	KF882727	Yes	Yes
	CBS 138.87	NBRC 30416, P6499	*Allium cepa*	Japan	1977	KF882627	KF882728	Yes	KF882673, KF882674
	CBS 126739	MAFF237666, TAC 97-13, NBRC 32965	*Allium cepa*	Japan	1997	KF882628	KF882729	Yes	Yes
	CBS 126738	MAFF 237665, TAC 97-5, NBRC 32964	*Allium victorialis*	Japan	1997	KF882629	KF882730	Yes	Yes
	CBS 126737	MAFF 237664, TAC 97-1, NBRC 32963	*Allium victorialis*	Japan	1997	KF882630	KF882731	Yes	Yes

Hybrid (type 2)	CBS 112969	ADC 03.38	*Allium cepa*	USA	-	KF882631	KF882732	Yes	KF882675, KF882676
Hybrid (type 3)	CBS 112968	P6207	*Allium cepa*	Switzerland	-	KF882632	KF882733	Yes	KF882677, KF882678
Phytophthora syringae	CBS 364.52	-	*Prunus armeniaca*	New Zealand	1952	HQ708406	AY564030	-	KF882679
	CBS 114110	-	Almond	Australia	2004	HQ708407	KF882734	FJ643562	KF882680
	CBS 110161	-	*Rhododendron*	Germany	1995	HQ708410	KF882735	FJ643561	KF882681

4.2.2 DNA isolation, PCR, cloning and sequencing

Phytophthora isolates were grown in clarified V8 broth (100 ml of clarified V8 juice, 1 g $CaCO_3$, 900 ml of sterile water), for 7-10 days at 15°C in the dark. The mycelial mats were harvested by filtration, blotted dry, frozen in liquid nitrogen and pulverized using mortar and pestle. DNA was extracted using Qiagen's DNeasy Plant Mini Kit (Hilden, Germany). The primers used in this study are shown in Table 4.2. PCR reactions for the nuclear ITS and *Ypt1* regions were performed in a 25 µl mix containing 2.5 µl 10× PCR buffer (Qiagen), 0.5 µl dNTPs (10 mM, Promega), 1 µl of each primer (10 uM), 0.15 µl Taq polymerase (5U/µl; Promega), 17.85 µl milli-Q water and 2 µl of DNA template (25 ng μl^{-1}).
For the mitochondrial genes (*Cox1* and *Nadh1*), the 25 µl reaction mixture contained 2.5 µl 10× PCR buffer (Qiagen), 3.5 µl $MgCl_2$ (25 mM, Qiagen), 0.5 µl dNTPs (10 mM, Promega), 0.5 µl of each primer (10 µM), 0.15 µl Taq polymerase (5U/µL; Promega), 15.35 µl milli-Q water and 2 µl of DNA template (25 ng μl^{-1}). The amplifications were done in a Flexcycler PCR Thermal Cycler (Analytikjena). For ITS and *Ypt1* the following program was used: initial denaturation for 10 min at 94°C; 35 cycles of denaturation for 1 min at 94°C; annealing for 1 min at 60°C; extension for 1 min at 72°C; final extension for 10 min at 72°C. For the mtDNA genes, another program was used: initial denaturation for 10 min at 94°C; 40 cycles of denaturation for 1 min at 94°C; annealing for 30 sec at 52°C; extension for 1 min at 72°C; final extension for 10 min at 72°C. To reduce the impact of PCR mediated recombination (Cronn et al., 2002) in the ITS region as was detected in our study, an improved PCR protocol was designed following the instructions suggested by Lahr and Katz (2009). More specifically, a new forward primer (ITSPA) was designed to reduce the size of the template to around 350 bp, now containing only the last 5 polymorphic base pairs between the *P. porri* and *P. primulae*/*P.* taxon parsley ITS sequences (Figure 4.2 B). Moreover, the amount of template was reduced to the absolute minimum needed for sufficient amplification and a proofreading polymerase (Phusion, NEB) was used. The reaction was performed in a 40 µl mix containing 8 µl Phusion HF buffer (5×, NEB), 0.8 µl dNTPs (10 mM, Promega), 1 µl of each primer (10 mM), 0.4 µl Phusion polymerase (20 U/mL, NEB), 27.8 µl milli-Q water and 2 µl of DNA template (0.2 ng μl^{-1}). The thermal cycler was programmed as follows: initial denaturation for 30 sec at 98°C; 30 cycles of denaturation for 10 sec at 98°C; annealing for 30 sec at 55°C;

extension for 20 sec at 72°C; final extension for 10 min at 72°C.
All PCR samples were held at 4°C before analysis by gel electrophoresis on 1% TAE gels containing ethidium bromide and evaluation under UV fluorescence. Before sequencing, PCR products were purified using the QiaQuick PCR Purification Kit (Qiagen). Both strands were sequenced by LGC Genomics (Berlin), using the same primers as for PCR. Cloning of ITS sequences was done using the CloneJET^{TM} PCR Cloning Kit (Fermentas). Ten to twenty clones per isolate were sequenced for analysis. All newly generated sequence data have been submitted in GenBank (KF882609 - KF882735).

Table 4.2: List of primers used in this study

Locus	Primer (F or R)	Sequence (5' - 3')	Reference
ITS	ITS1 (F)	TCC GTA GGT GAA CCT GCG G	White et al. (1990)
	ITS4 (R)	TCC TCC GCT TAT TGA TAT GC	White et al. (1990)
	ITSPA (F)	TTG TGG AGG CTG CCT GTA TG	This study
Ypt1	Ypt1F (F)	CGA CCA TYG GYG TKG ACT TT	Schena and Cooke (2006)
	Ypt5R (R)	GCA GCT TGT TSA CGT TCT CR	Schena and Cooke (2006)
Cox1	Oom-CoI-Lev-up (F)	TCA WCW MGA TGG CTT TTT TCA AC	Bala et al. (2010)
	FM-85-mod (R)	RRH WAC KTG ACT DAT RAT ACC AAA	Bala et al. (2010)
Nadh1	NADHF1 (F)	CTG TGG CTT ATT TTA CTT TAG	Kroon et al. (2004)
	NADHR1 (R)	CAG CAG TAT ACA AAA ACC AAC	Kroon et al. (2004)

4.2.3 Phylogenetic analysis

Alignments for the nuclear *Ypt1* and for the two mitochondrial (*Cox1* and *Nadh1*; concatenated) sequences, were made using MUSCLE (`http://www.ebi.ac.uk/Tools/msa/muscle/`), and manually edited afterwards using BioEdit. The two mtDNA genes represent about 4% of the mitochondrial genome, taking *P. ramorum* (clade 8c) as a reference for the mitochondrial genome size (Martin et al., 2007). Phylogenetic analysis was done with the Maximum Likelihood algorithm using MEGA 5.2 (Tamura et al., 2011). Model testing was done using the software implemented in MEGA 5.2. For the mtDNA (*Cox1* and *Nadh1*) alignment, the HKY + G + I substitution model was chosen and for *Ypt1*, the K2 + I model was used. Bootstrapping was done with 1000 replicates. All alignments and phylogenetic trees are publicly available in Treebase (study number S14964; `http://purl.org/phylo/treebase/phylows/study/TB2:S14964`).

4.2.4 DNA content analysis by flow cytometry

DNA contents were measured by flow cytometry for all isolates listed in Table 4.1, except CBS 112969. *Phytophthora* isolates were taken from storage at -80°C and grown in test tubes containing 5 mL clarified V8 broth for 6-10 days. The mycelium was then harvested and washed three times with sterile water. *Raphanus sativus* cv. Saxa was chosen as the internal DNA reference standard (with a genome size of 2C = 1.11 pg (Doležel et al., 1992). Nuclei extraction was done using the Cystain PI absolute P kit (Partec, Münster, Germany). For each sample approximately 0.5 cm^2 of young *Raphanus* leaf tissue and a small amount of *Phytophthora* mycelium (around 1 mg of dry blotted mycelium) were co-chopped with a razor blade (Gilette) (Galbraith et al., 1983) in a Petri dish containing 500 µl extraction buffer. After chopping, the suspension was filtered through a 10 µm filter (CellTrics, Partec, Germany) and 2 ml of a propidium iodide staining solution was added. The samples were incubated overnight in the dark at 4°C. Measurements were done on a Partec PAS III flow cytometer (Partec, Germany) equipped with a 20 mW solid state laser (Sapphire 488-20) emitting at a fixed wavelength of 488 nm. The data were analyzed using Flomax software (Partec Münster, Germany). DNA content was calculated using the ratios between the peak positions of the *Phytophthora* sample and the *Raphanus* standard. The genome sizes are shown as pg DNA/2C in which 2C corresponds to the complete DNA content of the nucleus, irrespective of ploidy (Greilhuber and Dolezel, 2009). Histograms shown in this paper were made using Summit v4.3. The isolates were measured three times on different days over the course of three years and each time they were recovered from the -80°C stock. The coefficient of variation between the three repeated measures was on average 4.7%.

4.2.5 Oospore production and isolation

Oospore production was assessed for all hybrid isolates and for the parental species *P. porri*. Oospore production was induced by incubating a *Phytophthora* culture in a Petri dish containing V8 agar for one month at 15°C. Isolation of the oospores was done by digesting the mycelium using lysing enzymes from *Trichoderma harzianum* (Sigma). Ten ml of a solution containing 50 mg lysing enzymes was filter sterilized and pre-incubated overnight at 28°C. After this step, the solution was added to the Petri dish containing a

Phytophthora culture and incubated for 2 days at 15°C. Hereafter, the solution containing oospores and mycelial fragments was scraped off the agar surface using a sterile spreader and filtered through a 70 µm filter (Cell Strainer, BD). Further removal of hyphal fragments was done by several rounds of centrifugation at low speed (1000 g), removing the supernatant and washing the pellet with sterile water. Oospores were visually examined under an Olympus BX51 microscope and the number of aborted oospores was counted.

4.3 Results

4.3.1 Hybrid characterization

Polymorphic ITS sequences

The ITS region of 48 isolates (see Table 4.1) was amplified by primers ITS1 and ITS4 (Table 4.2) and sequenced. The ITS sequence of 12 isolates showed double peaks in the DNA sequence chromatogram, which indicates that these isolates have a putative hybrid origin. Nine of these isolates originated from diseased *Allium* spp., mostly onion (*Allium cepa*). The other three isolates were obtained from diseased *Parthenium argentatum*, *Chrysanthemum* sp. and *Pastinaca sativa*. The isolates mainly originated from Japan (6 isolates) and Australia (3 isolates). The other three isolates were found in the USA, UK and Switzerland (Table 4.1).

For 11 of these isolates, the polymorphic base pairs were found exactly at those positions (7 on a total of 806 bp) where the ITS sequence of *P. porri* differs from that of *P. primulae*/*P.* taxon parsley (with the latter two having identical ITS sequences). These 11 hybrid isolates are shown in Figure 3.1, along with the possible parental species *P. porri* and *P. primulae*/*P.* taxon parsley, which showed no intraspecific or intra-isolate variation in ITS. Six isolates were polymorphic (indicated with a green colour in Figure 3.1) at all seven distinctive base pairs. The other five isolates showed polymorphisms in only some of the distinctive base pairs (non-polymorphic sites are indicated with a blue or yellow colour in Figure 3.1). The non-polymorphic base pairs in a given isolate all corresponded to one of the two parental types, as if the ITS type is reverting back to one of the two parental states.

The 12th putative hybrid, isolate CBS 112968, showed intra-isolate polymorphisms at

other positions in the ITS region and appeared not to be related to the other hybrid isolates. Instead, its ITS sequence was closely related to *P. cichorii*.

Species	Isolate(s)	Host plant	ITS polymorphisms[a]						
			119	147	639	687	690	736	777
P. porri	8 isolates	*Allium porrum*	C	/	C	T	C	A	T
P. primulae	6 isolates	*Primula* spp.	T	C	T	C	T	G	G
P. taxon parsley	2 isolates	*Petroselinum crispum*							
Hybrids	P6817	*Allium cepa*							
	CBS 138.87*	*Allium cepa*							
	CBS 126739	*Allium cepa*							
	CBS 112969*	*Allium cepa*			C				
	CBS 140.87	*Allium cepa*	C	/	C		C		
	CBS 139.87	*Allium grayi*	T	C		C	T	G	G
	CBS 126738	*Allium victorialis*							
	CBS 126737*	*Allium victorialis*							
	CBS 112966	*Chrysanthemum*	T	C		C	T	G	G
	CBS 114101	*Parthenium argentatum*							
	CBS 114040*	*Pastinaca sativa*	T			C	T	G	G

Figure 4.1: ITS polymorphisms for *P. porri*, *P. primulae*, *P.* taxon parsley and related hybrid isolates.
[a] A green colour indicates a polymorphic position, i.e. that both parental states (blue and yellow) were present in the ITS amplicon
* ITS cloning was done for these isolates: see Figure 4.2

Recombinant ITS types in the hybrids point to genetic recombination between the different parental genomes

By cloning and sequencing the polymorphic ITS amplification products, it is possible to identify the distinct haplotypes coming from the different parents. We cloned the ITS PCR product of four putative hybrids and analyzed the sequence of 10 clones per isolate (see Figure 4.2 A). Interestingly, we found that most of the clones showed recombinant sequences between the *P. porri* and *P. primulae*/*P.* taxon parsley parental haplotypes, suggesting that genetic recombination between the different parental genomes has occurred. ITS products for the possible parental species *P. porri* and *P.* taxon parsley were also cloned. Here, all clones had identical sequences, aside from an occasional random

SNP, which was never at the distinctive positions. To assess the possible occurrence of recombination caused by a PCR artefact, which has been reported in studies on plant hybrids (Cronn et al., 2002), a PCR reaction was run on the combined genomic DNA of *P. porri* and *P.* taxon parsley. From this experiment, it is clear that recombinant ITS clones can be formed due to a PCR artefact (Figure 4.2 A). However, the average number of crossovers per clone (shown in Figure 4.2 as 'average X per clone') was always higher in the hybrids compared to the combined genomic DNA experiment. To eliminate the bias caused by this PCR artefact, we optimized the PCR protocol following the instructions suggested by Lahr and Katz (2009). This protocol was again tested on a mixture of DNA of the two parental states (*P. porri* + *P.* taxon parsley) and twenty clones were sequenced (see Figure 4.2B). Here, no recombinant ITS clones were detected. In the hybrid isolates, however, again different types of recombinant ITS clones were found, leaving no doubt that this is a real biological phenomenon.

Sequencing of *Ypt1* haplotypes to determine the parental origin of the hybrids

The ITS region is located in the rRNA genes that are repeated in tandem arrays of several hundreds of copies. Because of this organization, the ITS region is sensitive to concerted evolution, i.e. the homogenization of the DNA sequence of the different repeats, which makes this region less suitable to study the parental origin of the interspecific hybrids. For this reason, we decided to sequence the highly variable Ras-related *Ypt1* region (Schena and Cooke, 2006), which is not sensitive to concerted evolution since it is a single copy gene.

We sequenced the *Ypt1* region of 54 clade 8b isolates (see Table 4.1), including the hybrids. The resulting alignment length was 451 bp and a phylogenetic tree is shown in Figure 4.3. The interspecific variability in *Ypt1* sequence was much higher compared to ITS. Despite the high interspecific variability, the *Ypt1* locus showed almost no intraspecific variation and thus was very distinctive at the species level. The *Ypt1* sequences of *P. primulae* and *P.* taxon parsley, however, were identical. Most hybrid isolates showed polymorphic *Ypt1* sequences as observed by double patterns in the DNA sequence chromatogram. The only exception was isolate CBS 114040, which showed a sequence identical to that of *P. primulae*/*P.* taxon parsley. Remarkably, a polymorphic *Ypt1* sequence was also observed

A

Species	Isolate(s)	Clone type	Frequency	ITS polymorphisms						
				119	147	639	687	690	736	777
P. porri	CBS127101	1	10/10	C	-	C	T	C	A	T
P. taxon parsley	CBS114156, BPIC 2584	1	10/10	T	C	T	C	T	G	G
P. porri + *P.* taxon parsley	CBS127101 + CBS 114156	1	6/20	T	C	T	C	T	G	G
	Average X per clone = 0.6	2	3/20	C	-	C	T	C	A	T
		3	4/20	T	C	C	T	C	A	T
		4	4/20	C	-	T	C	T	G	G
		5	1/20	T	C	C	T	C	G	G
		6	1/20	C	-	C	T	C	G	G
		7	1/20	T	C	T	C	T	G	T
Hybrids	CBS 114040	1	7/10	T	C	T	C	T	G	G
	Average X per clone = 0.6	2	3/10	T	C	C	C	T	G	G
	CBS 138.87	1	3/10	C	-	C	T	C	A	T
	Average X per clone = 1.0	2	2/10	T	C	C	C	T	G	G
		3	2/10	T	C	C	T	C	A	T
		4	1/10	C	-	C	C	T	G	G
		5	1/10	T	-	C	T	C	A	T
		6	1/10	C	C	C	T	C	A	T
	CBS 126737	1	6/10	T	C	C	C	T	G	G
	Average X per clone = 2.0	2	1/10	T	-	C	C	T	G	G
		3	1/10	T	C	C	C	T	A	T
		4	1/10	T	C	C	T	C	G	T
		5	1/10	T	C	T	C	T	G	G
	CBS 112969	1	4/10	C	-	C	T	C	A	T
	Average X per clone = 1.3	2	1/10	C	C	C	T	C	A	T
		3	1/10	C	-	C	T	C	G	T
		4	1/10	C	-	C	C	T	A	G
		5	1/10	C	-	C	C	T	G	G
		6	1/10	C	C	C	C	T	G	G
		7	1/10	T	C	C	C	T	G	G

B

Species	Isolate(s)	Clone type	Frequency	ITS polymorphisms						
						639	687	690	736	777
P. porri + *P.* taxon parsley	CBS127101 + CBS 114156	1	11/21			T	C	T	G	G
		2	10/21			C	T	C	A	T
Hybrids	CBS 138.87	1	14/20			C	T	C	A	T
	Average X per clone = 0.35	2	1/20			T	C	T	G	G
		3	3/20			C	C	T	G	G
		4	1/20			C	C	T	G	T
		5	1/20			C	C	T	A	T
	CBS 126737	1	3/20			T	C	T	G	G
	Average X per clone = 1.5	2	2/20			C	T	C	A	T
		3	10/20			C	C	T	G	G
		4	1/20			T	C	T	A	T
		5	1/20			C	T	C	G	G
		6	1/20			C	C	T	A	T
		7	1/20			T	T	C	A	T

Figure 4.2: Cloning of ITS PCR products of hybrid isolates and their possible parental species.
A, using Taq polymerase (with PCR artefact); B, using Phusion polymerase (without PCR artefact); X = recombination event

in isolate ICMP14653. This isolate was also collected from diseased onion, but had an ITS sequence identical to that of *P. primulae*/*P.* taxon parsley, without polymorphisms. The *Ypt1* gene was cloned for four hybrid isolates (CBS 138.87, ICMP14653, CBS 112968 and CBS 112969). For isolates ICMP14653, CBS 138.87 and CBS 112969, one haplotype

was very similar to *P. primulae*/*P.* taxon parsley (0-2 SNPs) and the other haplotype was very similar to that of *P. porri* (2 SNPs). For isolate CBS 112968, two different haplotypes were recovered that were closely related but distinct from *P. cichorii*. The two distinct haplotypes for these isolates are shown in the phylogenetic tree in Figure 4.3. All other hybrid isolates showing a polymorphic *Ypt1* sequence are not shown in Figure 4.3.

mtDNA sequencing shows that at least three different hybridization events have occurred in clade 8b

Since mitochondria are inherited maternally through the oogonia (Erwin and Ribeiro, 1996), sequencing of the mtDNA can identify the species that acted as the maternal parent in an interspecific cross. We sequenced two genes (*Cox1* and *Nadh1*) for 53 isolates and the concatenated alignment had a total length of 1539 bp. The multilocus phylogenetic tree is shown in Figure 4.4.

Phytophthora taxon parsley and most of the hybrid isolates (indicated as hybrid type 1 in Figure 4.4) form a cluster with high bootstrap support. Their closest relative was *P. primulae* which formed a separate cluster (99.7% sequence similarity, 5-6 SNPs). The hybrid isolate CBS 112969 (hybrid type 2 in Figure 4.4) had a different mtDNA sequence that showed most similarity to *P. primulae* (99.0% sequence similarity, 15 SNPs), suggesting that it was formed in a different hybridization event with an unknown species related to *P. primulae* acting as the maternal parent. However, the two *Ypt1* haplotypes of hybrid type 1 and 2 both corresponded to *P. porri* or *P. primulae*, indicating that both of these species were involved in their formation. An alternative explanation is that both hybrid type 1 and 2 originated in a hybridization event between *P. porri* and *P. primulae*, but have accumulated mutations in their mtDNA over time. However, in this scenario, it is hard to explain how mutations could have accumulated in the mtDNA genes, but not in *Ypt1*.

Both *P.* taxon parsley isolates and hybrid type 1 isolate CBS 114040 have one shared SNP that is not present in the other hybrid type 1 isolates, nor in the closest ancestor *P. primulae*, indicating that *P.* taxon parsley and CBS 114040 might be descendants of the hybrid type 1, supporting the *P. primulae*/*P. porri* hybridization theory. The fact that *P.* taxon parsley contains only one ITS and *Ypt1* haplotype could be interpreted as

a sign of non-hybrid origin. However, other hybrid isolates were found to have only one haplotype at one of the two nuclear loci. Only one ITS haplotype was shown for hybrid type 1 isolate ICMP14653, and only one *Ypt1* haplotype was shown for hybrid type 1 isolate CBS 114040. Therefore, it should be possible that in *P.* taxon parsley, additivity in both ITS and Ypt1 is lost. Isolate CBS 114040 could be an intermediate between hybrid type 1 and *P.* taxon parsley, since it contains one *Ypt1* haplotype (identical to *P.* taxon parsley/*P. primulae*) and almost lost all polymorphism in the ITS sequence (only two dimorphic sites).

The hybrid isolate CBS 112968 (hybrid type 3 in Figure 4.4), had a sequence most similar to that of *P. cichorii* (98.8% sequence similarity, 18 SNPs), demonstrating that this hybrid was formed in a third hybridization event with an unknown species related to *P. cichorii* acting as the maternal parent.

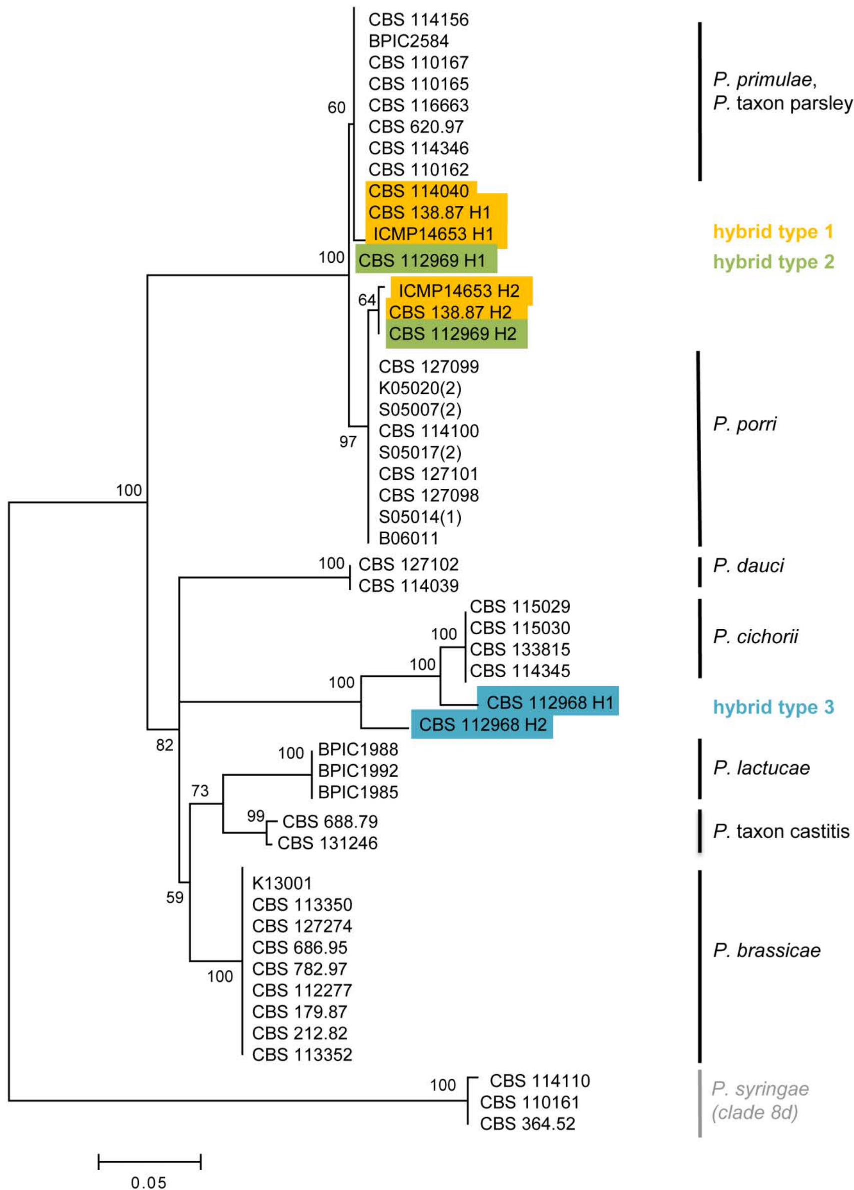

Figure 4.3: *Ypt1* phylogeny of *Phytophthora* clade 8b.
Phylogenetic tree derived from an alignment of *Ypt1* sequence data of 42 clade 8b isolates. The Maximum Likelihood bootstrap support values are shown for all branches. The tree is rooted with three *P. syringae* isolates (clade 8d). For hybrid isolates CBS 138.87, ICMP14653 (hybrid type 1), CBS 112969 (hybrid type 2) and CBS 112968 (hybrid type 3) the different ITS haplotypes for the three hybrid types are shown in yellow, green and blue, respectively.

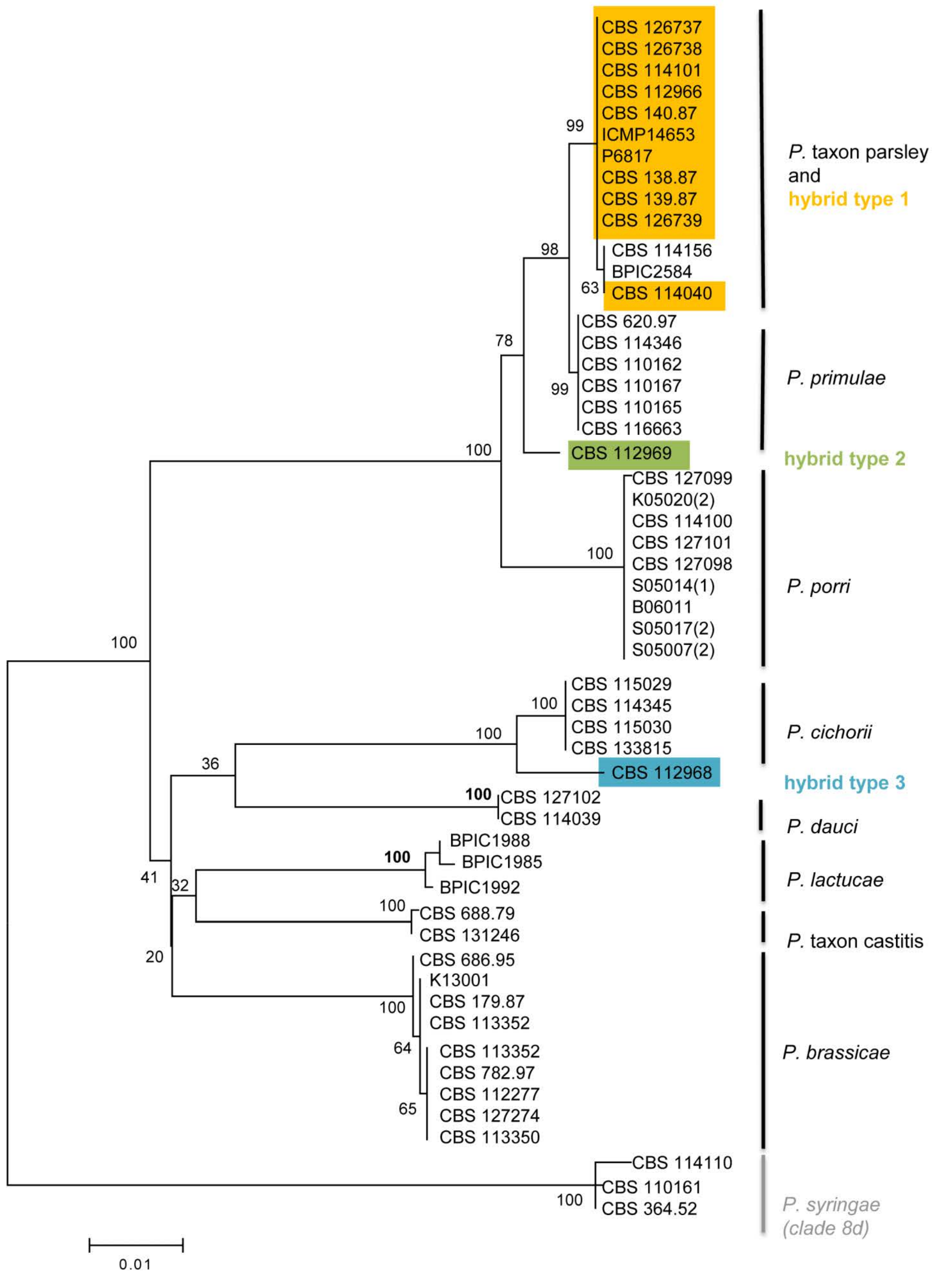

Figure 4.4: Multilocus mtDNA (*Cox1* + *Nadh1*) phylogeny of *Phytophthora* clade 8b.
Phylogenetic tree derived from a multilocus (concatenated) alignment of *Cox1* and *Nadh1* sequence data of 50 clade 8b isolates. The Maximum Likelihood bootstrap support values are shown for all branches. The tree is rooted with three *P. syringae* isolates (clade 8d). Hybrid type 1 is shown in yellow, hybrid type 2 in green and hybrid type 3 in blue.

DNA content measurements suggest polyploidy in different *Phytophthora* clade 8b species and are consistent with (allo)polyploidy in the hybrids

DNA content measurements were done for all isolates listed in Table 4.1 (except for hybrid type 2 isolate CBS 112969 for which we could not get stable measurements) and are presented in Table 4.3.

Most *P. porri* isolates had a similar nuclear DNA content with a mean value of 0.270 pg DNA/2C (SE = 0.003, n = 14). One isolate (S05014(1)) contained approximately half of this DNA content (0.142 pg DNA/2C), suggesting polyploidy in this species, with the small DNA content most likely representing the diploid state. Moreover, four isolates contained a mixture of nuclei with two different DNA contents, with one nucleus type having a mean DNA content of 0.142 pg DNA/2C (SE = 0.002, n = 4) and the other having a mean DNA content of 0.273 pg DNA/2C (SE = 0.005, n = 4), suggesting that they are diploid/polyploid (or mixoploid and probably diploid/tetraploid) heterokaryons. Histograms of the three different ploidy types are shown in Figure 4.5. The diploid/polyploid (or mixoploid) heterokaryons showed markedly different histograms compared to those of the diploid and tetraploid isolates. In diploid and tetraploids, the first peak corresponding to the G1 mitotic nuclei, was always the largest peak. The second peak, corresponding to the G2 nuclei (which are the dividing nuclei that contain a doubled DNA content), was always about 10 to 20% of the size of the G1 peak. In the diploid/polyploid heterokaryons, the relative proportion between the first two peaks was different. The second peak was much larger (sometimes larger than the first peak), and most of the times also a third peak was visible, corresponding to the G2 nuclei of the second population.

For *P. primulae*, all isolates measured had a similar DNA content with a mean value of 0.269 pg DNA/2C (SE = 0.005, n = 6). The two *P.* taxon parsley isolates both contained two types of nuclei and are assumed to be diploid/polyploid (probably diploid/tetraploid) heterokaryons. One isolate (BPIC2584) had a DNA content comparable to the heterokaryotic *P. porri* isolates described above. The other isolate (CBS 114156) had a considerably higher DNA content. The 11 hybrid type 1 isolates had highly variable DNA contents ranging from 0.146 to 0.388 pg DNA/2C. There is a positive correlation between the relative amount of polymorphic positions in the ITS PCR amplicon (coloured green in Figure 4.1) and the DNA content for a given hybrid (type 1) isolate (Pearson's r, r = 0.690, n = 11, p = 0.019). This suggests that the hybrids evolve from a high DNA-content, allopoly-

ploid state (such as in isolate CBS 126737) to a low DNA-content, diploid or diploid-like state (such as in isolate CBS 114040).

Most *P. brassicae* isolates have a similar DNA content with a mean of 0.277 pg DNA/2C (SE = 0.003, n = 6), one isolate had a much lower DNA content (0.206 pg DNA/2C) and three isolates had a much larger DNA content of 0.401 pg DNA/2C (SE = 0.010, n = 2). For *P. cichorii*, the average DNA content was higher, with a mean value of 0.361 pg DNA/2C (SE = 0.011). The hybrid type 3 (isolate CBS 112968), had a DNA content of 0.281 pg DNA/2C. *Phytophthora lactucae* had a mean DNA content of 0.246 pg DNA/2C (SE = 0.002, n = 3), *P. dauci* had a mean DNA content of 0.269 pg DNA/2C (SE = 0.004, n = 2) and *P.* taxon castitis had a mean DNA content of 0.226 pg DNA/2C (SE = 0.010, n = 2). For comparison, we also measured the DNA content of *Phytophthora syringae*. This species belongs to clade 8d (Grünwald et al., 2011) and is one of the closest relatives to the clade 8b species. Its mean DNA content was 0.176 pg DNA/2C (SE = 0.006, n = 3).

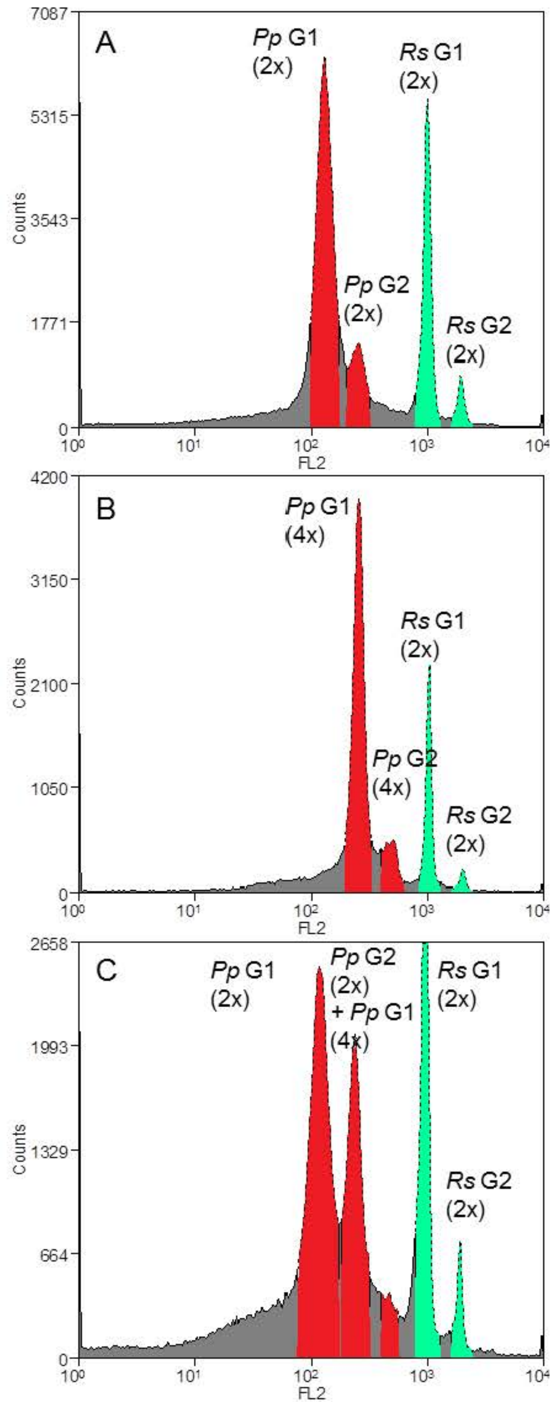

Figure 4.5: Histograms of the three *P. porri* DNA ploidy levels.
A, diploid (S05014(1)); B, tetraploid (K06007(2)) and C, mixoploid (B06011)

Table 4.3: DNA content measurements of the different *Phytophthora* clade 8b species and hybrids.
[a]SE is only shown where applicable, i.e. where more than one isolate was measured

Species	Isolate(s)	Host plant	Mean (SE)[a] DNA content per nucleus (pg DNA/2C)
P. porri	S05014(1)	*Allium porrum*	0.142
	B06011, S05007(2), S05017(2), K05020(2)		0.142 (0.002)/0.273 (0.005)
	CBS 181.87, CBS 802.95, CBS 114100, CBS 166662, CBS 127099, B06005(1), CBS 127101, K05025(1), B06008, S05009, K07015(4), S05012(2), K07015(2), S12001		0.270 (0.003)
P. primulae	CBS 620.97, CBS 110162, CBS 114346, CBS 110167, CBS 110165, CBS 116663	*Primula* spp.	0.269 (0.005)
P. taxon parsley	BPIC 2584	*Petroselinum crispum*	0.144/0.284
	CBS114156		0.171/0.347
hybrid type 1	CBS 114040	*Pastinaca sativa*	0.146
	CBS 112966	*Chrysanthemum*	0.222
	CBS 139.87	*Allium grayi*	0.223
	P6817	*Allium cepa*	0.237
	CBS 140.87	*Allium cepa*	0.252
	ICMP14653	*Allium cepa*	0.276
	CBS 114101	*Parthenium argentatum*	0.317
	CBS 138.87	*Allium cepa*	0.326
	CBS 126739	*Allium cepa*	0.337
	CBS 126738	*Allium victorialis*	0.339
	CBS 126737	*Allium victorialis*	0.388
P. brassicae	CBS 113350	*Brassica* spp.	0.206
	CBS 179.87, CBS 782.97, CBS 212.82, CBS 686.95, CBS 112277, CBS 112967		0.277 (0.003)
	CBS 113352, CBS 127274, K13001		0.401 (0.010)
P. dauci	CBS 127102, CBS 114039	*Daucus carota*	0.269 (0.004)
P. lactucae	BPIC1985, BPIC1988, BPIC1992	*Lactuca sativa*	0.246 (0.002)
P. cichorii	CBS114345, CBS133815, CBS115029, CBS115030	*Cichorium intybus*	0.361 (0.011)
P. taxon castitis	CBS 688.79, CH112	*Daucus carota, Fragaria×ananassa*	0.226 (0.010)
hybrid type 3	CBS 112698	*Allium cepa*	0.281
P. syringae	CBS 114110, CBS 110161, CBS 364.52	*Almond, Rhododendron, unknown*	0.176 (0.006)

High levels of aborted oospores in the hybrids point to meiotic problems

Oospore production was assessed in ten hybrid type 1 isolates as well as for hybrid type 2 and 3 and for three isolates of the parental species *P. porri*. In most hybrid type 1 isolates, oospore production was highly impaired. Three of the hybrid type 1 isolates did not produce oospores (CBS 138.87, CBS 112966 and CBS 114040); three isolates produced them in extremely low amounts (P6817, CBS 140.87 and CBS 126739). The other four isolates (ICMP 14653, CBS 114101, CBS 126738 and CBS 126737) produced oospores in higher amounts. The number of abortive oospores was high in all six hybrid type 1 isolates that produced oospores (between 26.7% and 100%, see Table 4.4), and significantly higher than in isolates of *Phytophthora porri* (2.8% - 8.6%). In Figure 3.6, examples of normal and aborted oospores are shown. Hybrid type 2 (CBS 112969) produced oospores, but a high amount was aborted (78.6%). Hybrid type 3 also produced oospores, but in this case only a low amount of aborted oospores was detected (8.6 %).

Table 4.4: Percentage of aborted oospores for the three hybrid types and for the parental species *P. porri*

Species	Isolate	Oospores counted	% of aborted oospores
hybrid type 1	CBS 114040	13	100
	CBS 112966	0	n.a.
	CBS 10.015	0	n.a.
	CBS 140.87	34	82.4
	ICMP14653	138	77.5
	CBS 114101	108	74.1
	CBS 138.87	0	n.a.
	CBS 126739	9	66.7
	CBS 126738	173	61.3
	CBS 126737	330	26.7
hybrid type 2	CBS 112969	84	78.6
hybrid type 3	CBS 112968	139	8.6
P. porri	K11003	171	7.6
	CBS 127101	70	8.6
	S05010	142	2.8

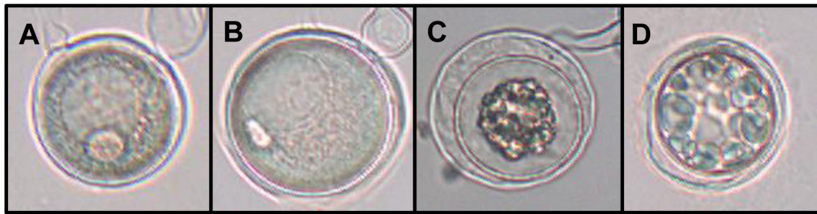

Figure 4.6: Normal and aborted oospores of hybrid type 1 (isolate CBS 126737). A,B: normal oospores; C,D: aborted oospores

4.4 Discussion

The increasing body of literature that is becoming available on hybridization in *Phytophthora* suggests that it could play an important role in the evolution of the genus. Natural interspecific hybridization events have been described in clade 1 (Bonants et al., 2000; Goss et al., 2011; Hurtado-Gonzales et al., 2009; Man In 't Veld et al., 1998; Man in 't Veld et al., 2006), clade 6 (Nagel et al., 2013), clade 7 (Brasier et al., 1999, 2004) and now in clade 8 (this study). From these previous studies, it is clear that interspecific hybridization has the potential to create new strains that have a new or expanded host range. The most striking example remains *Phytophthora alni*, which caused a new disease on alder trees across Europe and quickly became one of the most serious threats in riparian ecosystems. The pathogen was proven to be of recent hybrid origin and contains an evolving group of allopolyploid genotypes (Ioos et al., 2006). The clade 8b hybrids were genetically characterized by cloning and sequencing two nuclear regions (ITS and *Ypt1*) and sequencing of two mtDNA genes (*Cox1* and *Nadh1*). Hereby, three different types of hybrids could be distinguished with different parental species involved in their formation. The largest group (hybrid type 1, 11 isolates) originated from hybridization between *P. porri* and *P. primulae* or *P.* taxon parsley. Hybrid type 2 (isolate CBS 112969) was closely related to the hybrid type 1, but has a different mtDNA sequence related to *P. primulae*. Hybrid type 3 (isolate CBS 112968) is unrelated to the first two types, and was formed in a hybridization event between two unknown species closely related to *P. cichorii*.

There is a clear discrepancy between the nuclear (*Ypt1*) and mitochondrial (*Cox1* + *Nadh1*) phylogenies. While most clade 8b species show a much faster mutation rate in

the *Ypt1* gene (as visualized by longer branch lengths) compared to the mtDNA genes, the reverse is true for *P. porri* and *P. primulae*. This demonstrates that gene flow (hybridization events) between *P. porri* and *P. primulae* is common in evolutionary terms.
We have measured DNA contents for over 50 isolates belonging to the different *Phytophthora* clade 8b species and for the hybrids. In *P. porri*, where 18 isolates were measured, we detected intraspecific DNA ploidy variation. The majority of the isolates were polyploid (probably tetraploid), one isolate was diploid, and four isolates were diploid/polyploid heterokaryons. Previous research in our lab (Declercq, 2009) has shown that isolates belonging to these three different ploidy types, have identical AFLP patterns. In the case of allopolyploid hybrids, AFLP patterns would differ significantly from the parental patterns (i.e. a combination of AFLP fragments from both parents; Bonants et al. (2000)). Therefore, the data suggest that in *P. porri*, a whole genome duplication has occurred, creating an autopolyploid. In *P. primulae*, no intraspecific DNA content variation was detected and the mean DNA content was similar to that of the polyploid *P. porri* isolates, suggesting that also *P. primulae* is polyploid. Both isolates of *P.* taxon parsley were diploid/polyploid (mixoploid) heterokaryons. In *P. brassicae*, three different DNA contents were found in the nine isolates that were analyzed. Most likely, these DNA content variations also represent ploidy variations. In the other clade 8b species, no considerable intraspecific DNA content variation was detected, but DNA contents were always in the same range as for the polyploid *P. porri* isolates. It is difficult to infer ploidy levels for these species, since DNA content does not always correlate with ploidy level when comparing different species, even if they are closely related (Suda et al., 2006). Chromosome counts and/or genome sequencing will be needed to verify the ploidy level of these species. *P. syringae* (clade 8d), one of the closest relatives to the clade 8b species, had a DNA content of 0.176 pg DNA/2C. Isolates of *P. ramorum* (clade 8c) have also been evaluated for DNA content by flow cytometry using the same protocol as was used in our study (Vercauteren et al., 2011). These isolates had a stable DNA content of 0.146 pg DNA/2C (SE = 0.002, n = 3), which is assumed to represent the diploid state.
The hybrid type 1 isolates show a large variation in DNA content. This variation in DNA content was positively correlated with additivity (i.e. the presence of two alleles) in the nuclear loci. The isolates with the highest DNA content showed complete additivity in both nuclear loci, which is what is to be expected from a stable or recently formed

allopolyploid. The isolates with the smallest DNA contents show almost no additivity in ITS, and in one isolate (CBS 114040) only one *Ypt1* haplotype is still present. Processes that can cause this loss of additivity include chromosome loss, non-reciprocal translocations, gene conversion and unequal crossing over (Gaeta and Pires, 2010; Renny-Byfield et al., 2013).

Recombinant ITS sequences between the two parental types were detected by cloning and sequencing the ITS regions of different hybrid isolates and are the result of homeologous recombination (recombination between the different parental genomes). Theoretically, these recombinations can be due to either asexual, mitotic recombination or gene conversion, or sexual recombination (meiotic cross-over). Asexual or mitotic recombination is associated with double strand break repair (LaFave and Sekelsky, 2009). Mitotic recombination and gene conversion causing loss of heterozygosity have been described several times in *Phytophthora* (Chamnanpunt, 2001; Lamour et al., 2012; Randall et al., 2014). However, mitotic recombination or gene conversion has not been described between homeologous chromosomes in allopolyploids. In *Phytophthora*, either mitotic or meiotic recombination can be transferred to the offspring, since there is no predetermined germline. Although it is unclear to what extent homeologous recombination or gene conversion (either mitotic or meiotic) occurs in other parts of the genome, it can be an important process of genome stabilization of polyploids after hybridization but it can also create novel genetic variation as has been described for plant hybrids (Gaeta and Pires, 2010).

Our study raised some concern on the use of ITS as the universal barcode for identification of *Phytophthora*, since hybridization events can be easily overlooked. The ITS region is located in the rRNA genes that are repeated in tandem arrays of several hundreds of copies. Because of this organization, the ITS region is sensitive to concerted evolution, i.e. the homogenization of the DNA sequence of the different repeats. From plant research, it is known that the ITS can evolve in three different ways after interspecific hybridization: (1) unidirectional concerted evolution leads to loss of one of the parental copies and fixation of the other (2) formation of a new ITS type that represents a mixture of the two parental types, (3) presence of both ITS types, which is the case in young hybrid taxa, although this situation can also be stable in older hybrid taxa (Kovarik et al., 2004, 2005; Wendel et al., 1995). In the clade 8b hybrids, unidirectional concerted evolution is ongoing, keeping one of the two parental haplotypes and in this way erasing the footprints

of hybridization.

Most *Phytophthora* species show a certain degree of host specificity, and this is also the case for the clade 8b species. Hence, the question remains how and where different species can meet intimately, a requirement for interspecific hybridization to occur. Since the clade 8b species show a clear host preference for winter grown field vegetables that are commonly used in crop rotation systems (such as leek, carrot, cabbages, lettuce, parsley, chicory), it is likely that the different species can be present on the same field. Moreover, all three hybrid types, formed in hybridization events with different parental species, have been isolated from onion (*Allium cepa*). This led us to hypothesize that *Allium cepa* might be a common susceptible host of the different clade 8b species involved in the hybrids' formation, offering a hot spot for interspecific hybridization to occur. Pathogenicity tests with the different clade 8b species on onion, could shed more light on this hypothesis (see Chapter 5).

Of the hybrid type 1, six isolates were derived from *Allium cepa*, the other isolates were derived from *Allium victorialis*, *Allium grayi*, *Pastinaca sativa*, *Chrysanthemum* sp. and *Parthenium argentatum*, indicating a clear expansion of host range compared to the parents that were so far only detected on leek (*P. porri*) and parsley (*P.* taxon parsley).

As a result of globalization, plants and therefore their pathogens are now being traded all over the world on a large scale. This evidently increases the chance of interspecific hybridization since closely related species or different genotypes of the same species that have been geographically isolated are brought together while their reproductive barriers might be incomplete (Brasier, 2000).

For the reasons outlined above, the implications of hybridization on the adaptive potential and pathogenicity of *Phytophthora* spp. deserve to be studied in more detail. An outstanding topic of discussion in this respect is the fate of genes involved in plant pathogen interaction. In the past few decades, it has become clear that *Phytophthora* pathogens are extremely flexible and this has made it a very challenging task for plant breeders to develop crops with durable resistance against *Phytophthora*. A lot of attention has been given to the biology of the RXLR effectors that have been discovered recently (Whisson et al., 2007; Haas et al., 2009). These effector genes have been coined as one of the major determinants of host specificity. From whole genome sequencing, we know that a single pathogenic *Phytophthora* isolate can contain high numbers of different and often unique

RXLR effector genes (563 different RXLR effector genes predicted from the *P. infestans* genome sequence (Haas et al., 2009)). It has also been discovered that the genome of *P. infestans* (with its 250 Mbp the largest oomycete and chromalveolate genome sequenced up till now) consists of 74% repetitive sequence, contributing to the flexibility of the genome.

It is easy to imagine how (allopolyploid) hybridization and its consequences could have helped to build up the enormous effector repertoires that we find in *Phytophthora* pathogens today. The fact that effector genes are mainly contained in gene sparse regions in the DNA enables recombination between effector gene regions, without high risks of lethality (see Chapter 7).

Intra-specific hybridization between divergent races of the same species could also enhance the pathogen's pathogenic potential by expansion and/or recombination of effector gene repertoires. In *P. ramorum*, a heterothallic species, crosses have been induced between different genotypes that have diverged as a consequence of geographical isolation (Boutet et al., 2010). The resulting oospore progenies were characterized by DNA content estimation and microsatellite genotyping (Vercauteren et al., 2011). A large proportion of the progeny showed DNA contents that were considerably larger (up to 70%) than their parental DNA content. Microsatellite patterns also indicated polyploidy and aneuploidy in the progeny.

The same processes might be responsible for the increased pathogenic potential observed for *P. infestans* in Europe since the 1980s, when sexual reproduction of the pathogen was enabled by the introduction of the A2 mating type from its center of origin to Europe. In a recent study (Yoshida et al., 2013), herbarium samples of the *P. infestans* strains that caused the Irish potato famine in the 19th century were collected and their genomes were sequenced. These genomes were compared to the genomes of recent aggressive *P. infestans* strains that have outcompeted the old strains. An important difference between the old strains (that were dominant for about 50 years) and the new strains, is the rise in ploidy level. The old strains were found to be diploid, while the new strains are mostly triploid and sometimes tetraploid, which was estimated based on allele frequency from the resequencing alignments. It is unclear what caused this rise in ploidy level, but in our opinion, hybridization between divergent *P. infestans* genotypes, seems to be a likely scenario.

In conclusion, we have found that interspecific hybridization has occurred at different times in clade 8b with different parental species involved. The combination of sequencing different genes and estimating DNA content by flow cytometry provided valuable insights in the processes involved in and after interspecific hybridization. A recently formed hybrid will contain the complete complement of both parental genomes (allopolyploidy). Novel gene combinations such as a combined effector repertoire can enable the pathogen to invade new habitats or hosts. After allopolyploidization, different genetic processes such as chromosome loss and chromosomal rearrangements (which can be between the different parental genomes through homeologous recombination), can enable the pathogen to further adapt to a new habitat or host. As can be seen in the hybrid type 1 isolates, the progeny of a single hybridization event can follow different evolutionary paths, which ultimately can result in the formation of new species. It is clear from our data that the footprints of hybridization can easily be lost. Therefore, it is possible that the occurrence and importance of hybridization in *Phytophthora* is highly underestimated. We hypothesize that the occurrence of DNA content variation and polyploidy in *Phytophthora* species is linked with (ancient) hybridization events.

Acknowledgements

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Addendum 1

4.5 Hybrid morphology and growth rates

4.5.1 Colony morphology of hybrids

Hybrid isolates were grown on V8 for 7 days at 18°C before pictures were taken. In Figure 4.7, the colony morphology of the hybrids is shown. Hybrid type 1 isolates CBS 138.87 and CBS 139.87 are not represented in Figure 4.7. These isolates showed very irregular growth rates and were both lost in culture during the course of this work. The hybrid isolates show a more diverse colony morphology compared to the parental species (see Chapter 3). Some isolates showed very little aerial mycelium while others had a dense and fluffy aerial mycelium.

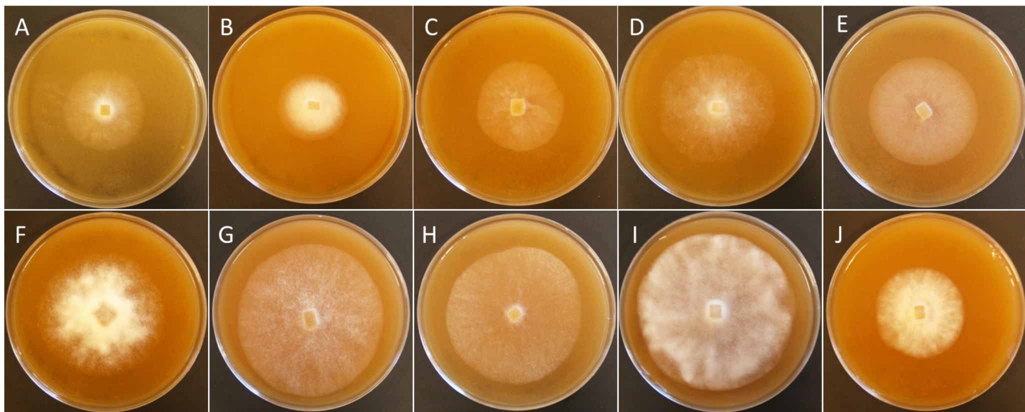

Figure 4.7: Colony morphology of hybrid isolates.
A-H, hybrid type1; I, hybrid type 2, J, hybrid type 3. A, CBS 114040; B, CBS 112966; C, P6817; D, CBS 140.87; E, CBS 114101; F, CBS 112739; G, CBS 112738; H, CBS 112737; I, CBS 112969; J, CBS 112968

4.5.2 Hybrid type 1 growth rates and correlation with DNA content

Growth rate was determined on V8 medium in mm/day after 6 days of incubation at 15°C in the dark. Two replicates per isolate were measured. Growth rates were assessed for all hybrid type 1 isolates except CBS 139.87 (this isolate was lost in culture) and the parental species *P. porri* (14 isolates) and *P.* taxon parsley (2 isolates). The distribution of growth rates for isolates of each group/species is presented in Figure 4.8A in the form of a box plot. From this figure it can easily be seen that the hybrid isolates have a much larger range in growth rate than the parental species *P. porri* and *P.* taxon parsley. One hybrid isolate (CBS 126737) showed a higher growth rate than any of the isolates of the parental species, indicating heterosis or hybrid vigour. Moreover, there was a significant correlation (Figure 4.8B) between the growth rate (mm/day) of the hybrids and their DNA content (Pearson's r, r = 0.683, n = 10, p = 0.029).

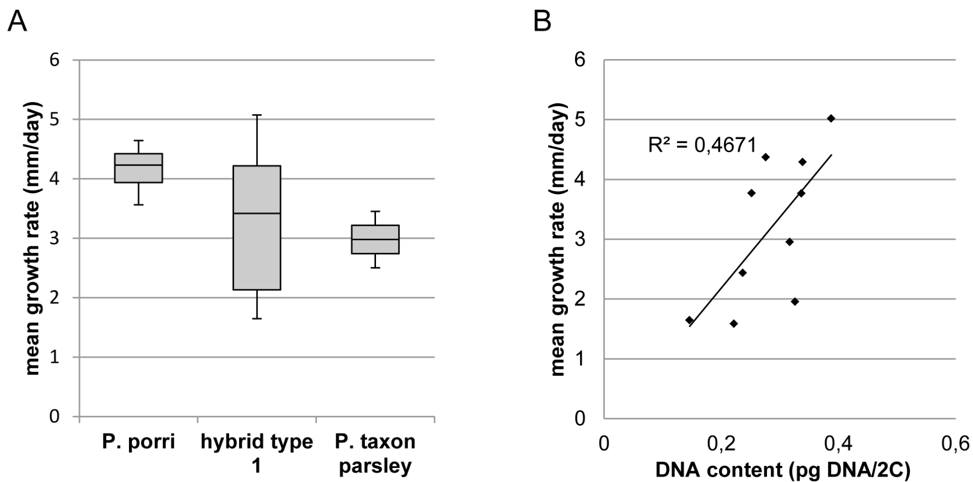

Figure 4.8: Hybrid type 1 growth rates and correlation with DNA content. A, Box plot distribution of growth rates of *P. porri* (14 isolates), hybrid type 1 (10 isolates) and *P.* taxon parsley (2 isolates). B, correlation between DNA content (pg DNA/2C) and mean growth rate of 10 hybrid type 1 isolates.

Addendum 2

4.6 Optimization of the flow cytometry protocol for *Phytophthora* genome size determination: a work in progress

4.6.1 Introduction

Flow cytometry is a technique to count and measure different aspects of particles (such as cells or nuclei) in a fluid stream. A typical flow cytometer consists of three parts: fluidics, optics and electronics. In brief, using hydrodynamic focusing, the particle fluid stream is manipulated by a sheath fluid so that the particles go through the optical system (laser, lamp or light emitting diode) one by one. The excitation of the particles is deflected and passed by a set of filters and mirrors to break it in to different wavelengths of the emitted fluorescence (FL) and scattered laser light (SSC and FSC). By use of photomultiplier tubes, the incoming photons are converted and multiplied into an electric signal which is sent to the computer. On the computer, the data is shown as single-parameter histograms or two-parameter dot plots.

Flow cytometry can be used for a very broad array of applications ranging from the detection and physiological status assessment of blood cells in blood or micro-organisms in water suspensions to nuclear DNA content determination. Up till now, the devices have been used mainly for medical purposes and are indispensable in routine clinical diagnostics. However, the method has promising applications in the fields of plant science and plant pathology (D'hondt et al., 2011; Doležel et al., 2007).

Flow cytometry for DNA content estimation of plants has been optimized to a set of best practices that generally deliver very robust results. The development of a set of internationally accepted reference standards (Suda and Leitch, 2010) has been crucial. Also, commercial kits for sample preparation have been developed that offer a high level of reproducibility. Next to this, a set of parameters are available to which data needs to conform to be considered reliable. Only recently, plant pathologists and mycologists have started using the technique for genome size and ploidy level estimation of fungi and oomycetes (Catal et al., 2010; Hamed and Gisi, 2013; Si-Ammour, 2002; Vercauteren

et al., 2011). Mainly, the protocols and reference standards developed for plant genome size determination have been adopted for use with Fungi and oomycetes. However, it is clear that these protocols and reference standards have their limitations, as will be discussed in this addendum, and that the technique needs to be specifically optimized for use with Fungi and oomycetes.

Fungi and oomycetes generally have much smaller genomes than plants (19 to 160 Mbp for Fungi and 37 to 280 Mbp for oomycetes (Raffaele and Kamoun, 2012) versus 126 Mbp to ± 300 000 Mbp for angiosperms (`http://data.kew.org/cvalues/`). Hence, most of the available plant reference standards have a much larger genome size than the fungus or oomycete being measured, and therefore they cannot be used or can only be used when measuring on a logarithmic scale, lowering the resolution of the data. Therefore, there exists a need for small genome size fungal and oomycete reference standards. Also, a good reference standard should ideally be of the same taxonomic kingdom because of differences in GC content and the possible presence of specific secondary metabolites that can interfere with the measurements. Specific fungal or oomycete standards have not been developed and different plant or animal standards have been used with varying results. For *Phytophthora* genome size determination, both plant and animal standards have been used (*Arabidopsis thaliana* ecotype Col-0) (Si-Ammour, 2002), chicken red blood cells (Catal et al., 2010), *Raphanus sativa* cv. Saxa (Vercauteren et al., 2011) and *Solanum lycopersicum* cv. Baby (Hamed and Gisi, 2013).

In flow cytometry applications, genome size data is typically presented as pg DNA/2C which represents the absolute DNA content of the nucleus irrespective of ploidy or 'holoploid' DNA content (Greilhuber et al., 2005). The C-value terminology (with C from constant) was derived long ago under the assumption that DNA contents within a single species follow a set of multiplications of a constant number (1C = onefold, 2C = twofold, etc.). The terminology is still used today although it can be confusing, especially in polyploid species. See Greilhuber and Dolezel (2009) for a clear overview of the terminology. A conversion between C-value and Mbp can be made using the following calculation: 1 pg DNA = 978 Mb. Genome sizes derived from sequencing studies are generally shown in Mbp and these numbers are equivalent to the 1C value (total DNA content of one chromosome complement), while in flow cytometry studies, the 2C value (in pg), representing the complete DNA content of the nucleus, irrespective of ploidy, is generally given. In

Figure 4.9, an overview of a typical flow cytometry histogram output for genome size determination is given.

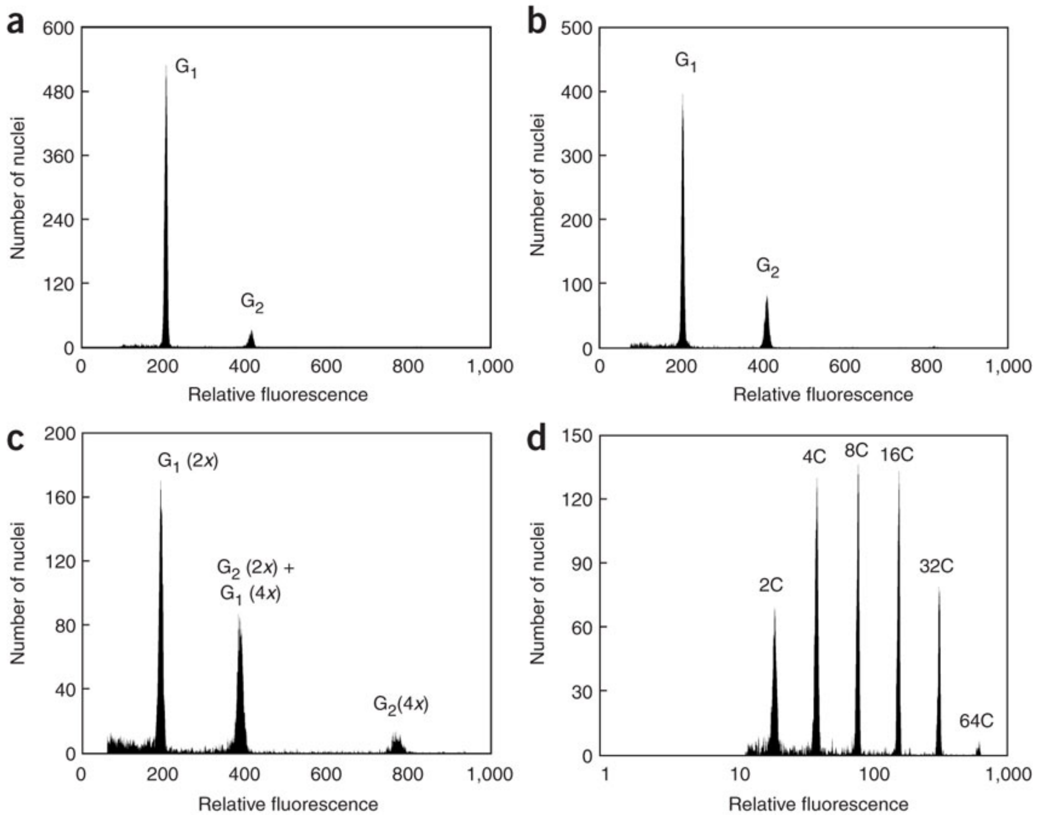

Figure 4.9: Histograms of relative fluorescence intensities (relative nuclear DNA contents) obtained after the analysis of isolated plant nuclei using DAPI staining.
A, Analysis of nuclei isolated from a leaf of *Pisum sativum* yielded a historgram with a single dominant peak of G1-phase nuclei. B, The analysis of an older leaf of *Zea mays* revealed a higher proportion of nuclei within G2 phase. C, a similar histogram is obtained after analyzing a mixoploid plant of *Kochia scoparia* (2n = 2x + 4x) with diploid and tetraploid cells. D, The analysis of endopolyploidy in *Senecio rowleyanus* was facilitated by the use of a logarithmic scale, permitting visualization of higher numbers of (endo)ploidy level. Reproduced from Doležel et al. (2007) with permission from the publisher.

In this addendum, we describe the optimization of the protocol used by Vercauteren et al.

(2011) and discuss the advantages and limitations of the method.

4.6.2 Materials and methods

Selection of a suitable internal reference standard

Both *Raphanus sativa* cv. Saxa (young leaf; 2C = 1.11 pg DNA; Doležel et al. (1992)) as *Arabidopsis thaliana* ecotype Col-0 (inflorescence; 2C = 0.412 pg DNA; Schmuths et al. (2004)) were tested as internal reference standard. For *Arabidopsis thaliana*, we used the inflorescence since nuclei in this tissue do not show endopolyploidization in contrast to the nuclei in the leaves (Galbraith et al., 1991).

Sample preparation

Phytophthora isolates were taken from storage at -80°C and grown for 6-10 days in test tubes containing 5 mL clarified V8 broth. The mycelium was then harvested and washed three times with sterile water. Different amounts of *Phytophthora* mycelium and internal standard tissue were tested for determining the optimal ratio. Nuclei extraction was done using the Cystain PI absolute P kit (Partec, Germany). The internal standard plant tissue and the dry-blotted *Phytophthora* mycelium were co-chopped for 1 min with a sharp razor blade (Gilette; Galbraith et al. (1983)) in a Petri dish containing 500 µl extraction buffer. After chopping, the suspension was filtered through a 10 µm filter (CellTrics, Partec, Germany) and 2 ml of a propidium iodide staining solution (Cystain PI absolute P kit) was added. The samples were either measured 2 hours after preparation or incubated overnight in the dark at 4°C. Measurements were done on a Partec PAS III flow cytometer (Partec, Germany) equipped with a 20 mW solid state laser (Sapphire 488-20) emitting at a fixed wavelength of 488 nm or on a CyFlow flow cytometer (Partec, Germany) equipped with a 100 mW diode laser (emitting at 532 nm).

Instrument settings

Before use, the instrument was calibrated using Fluoresbrite®Yellow Green microspheres (3 µm; PolyScience, Warrington, Pennsylvania). The sample was run at a speed of ca. 30-50 particles per second. At least 3000 nuclei were measured per relevant (G1) peak.

Data analysis

The data were analyzed using Flomax software (Partec, Germany). DNA contents were calculated using the ratio between the mean peak positions of the *Phytophthora* sample and the *Raphanus* or *Arabidopsis* reference standard. Histograms shown in this thesis are made with Summit v4.3.

4.6.3 Results and discussion

Selection of a suitable reference standard

For most measurements (including all measurements in Chapter 4), *Raphanus sativa* cv. Saxa was used as internal standard. The data gathered provides sufficient resolution to support our conclusions on DNA content variation and DNA ploidy variation (see section 4.3). However, when one wants to use the data for absolute genome size determination, the use of *Raphanus* as reference standard has some limitations. First, the difference in genome size between the *Phytophthora* clade 8b species with the smallest DNA contents and *Raphanus sativa* cv. Saxa is almost eightfold (0.14 pg versus 1.11 pg DNA/2C), obligating the use of a logarithmic scale instead of a linear scale (see Figure 4.10 A-B). In Figure 4.10 B, it can be seen that when the linear scale is used, the *Phytophthora* measurements have to be done on low channels, and even then the *Raphanus* G2 peak does not fit on the scale. The signal amplifiers only work in a linear mode in a certain range of signal intensity (on both linear and logarithmic scales). On a logarithmic scale, however, the smaller (non-linear) signals are spread over a wider number of channels and therefore measuring has to be done at much higher channels in order to obtain a linear response. Conversely, the use of *Arabidopsis thaliana* ecotype Col-0 as reference standard permits the use of a linear scale since the genome size is comparable to that of *Phytophthora* spp. (see Figure 4.10 C). However, the use of *Arabidopsis thaliana* ecotype Col-0 also had some limitations. First, a number of publications report the intraspecific genome size variability (up to 1.1 fold, Schmuths et al. (2004)). However, this problem should be avoided when measurements are done using the same *A. thaliana* ecotype. Second, in our study, many *Phytophthora* isolates measured had genome sizes very close to that of *Arabidopsis thaliana* ecotype Col-0, sometimes causing difficulties in interpretation because of overlapping peaks (Figure 4.10 C). When we calibrated both standards against

each other (Figure 4.10 D) we found an error of 6%. This is a recurrent problem that has been described previously (Johnston et al., 1999; Praça-Fontes et al., 2011a,b) and is due to several factors including non-linearity between reference standards with distant genome sizes.

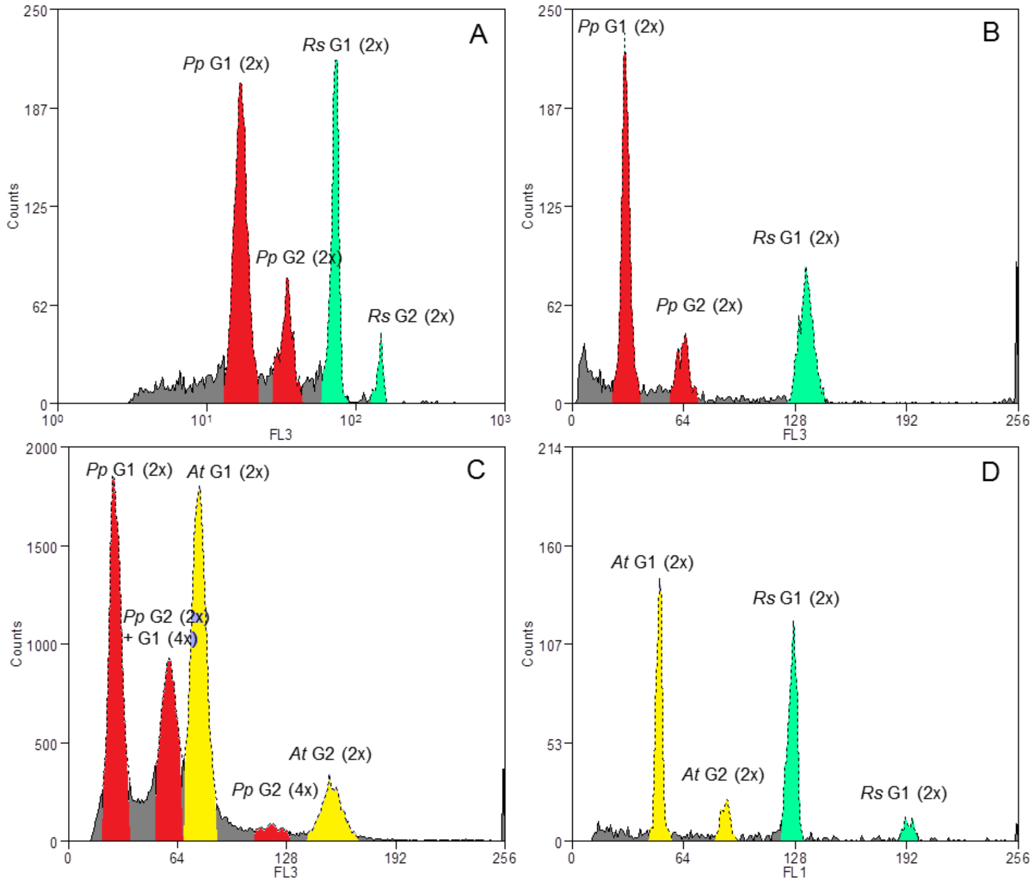

Figure 4.10: Representative histograms of DNA content measurements in *Phytophthora*.
A-B, *Phytophthora* (red) DNA content measurement using *Raphanus* (green) as internal reference standard, measured on a logarithmic scale (A) or linear scale (B); C, *Phytophthora* (red) DNA content measurement using *Arabidopsis* (yellow) as internal reference on a linear scale; C, calibration of both internal standards on a linear scale.

Sample preparation

Different amounts of reference standard (*Raphanus sativa* leaf tissue or *Arabidopsis thaliana* inflorescence tissue) and *Phytophthora* mycelium combinations were tested. The optimal combination was using a minimal amount of *Phytophthora* mycelium (about 1 mg of dry blotted mycelium) and around 5 mg of plant tissue (corresponding to about 0.5 cm^2 of leaf tissue for *Raphanus* and less than one complete inflorescence of *Arabidopsis*). Using this combination, comparable peak heights (or more precisely a comparable amount of particles per relevant peak) were derived on the histogram as shown in Figure 2. It should be noted that some variation exists depending on the age of the *Phytophthora* and *Raphanus* tissue. Best results are obtained using actively growing, young mycelium.
For *Raphanus*, best results are obtained using the youngest leaf. For optimal chopping, it is important that the plant tissue retains its turgor, so chopping should be done immediately after the tissue is removed from the plant.

Data analysis

It is common practice for DNA content measurements in plants to obtain peaks with a coefficient of variation (CV) as low as possible (<3%). Meaningful CV values can only be calculated when measuring on a linear scale. In our *Phytophthora* measurements (on the linear scale), we never obtained such low CV values. For example in Figure 4.10 B, the CV for the *Raphanus* G1 peak is 2.79%, while for the *Phytophthora* G1 peak, it is 7.68%.

4.6.4 Conclusions

The flow cytometry protocol originally developed for plants (Doležel et al., 2007), proves to be very useful for DNA content and ploidy level estimation of *Phytophthora* species. However, there exists a need for new reference standards suitable for use with small genomes and more related to *Phytophthora*.
As discussed above, both plant reference standards used in this work have some limitations. *Raphanus* obliges the use of the logarithmic scale, which is not ideal. With *Arabidopsis*, the linear scale can be used. However, since the DNA content of *Arabidopsis* was very close to that of the *Phytophthora* species we investigated, it had some limitations

for our studies. Moreover, both reference standards are plants and thus totally unrelated to *Phytophthora* taxonomically. Possible candidate reference standards belonging to the Chromalveolata include the brown algae (*Phaeophyceae*). The model organism of the brown algae, *Ectocarpus siliculosus* has recently been sequenced and its genome size has been estimated at 214 Mbp (Cock et al., 2010) which is in the desired range (between 1.2 and 2.5 times the genome size of the organism of study) for *Phytophthora* genome size measurements. Further experiments will have to determine if the nuclei extraction and sample preparation are as straightforward as with plants. Also, the presence of secondary metabolites could constrain the use of this organism as a reference standard, but this remains to be tested.
To further improve the resolution of the *Phytophthora* measurements, the composition of the buffers used for sample preparation could be optimized. Optimal buffer conditions will most likely lower the CV values of the *Phytophthora* peaks.

Acknowledgements

The authors would like to thank Danny Geelen and Nico De Storme for the suggestion to use *Arabidopsis* inflorescence tissue as a reference standard and for providing *Arabidopsis* plant material for our experiments.

In the fields of observation chance favors only the prepared mind.

Louis Pasteur

5

Virulence screening of *Phytophthora* clade 8b species and hybrids on multiple hosts

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Abstract

In previous chapters, *Phytophthora* clade 8b was expanded to a group of six host-specific species that are specifically adapted to attack winter-grown crops (mostly vegetables). Next to this, two new taxa and three different hybrid types were described, with different parental species involved in their formation. For one of the hybrid types, DNA content determination for 11 isolates by flow cytometry suggested that these isolates represent a range of evolving allopolyploid genotypes. It was hypothesized that hybridization and polyploidy can cause shifts in host range and can act as a driving factor of speciation. Pathogenicity tests of the hybrid and non-hybrid species on multiple hosts to determine the host range of the hybrid and non-hybrid isolates are described in this chapter. The eleven different clade 8b species, taxa and hybrids and *P. syringae* (clade 8d), were tested for their pathogenicity against Chinese cabbage, lettuce, leek and onion. The data shows that all three hybrid types generally show higher virulence levels than their putative parents, and other non-hybrid clade 8b species. Moreover, from the data gathered it is clear that onion (*Allium cepa*) is a common susceptible host to most of the clade 8b species and could therefore serve as a hot spot for interspecific hybridization to occur in the field.

5.1 Introduction

Phytophthora is a genus of filamentous plant pathogens belonging to the Stramenopiles. Many destructive pathogens belong to this genus such as *P. infestans*, *P. ramorum* and *P. cinnamomi*, causing potato late blight, sudden oak death and forest dieback, respectively. The genus consists of more than 120 species of which half were described during the past decade (Kroon et al., 2012), and many more remain to be described (Brasier, 2009). The genus is currently divided into 10 phylogenetic clades (Martin et al., 2014). Most of the clades are subdivided into two or more subclades. Next to this, more and more reports on natural interspecific *Phytophthora* hybrids (Man In 't Veld et al., 1998; Brasier et al., 1999; Bonants et al., 2000; Man in 't Veld et al., 2006; Nirenberg et al., 2009; Goss et al., 2011; Nagel et al., 2013) as well as artificial hybrids created in the lab (Ersek et al., 1995; Goodwin and Fry, 1994; Donahoo et al., 2008) in *Phytophthora* are accumulating. Interspecific hybrids often show a new or expanded host range compared to the parents (Schardl and Craven, 2003; Olson and Stenlid, 2002; Brasier, 2000, 2001). In Chapter 3, three new species (*P. dauci*, *P. lactucae* and *P. cichorii*) belonging to the phylogenetic clade 8b were officially described. The addition of these new species to the previously described *P. porri*, *P. primulae* and *P. brassicae* expanded the clade to a group of six species. Next to this, two new taxa were described (*P.* taxon parsley and *P.* taxon castitis) that possibly also represent separate species but could not be officially described yet due to the limited number of isolates available and the fact that some of the isolates did not produce sexual structures. With the description of these new species, some interesting common ecological features of this clade emerged. First, all species in this group preferably infect herbaceous crops (mostly vegetables) at low temperatures during winter seasons. Second, most of the species show a high degree of host specificity in the field and their names have been given accordingly. Chapter 4 described the occurrence of three different types of interspecific hybrids, arising from hybridization between different clade 8b species including *P. porri*, *P.* taxon parsley and *P. cichorii.* More specifically, a total of 13 hybrid isolates were collected from different hosts, including onion (*Allium cepa*, *A. victorialis* and *A. grayi*; 10 isolates), parsnip (*Pastinaca sativa*; 1 isolate), chrysanthemum (1 isolate) and Guayule (*Parthenium argentatum*; 1 isolate). Interestingly, all three hybrid types have been isolated from *Allium cepa.* From this observation, the hypothesis was born that *A. cepa* could be a common susceptible host for the different clade 8b species

and that it could thus serve as a hot spot for interspecific hybridization. In this chapter, we verified this hypothesis by performing pathogenicity tests of the eleven different clade 8b species, taxa and hybrids and their close relative *P. syringae* (clade 8d; Grünwald et al. (2011)) on onion and three other hosts (leek, lettuce and Chinese cabbage). Moreover, as stated above, interspecific hybrids are believed to have the potential to invade new or expanded host ranges compared to the parents but controlled experiments to test this hypothesis are scarce. *Phytophthora* clade 8b offers a good system for this type of experiments because of the different host-specific pathosystems. From the data gathered, it can be concluded that onion is a common susceptible host for most clade 8b species while the other hosts tested are only susceptible to one or a few of the clade 8b species, supporting the hypothesis stated above. Moreover, we show that the hybrids (all three types) generally show higher virulence levels than their putative parents and the other non-hybrid species.

5.2 Materials and methods

5.2.1 Isolate collection and maintenance

All isolates used in this study were freshly isolated from diseased plants, or derived from different culture collections around the world (see Table 5.1). The isolates were maintained routinely on V8 agar (see section 3.2) or on Corn Meal Agar (Beckton Dickinson). For long term storage, isolates were kept on V8 plugs at -80°C in 10% glycerol.

5.2.2 Pathogenicity tests

For the four different plant hosts (lettuce, Chinese cabbage, leek and onion), cultivars were chosen with known susceptibility to *Phytophthora* or where no such susceptibility was documented, a pre-screening was performed after which a susceptible cultivar was chosen. For the pathogenicity tests with lettuce (cv. Cobham Green) and Chinese cabbage (cv. Bilko), seeds were pre-germinated for two days in square Petri dishes (Greiner; $120 \times 120 \times 17\,\text{mm}$) containing Murashige and Skoog medium (MS) including vitamins. After this germination period, the pathogen was added by putting a mycelial plug (diam-

Table 5.1: *Phytophthora* isolates used for pathogenicity experiments

Species	Code	Host (isolated from)	Origin	Year of isolation
P. porri	S12001	*Allium porrum*	Belgium	2012
P. primulae	CBS 114346	*Primula polyantha*	New Zealand	2003
***P.* taxon parsley**	BPIC 2584	*Petroselinum crispum*	Greece	2006
***P.* taxon castitis**	CBS 131246	*Fragaria*×*ananassa*	Sweden	1995
P. dauci	CBS 127102	*Daucus carota*	France	2009
P. brassicae	K13001	*Brassica oleraceae*	Belgium	2013
P. lactucae	BPIC 1987	*Lactuca sativa*	Greece	2001
P. cichorii	CBS 133815	*Cichorium intybus*	UK	1999
Hybrid type 1	ICMP14653	*Allium cepa*	New Zealand	2002
Hybrid type 2	CBS 112969	*Allium cepa*	USA	-
Hybrid type 3	CBS 112968	*Allium cepa*	Switzerland	-
P. syringae	CBS 114110	Almond	Australia	2004

eter 5 mm) from a seven day old V8 culture at one cm of each side of the stem base. For pathogenicity tests with leek (cv. Tadorna) and onion (cv. Cassiopea), seeds were pre-germinated for 10 days in square Petri dishes containing MS medium including vitamins. Inoculation was done by putting two mycelial plugs against the same leaf of each seedling. The Petri dishes were sealed with parafilm and incubated vertically in a growth chamber at 18°C (± 2°C), with an 8/16 light/dark regime. Symptoms were evaluated 14 days post-inoculation (DPI) using the following disease scales: Chinese cabbage and lettuce: 0 = no symptoms, 1 = starting lesion on stem base, 2 = complete necrosis of stem base and/or plant dead; leek and onion: 0 = no symptoms, 1 = less than 50% of leaf infected (papery white lesion), 2 = more than 50% of leaf infected (papery white lesion). Each host plant was infected with 12 different species (see Table 5.1) and a non-infected control was added (13 treatments in total). For most *Phytophthora* species, a pre-screening was done with several isolates on their respective hosts, after which a virulent isolate that also showed good growth on MS medium was chosen for tests with all hosts. In this pre-screening, the effect of a sterile V8 plug (without *Phytophthora*) was tested on each host, which yielded no disease symptoms on any of the four host plants (results not shown). When a pre-screening was not performed (for *P.* taxon castitis, *P.* taxon parsley, *P. primulae* and *P. syringae*), an isolate was chosen based on year of isolation and growth on MS medium. Each treatment was done with at least 18 plants (n $\geq$ 18) and was repeated twice (Chinese cabbage and lettuce) or three times (leek and onion). Statistical analysis

was done using SPSS version 22 on pooled data for the different repeats. The number of plants was different for the four experiments: for Chinese cabbage, each treatment was done on 42 plants ($n = 42$) except for *P. cichorii* ($n = 36$); for lettuce at least 42 plants were tested ($n \geq 42$) for each species except *P. cichorii* ($n = 24$); for leek at least 62 plants were tested ($n \geq 62$) for each species instead *P. cichorii* ($n = 42$) and *P. dauci* ($n = 47$) and for onion, at least 59 plants ($n \geq 59$) was were tested per species except for *P. cichorii* ($n = 42$) and *P. dauci* ($n = 45$). The non-parametric Kruskal-Wallis test for k independent samples was used, after which pair-wise comparisons were performed for all treatments using Mann-Whitney U tests at a confidence level of $p = 0.05$.
For each host plant/*Phytophthora* combination, the mean disease index (DI) was calculated using the following formula:

$$DI = \frac{\sum \text{Disease class} \times \text{number of plants within that class}}{\text{Total number of plants within treatment}}$$

5.2.3 Microscopy

Phytophthora infection was checked for each host-treatment combination. After evaluation (14 DPI), diseased plantlets were put in a lactophenol-Trypan blue (0.1 g Trypan blue in 100 ml lactophenol) solution overnight. The next day, the plantlets were transferred to chloral hydrate for destaining for a maximum duration of one week. After destaining, the tissue was mounted in 70% glycerol and was examined immediately under an Olympus BX51 microscope.

5.3 Results

5.3.1 Pathogenicity tests

The results from the different repeats were very similar and therefore all data for the specific host plants were pooled. The results are shown in stacked bar charts (with the number of plants in each disease class shown relative to the total) in Figure 5.1. For each specific host plant/*Phytophthora* combination, the mean disease index (DI) is shown above each bar. Representative disease pictures for each host plant are shown in Figure

5.2. On Chinese cabbage, only *P. brassicae* could invoke strong disease symptoms. Of the other species, only hybrid type 1 could cause disease that was significantly different from the control. However, for hybrid type 1, disease only occurred in 10% of the plants, while for *P. brassicae*, all plants were infected. On lettuce, the species that was most virulent was *P.* taxon castitis, which is the closest relative of *P. lactucae* in the clade 8b phylogeny. Six other species (*P. primulae*, *P. lactucae*, *P. porri* and all three hybrid types) could invoke disease symptoms on lettuce, significantly different from the control, although the disease incidence was much lower compared to *P.* taxon castitis.
In leek, *P. porri* had the highest disease index (DI = 0.78) although this was not significantly different from the disease caused by *P. brassicae* (DI = 0.65) and hybrid type 2 (DI = 0.63). All other species could also invoke disease symptoms on leek that significantly differed from the control.

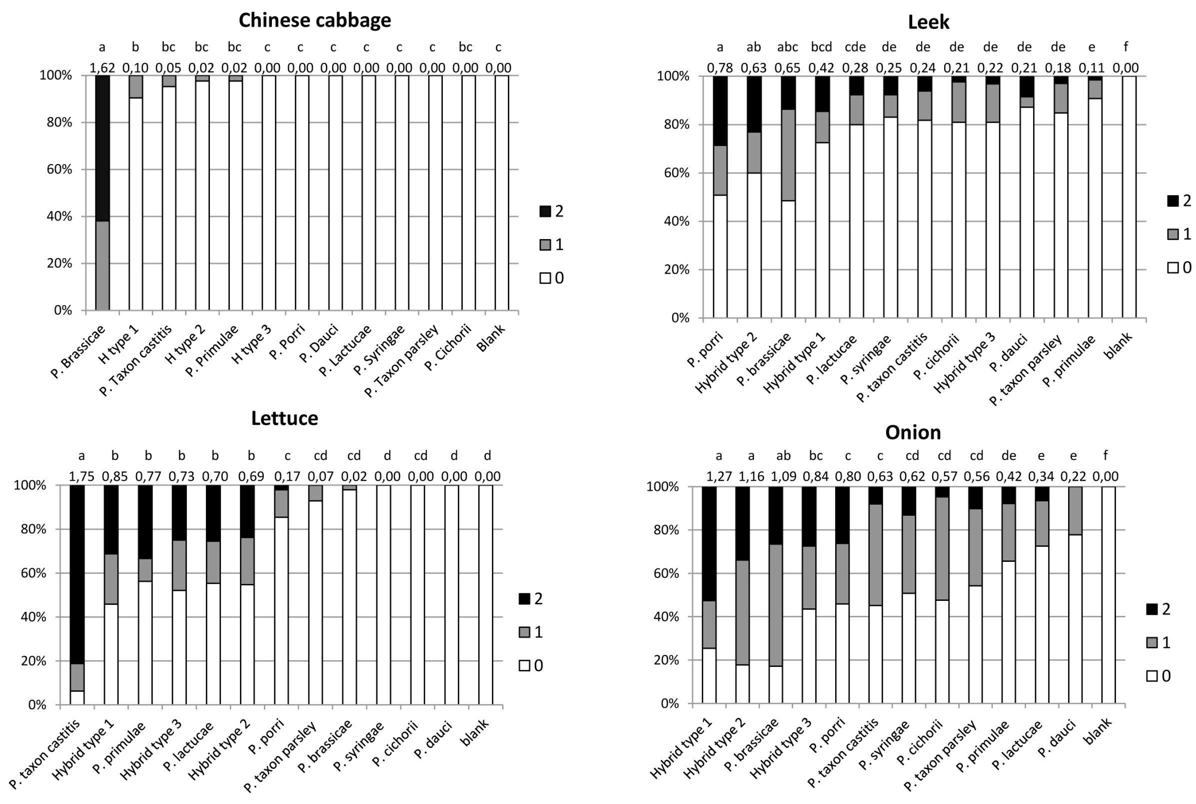

Figure 5.1: Disease symptoms on four host plants (A, Chinese cabbage; B, lettuce; C, leek; D, onion) challenged with 12 *Phytophthora* species arranged from highest to lowest disease index (DI).
'Blank' represents a non-infected control. The graph represents pooled data from two (Chinese cabbage and lettuce) or three (leek and onion) repeated experiments. Disease was scored using the following disease scales: Chinese cabbage and lettuce: 0 (white) = no symptoms, 1 (grey) = starting lesion on stem base, 2 (black) = complete necrosis of stem base and/or plant dead; leek and onion: 0 (white) = no symptoms, 1 (grey) = less than 50% of leaf infected (papery white lesion), 2 (black) = more than 50% of leaf infected (papery white lesion). Statistical analysis was done using non-parametric Kruskal-Wallis tests for 13 independent treatments, after which pair-wise comparisons were performed for all treatments using Mann-Whitney U tests. Treatments with the same letter are not significantly different from each other at a confidence level of $p = 0.05$.

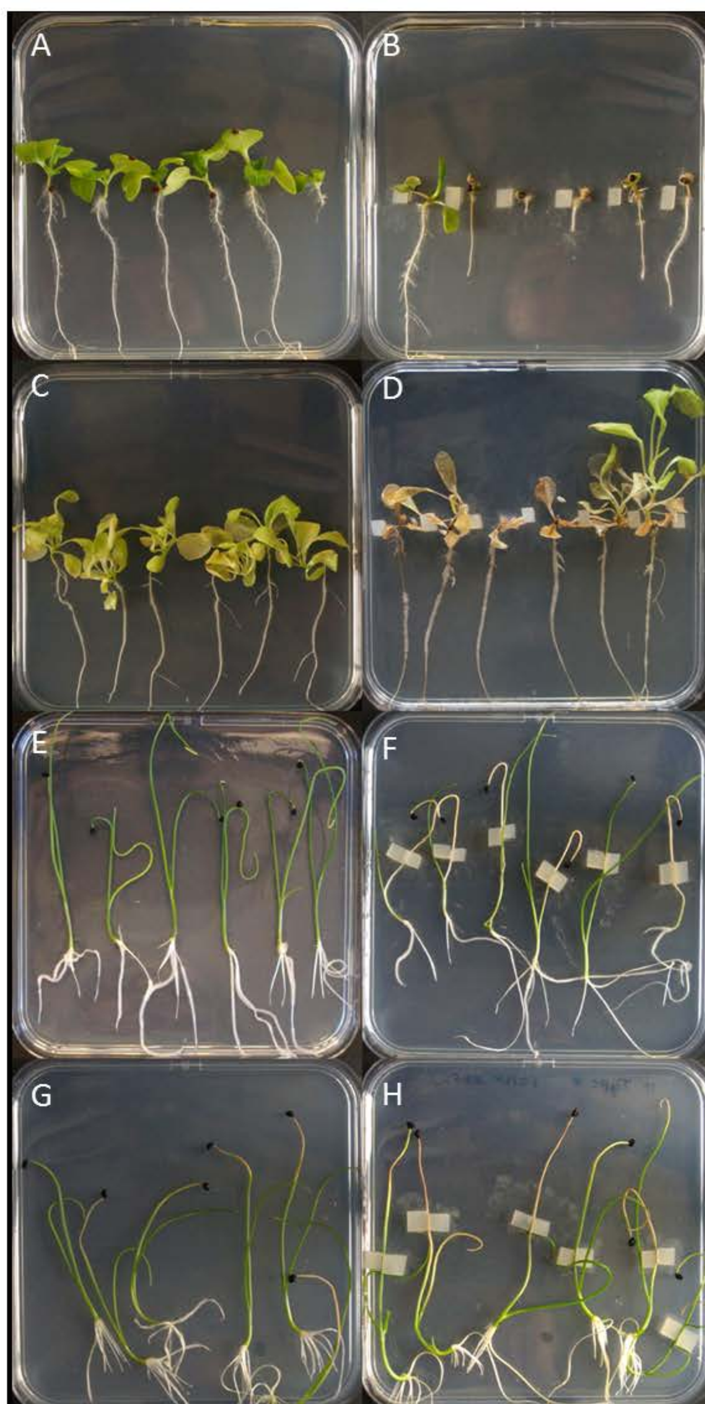

Figure 5.2: Typical disease symptoms on the four host plants.
A, Chinese cabbage/blank; B, Chinese cabbage/*P. brassicae*; C, lettuce/blank; D, lettuce/*P.* taxon castitis; E, leek/blank; F, leek/*P. porri*; G, onion/blank; H, onion/hybrid type 1.

In onion, all species tested could also invoke disease symptoms (significantly different from the control), however the average DI (mean of DI of all species on a specific host plant) was much higher than for leek (mean DI = 0.66 versus 0.32 for leek).
The species that caused the strongest disease on onion were hybrid types 1 and 2 and *P. brassicae*. Six other species could also invoke strong disease (DI between 0.56 and 0.84) in onion, namely hybrid type 3, *P. porri*, *P.* taxon castitis, *P. syringae*, *P. cichorii* and *P.* taxon parsley. In Table 5.2, all DI values are shown together with the presence/absence of hyphae/oospores as determined by microscopy.

5.3.2 Microscopy

The presence of hyphae and/or oospores in the host plant tissue was checked for each host plant/*Phytophthora* combination. Results are shown in Table 5.2. Pictures are shown in Figure 5.3. The results largely supported the results from the pathogenicity tests, i.e. for (almost) every host/treatment combination that yielded a significant disease score, hyphae or oospores were detected inside the tissue. However, there were some exceptions. For leek, disease symptoms significantly different from the control were macroscopically detected for all 12 species; however, only for the following species hyphae or oospores were detected inside the tissue: *P. porri* and hybrid type 1 (hyphae + oospores); hybrid type 2, hybrid type 3 and *P.* taxon parsley (hyphae only). For all other species, no hyphae were detected inside the tissue, or hyphal penetration was halted in an early stage by the plant. Hence, the macroscopic symptoms detected most likely represented an immune response from the plant instead of a true compatible interaction. For onion, hyphae and oospores were detected for the following species: hybrid type 1, hybrid type 2, *P. porri* and *P. primulae* (hyphae and oospores); hybrid type 3, *P.* taxon castitis, *P. syringae* and *P.* taxon parsley (hyphae only). No hyphae or oospores were detected inside the tissue for *P. brassicae*, *P. lactucae* and *P. dauci*, despite the fact that they caused significant macroscopic symptoms.

Table 5.2: Comparison of the disease index (DI) of all 52 different host/*Phytophthora* interactions arranged from highest to lowest mean DI.
The colouring scale is the same as used in Figure 5.1: green = 0% - 25% of max DI; yellow = 25% - 50% of max DI; orange = 50% - 75% of max DI; red = 75% - 100% of max DI. *o/h = oospores/hyphae. n.a. = no microscopy performed

	Chinese cabbage		**leek**		**onion**		**lettuce**	
	DI	**o/h present?**	**DI**	**o/h* present?**	**DI**	**o/h present?**	**DI**	**o/h present?**
P. brassicae	1.62	only hyphae	0.65	no	1.09	no	0.02	no
***P.* taxon castitis**	0.05	n.a.	0.24	no	0.63	only hyphae	1.75	only hyphae
Hybrid type 1	0.10	n.a.	0.42	both	1.27	both	0.85	both
Hybrid type 2	0.02	n.a.	0.63	only hyphae	1.16	both	0.69	both
Hybrid type 3	0.00	n.a.	0.22	only hyphae	0.84	only hyphae	0.73	only hyphae
P. porri	0.00	n.a.	0.78	both	0.80	both	0.17	both
P. primulae	0.02	n.a.	0.11	no	0.42	both	0.77	both
P. lactucae	0.00	n.a.	0.28	no	0.34	no	0.70	both
P. syringae	0.00	n.a.	0.25	no	0.62	only hyphae	0.00	no
***P.* taxon parsley**	0.00	n.a.	0.18	only hyphae	0.56	only hyphae	0.07	both
P. cichorii	0.00	n.a.	0.21	no	0.57	no	0.00	no
P. dauci	0.00	n.a.	0.21	no	0.22	no	0.00	no
blank	0.00	no	0.00	no	0.00	no	0.00	no

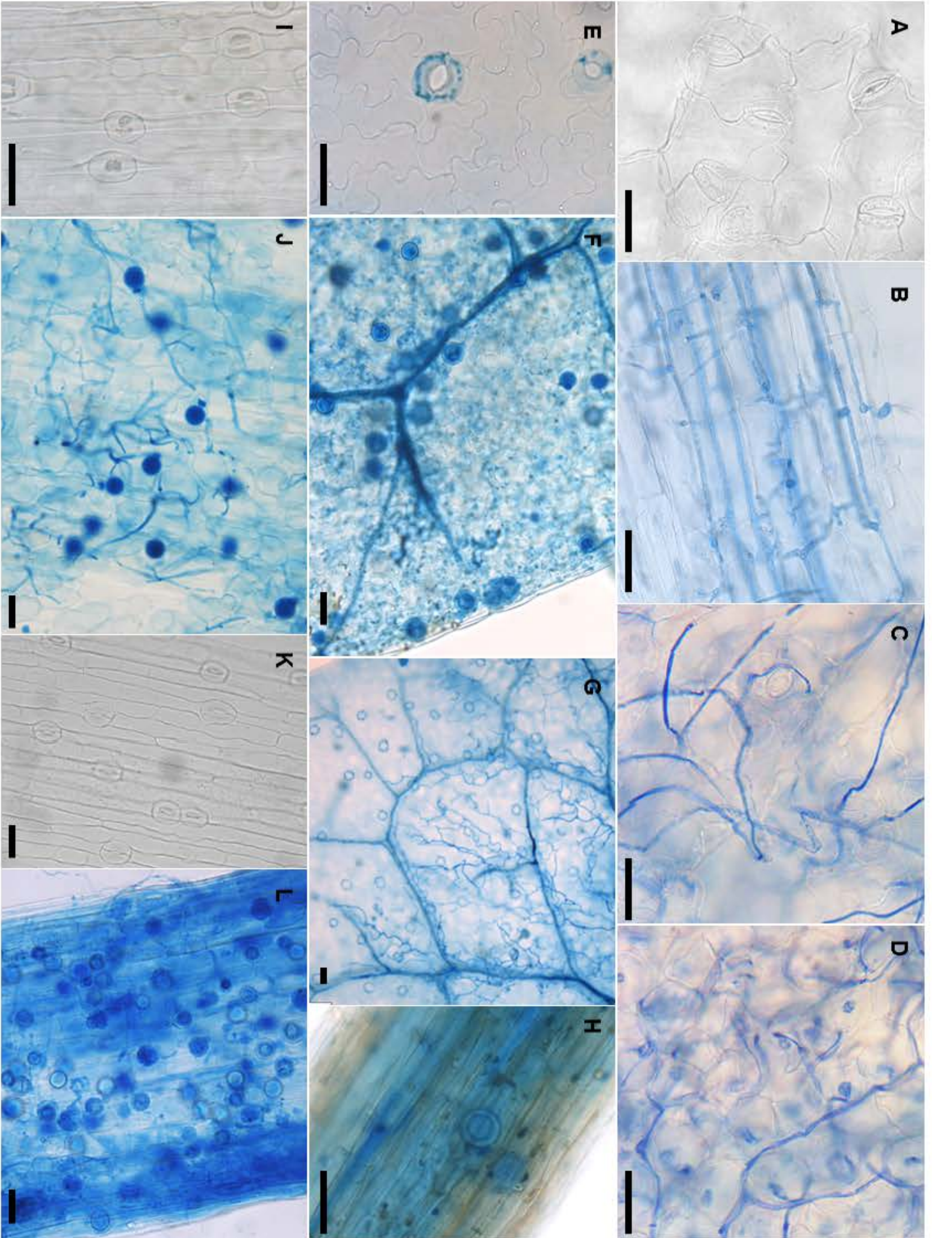

Figure 5.3: Trypan-blue staining for the presence of oospores and/or hyphae in the four hosts.
A, Chinese cabbage/blank; B-D, Chinese cabbage/*P. brassicae*; E, lettuce/blank; F, lettuce/*P. primulae*, G-H, lettuce/*P. lactucae*; I, onion/blank; J, onion/hybrid type 1; K, leek/blank; L, leek/*P. porri*.

5.4 Discussion

Phytophthora clade 8b has been described as a host-specific group of species, and their names have been derived accordingly (see Chapter 3). Indeed, for most species, isolates have only been detected on their respective host plants or host plant family (*P. porri*, *P. primulae*, *P. brassicae*, *P. cichorii*, *P. dauci* and *P. lactucae*). Next to this, two taxa were described that also represent separate taxonomic entities but could not (yet) be officially described as an official species. *Phytophthora* taxon parsley is a close relative of *P. primulae*: the two species have an identical *Ypt1* and ITS sequence but their mtDNA (*Cox1* and *Nadh1*) is different (see Chapter 4). This is bizarre since the *Ypt1* shows a much faster rate of evolution compared to mtDNA genes, as visualized by longer branch lengths in the phylogeny (Figure 4.3 and Figure 4.4). A possible explanation for this discrepancy is that *P.* taxon parsley is itself of hybrid origin, but has lost additivity in its nuclear loci (ITS and *Ypt1*), which is theoretically possible since we have detected several hybrids that have lost additivity at one or the other locus (ITS and *Ypt1*). Hence, *P.* taxon parsley could be the product of a hybridization event (probably with the same origin as the hybrid type 1 isolates), that has adapted to a new host. Hybrid type 1 isolate CBS 114040 is identical to the *P.* taxon parsley isolates in all sequences, except for the presence of two polymorphic sites in its ITS that correspond to the ITS sequence of *P. porri*, revealing its hybrid origin. This isolate was also found on parsnip (*Pastinaca sativa*), which is one of the closest relatives of parsley in the Apiaceae family.
Phytophthora taxon castitis is another taxon described in the clade 8b group that forms a separate taxonomic entity (*ITS*, *Ypt1* and mtDNA (*Cox1* + *Nadh1*) is clearly distinct from all other clade 8b species) but could not be described as a separate species since the isolates did not produce any sexual structures, nor by itself nor in combination with mating type inducer strains (see Chapter 3). Only two isolates belonging to this taxon have been found, one on strawberry and one on carrot. In our pathogenicity tests it was shown to be very aggressive to lettuce, and mildly aggressive to onion. Therefore, this taxon does not seem to be adapted to a specific host plant or family.

In this chapter the *in vitro* pathogenic potential of 11 different clade 8b species, taxa and hybrids (*P. porri*, *P. brassicae*, *P. primulae*, *P. cichorii*, *P. dauci*, *P. lactucae*, *P.* taxon castitis, *P.* taxon parsley and hybrid types 1 to 3) and their close relative *P. syringae*

(clade 8d), was evaluated on four different host plants (Chinese cabbage, lettuce, leek and onion). The goal of our experiments was to test the following hypotheses: (i) hybrids can infect a broader host range than their parents; (ii) hybrids show equivalent or higher virulence levels compared to their parents and (iii) onion is a common susceptible host of several clade 8b species and can thus serve as a hot spot for interspecific hybridization.
Although the hybrids generally showed higher virulence levels than their putative parents, none of the hybrid isolates could infect a host species that couldn't be infected by either of the parents. However, the *in vitro* system used in these tests creates an environment which is very conducive for *Phytophthora* (high humidity and optimal temperature for growth), so it could be that *Phytophthora* species that are able to cause disease on certain host plants *in vitro*, can not do so under field conditions.
Onion seems to be a common susceptible host to different clade 8b species. Also in lettuce, different species could cause significant disease symptoms, but disease indexes were lower compared to onion.

In previous chapters, we described three new species and two new taxa (Chapter 3) as well as three different hybrid types (Chapter 4) in *Phytophthora* clade 8b. In the third chapter, we noted that speciation in this clade is clearly linked with the host plant: separate taxonomic entities in this clade are almost always associated with a single host plant. In the fourth chapter we detected three different hybrid types and hypothesized that hybridization and polyploidy is a driving factor of speciation in this clade. In more detail, a recently formed hybrid will contain the complete complement of both parental genomes (allopolyploidy). Novel gene combinations such as a combined effector repertoire can enable the hybrid to invade new habitats or hosts. After allopolyploidization, different genetic processes such as chromosome loss and chromosomal rearrangements (which can be between the different parental genomes through homeologous recombination), can enable the pathogen to further adapt to a new habitat or host. Polyploidization is also known to trigger epigenetic changes in plants (see chapter 7). In case of *Phytophthora* allopolyploids, differential gene expression of effector genes induced by epigenetic changes could lead to host range changes.

One of the most important things -
sometimes the hardest thing - is to have an
open mind.

Jane Goodall

6

Polyploidy and ecological adaptation in *Phytophthora* clade 8b

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Abstract

In the previous chapters, polyploidy was found to be a common feature of *Phytophthora* clade 8b. Next to this, the *Phytophthora* clade 8b species cause diseases that only occur during winter seasons in regions with temperate climates. In this chapter, different experiments were done to determine if polyploidy offers an fitness advantage to the pathogen to survive in cool climates. Specifically, effects of cold and frost on mycelial growth and survival were conducted for *P. porri.* Next to this, fungicide (Metalaxyl-M) resistance and pathogenic potential on leek in relation to ploidy level were evaluated. A main obstacle in these experiments is that only one diploid *P. porri* isolate was available for study and therefore, no strong conclusions could be drawn. However, clear differences in cold and frost tolerance between the diploid isolate and the polyploid isolates were detected. More diploid isolates will have to be collected from the fields if these findings are to be verified. No associations could be found between pathogenic potential or Metalaxyl resistance and ploidy level. However, there was a correlation between Metalaxyl resistance in Belgium and year of isolation: only *P. porri* cultures isolated since 2005 showed Metalaxyl resistance in our experiments.

6.1 Introduction

In Chapter 4, we have detected intra- and interspecific DNA ploidy variation among the different *Phytophthora* clade 8b species, based on DNA content measurements by flow cytometry. Intra-specific DNA ploidy variation was found in *P. porri* and *P. brassicae*. In each of those species, three different DNA ploidy levels were detected. Although no genome sequence is available for any of the clade 8b species, the lowest DNA contents measured (± 0.140 pg DNA/2C) are in the same order as those measured for *P. ramorum*, a close relative belonging to clade 8c (0.146 pg DNA/2C, Vercauteren et al. (2011)). This corresponds to diploidy in *P. ramorum* since the genome size was estimated at 65 Mb by sequencing (Tyler et al., 2006). The genome size as determined by flow cytometry is around 5 Mb larger (2C = 0.146 pg so 1C = 0.073 pg or 70 Mb using the following conversion: 1 pg = 978 Mb, (Doležel et al., 2003)). Genome sizes determined by sequencing are known to be estimated lower than their real value, because of difficulties in sequencing highly repetitive parts of the genome (Bennett, 2003). Therefore, we assume that the intraspecific ploidy variations in *P. porri* correspond to diploidy, tetraploidy and mixoploidy (diploid + tetraploid nuclei in the same mycelium) and those in *P. brassicae* to triploidy, tetraploidy and hexaploidy in *P. brassicae*. In the other clade 8b species and taxa, no intra-specific DNA ploidy variation was detected. However, the mean DNA contents of these species were all similar to the polyploid DNA contents detected in *P. porri* and *P. brassicae*. Therefore, we assume that polyploidy is a general feature of *Phytophthora* clade 8b. Interspecific hybrids between *P. porri* and *P. primulae* or *P.* taxon parsley were found to have a large variation in DNA content with the lowest DNA content corresponding to an inferred diploid DNA content and the largest DNA content between the inferred pentaploid and hexaploid DNA content. The hybrids with intermediate DNA contents are therefore assumed to be in the process of diploidization and are continuously evolving in a 'ratchet'-like manner (see Chapter 7 and Gaeta and Pires (2010)). The polyploidy in the non-hybrid species could either be the consequence of ancient hybridization events or of autopolyploidization events. In *P. porri*, evidence for autopolyploidy is presented in the form of identical AFLP profiles for diploid, mixoploid and tetraploid isolates (Declercq et al., 2009).

Polyploidy occurs in many taxonomic groups but has been mostly studied in plants,

specifically angiosperms. Seventy percent of angiosperms have polyploid ancestry based on evidence from cytogenetic analysis, morphological studies of fossils (Masterson, 1994) and more recently, whole genome sequencing and EST analysis (Cui et al., 2006). Since its discovery over a century ago, polyploidy was postulated to offer an ecological advantage because of three reasons: (1) polyploid crops that have been made for breeding purposes show altered morphological and growth characteristics (Ramsey and Schemske, 2002; Van Laere et al., 2010), (2) in nature, closely related diploid and polyploid species are found in distinct geographic ranges and (3) polyploid species are often associated with alpine and arctic habitats. Therefore, the general assumption has been that polyploidy is the *causal factor* of ecological differentiation. However, there is still very little evidence supporting this hypothesis (Otto, 2007; Paterson, 2005). Ecological studies have traditionally focused on simple comparisons of diploid and naturally occurring polyploid populations of plant species that diverged from each other in the distant past. However, using this approach, one cannot distinguish between ecological differentiation caused by polyploidy by itself and ecological differentiation caused by genic changes (natural selection) arising since the time of polyploid formation. Next to this, the use of polyploid species arising from hybridization (allopolyploidy) also confounds the effects of polyploidy with the effects of the merging of two different genomes. The comparison of neo-autopolyploids (mostly created in the lab) with their diploid counterparts, is a better experimental approach. Using this approach, important evidence has recently been provided by studies in wild yarrow (*Achillea* spp., Levin (2011); Ramsey (2011)). In the paper by Ramsey (2011) it is shown that natural hexaploids show a fivefold fitness advantage over tetraploids in the habitats where the hexaploids naturally occurred. The same experiments with neo-hexaploids reveal that 70% of this fitness advantage is achieved via genome duplication alone.

In this chapter, we focus on the intra-specific ploidy variation found in *P. porri* and evaluate if polyploidy offers an ecological fitness advantage over diploidy. Specifically, cold and frost tolerance, fungicide resistance and pathogenic potential on leek were evaluated.

6.2 Materials and methods

6.2.1 DNA content estimation using flow cytometry

DNA contents of 77 *Phytophthora* clade 8b (and clade 8d) isolates were determined using flow cytometry. See Chapter 4 for a detailed description of the protocol. All measurements in Table 6.1 were done using *Raphanus sativus* cv. Saxa as an internal reference standard.

6.2.2 Growth rate analysis

Growth rates were measured for 19 *P. porri* isolates. Two plugs of each isolate were transferred to two fresh V8 plates and incubated at 18°C. The next day, growth was marked on the Petri dish and the plates were incubated at 4°, 8°or 15°C. After 8 days, radial growth was measured in four directions on each plate. A mean growth rate (in mm/day) was calculated for each plate. Then, means and standard deviations for each isolate were calculated from the two plates. Analysis of Variance was performed using SPSS v. 22 to find significant differences ($p = 0.05$) between the growth rate means of the isolates at a certain temperature.

6.2.3 Survival after freezing treatment

The survival of 14 *P. porri* isolates (representing the three different DNA ploidy levels) was evaluated after a freezing treatment. For each isolate, 10 plugs (diameter 1 cm) were removed from the outer zone of an actively growing V8 culture and each plug was transferred to a single well in a 24 well plate in a sterile manner. The 24 well plates were then closed with parafilm and put at -20°C for 8 hours. Afterwards, plugs were thawed at room temperature and excess water was removed using sterile filter paper. The plugs were plated on fresh V8 plates and incubated at 15°C. For each plug, growth (0/1) was recorded daily for 27 days. For each isolate, DNA content was measured using flow cytometry before and after freezing.

6.2.4 Metalaxyl sensitivity

Sensitivity to the fungicide Metalaxyl-M was determined for 18 *Phytophthora porri* isolates using two different formulations: Ridomil 5G (5% Metalaxyl-M; Syngenta) and Santhal®(465.2 $\mathrm{g\,l^{-1}}$ Metalaxyl-M; Syngenta). Sensitivity was tested at three concentrations: 2, 10 and 100 $\mathrm{mg\,l^{-1}}$ Metalaxyl-M amended to V8 agar. Isolates were considered sensitive to Metalaxyl-M if no growth occurred, even at the lowest concentration of 2 $\mathrm{mg\,l^{-1}}$. Isolates were considered resistant to Metalaxyl-M when no growth inhibition compared to the control treatment (V8 without Metalaxyl-M) was observed at all concentrations.

6.2.5 Pathogenicity tests

Pathogenicity tests on leek (cv. Tadorna) were done with 11 *P. porri* isolates representing the three different ploidy levels. Seeds were pre-germinated for about 10 days in square Petri dishes (Greiner; 120 × 120 × 17 mm) containing Murashige and Skoog (MS) medium including vitamins. After pre-germination, seedlings of about 5 cm long were transferred to fresh MS plates. Inoculation was done by putting two mycelial plugs (diameter 5 mm) from the actively growing margin of a seven day old V8 culture against the same leaf of each seedling. The Petri dishes were sealed with parafilm and incubated vertically in a growth chamber at 18°C (± 2°C), with an 16:8 L:D regime. Symptoms were evaluated 14 days post-inoculation (DPI) using the following disease scale: 0 = no symptoms, 1 = less than 50% of leaf infected (papery white lesion), 2 = more than 50% of leaf infected (papery white lesion). A non-infected control was added to the experiment. Each treatment was done with 18 plants (n = 18) and the experiment was performed twice. For each leek/*Phytophthora* combination, the mean disease index (DI) was calculated using the following formula:

$$DI = \frac{\sum \text{Disease class} \times \text{number of plants within that class}}{\text{Total number of plants within treatment}}$$

6.3 Results

6.3.1 DNA content estimation using flow cytometry

Mean DNA contents of the clade 8b species were already shown in Chapter 4, Table 4.3, where the emphasis was on the large variation in DNA content found for the hybrids compared to the non-hybrid species. Next to this, intra-specific DNA content variation, most likely representing DNA ploidy variation, was found for *P. porri* and *P. brassicae*. In Table 6.1, the absolute DNA content measurements of the 77 isolates measured in this study are presented. In *P. porri*, three different DNA ploidy levels probably representing diploidy (1 isolate), tetraploidy (19 isolates) and mixoploidy (diploid/tetraploid; 5 isolates) were detected. One isolate had an outlying DNA content between the 2x and 4x values (CBS 181.87), and one isolate (K06005(1)) was found to have a DNA content corresponding to pentaploidy (5x). Assuming that the greatest common divisor of the three main DNA content categories represents one chromosome complement (1x), then 1x $\approx$ 0.07 pg DNA. For *P. brassicae*, intraspecific DNA ploidy variation was detected, assuming that the 1x value of *P. porri* is also valid for *P. brassicae*, these variations represent triploidy (3x; 1 isolate), tetraploidy (4x; 6 isolates) and hexaploidy (6x; 3 isolates). In Table 6.1, a colouring scheme is applied for easy visual examination (dark green = 2x, light green = 3x, yellow = 4x, orange = 5x, red = 6x, no colour = outlying DNA content).

Next to the five mixoploid *P. porri* isolates, both *P.* taxon parsley isolates were mixoploid. One isolate (BPIC2584) had a DNA content similar to that of the *P. porri* isolates (probably a diploid/tetraploid heterokaryon), whereas the other isolate (CBS 114156) showed a considerably higher DNA content, but again the DNA content of the second population was exactly twofold the DNA content of the first population, indicating mixoploidy.

Intraspecific DNA content variation was not detected for *P. primulae*, *P. dauci*, *P.* taxon castitis, *P. lactucae*, *P. cichorii* and *P. syringae*. However, it is possible that DNA content variation also occurs in these species but that this was not detected due to the low numbers of isolates analyzed. It is difficult to infer ploidy levels for the latter six species, since DNA content does not necessarily correlate with ploidy level when comparing different species, even if they are closely related (Suda et al., 2006). However, since *P. porri* and *P. primulae* are very closely related and have a very similar main DNA content (of

ca. 0.280 pg DNA/2C = 4x), we assume that probably also *P. primulae* is tetraploid. The other species also have DNA contents in the tetraploid range. Moreover, their DNA contents are significantly higher than that of close relatives *P. syringae* (with a mean of 0.176 pg DNA/2C; SE = 0.006; n = 3) and *P. ramorum* (mean DNA content 0.146 pg DNA/2C; SE = 0.002; n = 3; Vercauteren et al. (2011)), which are assumed to be diploid.

Table 6.1: Mean DNA contents of the 77 *Phytophthora* isolates measured in this study.
[a] Host plant from which the isolate was derived; [b] Number repeated measurements. DNA contents are shaded with a colour for easy visual examination: dark green = 2x, light green = 3x, yellow = 4x, orange = 5x, red = 6x, no colour = outlying DNA content. [c] The value of the second G1 peak in mixoploid individuals. [d] Inferred ploidy level assuming that 1x = 0.07 pg DNA (derived for *P. porri*).

species	isolate	host[a]	origin	year of isolation	n[b]	1st G1 peak (pg DNA/2C)	SD (pg DNA/2C)	2nd G1 peak[c] (pg DNA/2C)	SD (pg DNA/2C)	Inferred ploidy level[d]
hybrid type 1	CBS 114040	Pastinaca sativa	Australia	-	4	0.146	0.003			2x
	CBS 112966	*Chrysanthemum sp.*	UK	-	4	0.222	0.009			3x
	CBS 139.87	*Allium grayi*	Japan	1977	3	0.223	0.017			3x
	P6817	*Allium cepa*	Australia	1980	3	0.237	0.010			outlying
	CBS 140.87	*Allium cepa*	Japan	1977	4	0.252	0.014			outlying
	ICMP 14653	*Allium cepa*	New Zealand	2002	4	0.276	0.021			4x
	CBS 114101	*Parthenium argentatum*	Australia	1983	4	0.317	0.019			5x
	CBS 138.87	*Allium cepa*	Japan	1977	3	0.326	0.001			5x
	CBS 126739	*Allium cepa*	Japan	1997	5	0.337	0.032			5x
	CBS 126738	*Allium victorialis*	Japan	1997	3	0.339	0.024			5x
	CBS 126737	*Allium victorialis*	Japan	1997	4	0.388	0.023			6x
hybrid type 2	CBS 112969	*Allium cepa*	USA	-	3	unstable				n.a.
hybrid type 3	CBS 112968	Allium cepa	Switzerland	-	4	0.281	0.010			4x
Phytophthora primulae	CBS 110165	*Primula sp.*	Germany	1998	3	0.245	0.009			outlying
	CBS 114346	*Primula polyantha*	New Zealand	2003	3	0.268	0.005			4x
	CBS 620.97	*Primula acaulis*	Germany	1997	4	0.269	0.014			4x
	CBS 116663	*Primula sp.*	Netherlands	1999	2	0.271	0.004			4x
	CBS 110162	*Primula sp.*	Germany	1997	3	0.272	0.02			4x
	CBS 110167	*Primula eliator*	Germany	1999	3	0.276	0.012			4x
	CBS 110164	*Primula sp.*	Germany	1998	1	0.278				4x
Phytophthora brassicae	CBS 113350	*Brassica oleraceae*	Netherlands	1994	2	0.206	0.02			3x
	CBS 112967	*Brassica oleraceae*	UK	-	2	0.264	0.014			4x
	CBS 112277	*Brassica oleraceae*	New Zealand	2001	2	0.268	0.021			4x

	CBS 212.82	*Brassica oleraceae*	Netherlands	1982	3	0.272	0.010			4x
	CBS 179.87	*Brassica oleraceae*	Netherlands	1987	2	0.273	0.020			4x
	CBS 686.95	*Brassica oleraceae*	Netherlands	1995	1	0.278				4x
	CBS 782.97	*Brassica chinensis*	Netherlands	1994	4	0.281	0.003			4x
	CBS 113352	*Brassica oleraceae*	Netherlands	1995	3	0.373	0.034			outlying
	CBS 127274	*Brassica oleraceae*	Belgium	2010	4	0.411	0.019			6x
	K13001	*Brassica oleraceae*	Belgium	2013	1	0.412				6x
Phytophthora dauci	CBS 114039	*Daucus carota*	Australia	2003	1	0.265				
	BorfSP370	*Daucus carota*	France	2009	3	0.274	0.008			
Phytophthora taxon castitis	CBS 688.79	*Daucus carota*	Canada	1978	2	0.215	0.017			3x
	CH112	*Fragaria times ananassa*	Sweden	1995	3	0.235	0.003			outlying
Phytophthora lactucae	BPIC 1992	*Lactuca sativa*	Greece	2003	3	0.232	0.021			
	BPIC 1991	*Lactuca sativa*	Greece	2003	2	0.235	0.005			outlying
	BPIC 1987	*Lactuca sativa*	Greece	2002	1	0.238				outlying
	BPIC 1986	*Lactuca sativa*	Greece	2002	2	0.240	0.002			outlying
	BPIC 1988	*Lactuca sativa*	Greece	2002	3	0.247	0.015			outlying
	BPIC 1985	*Lactuca sativa*	Greece	2002	3	0.250	0.014			outlying
Phytophthora cichorii	CBS 115030	*Cichorium intybus*	Netherlands	2004	3	0.326	0.026			5x
	CBS 114345	*Cichorium intybus*	Netherlands	2003	3	0.365	0.023			outlying
	CBS 115029	*Cichorium intybus*	Netherlands	2004	3	0.372	0.03			outlying
	SCRACE5388	*Cichorium intybus*	UK	1999	2	0.384	0.010			outlying
Phytophthora porri	S05014(1)	*Allium porrum*	Belgium	2005	4	0.142	0.005			2x
	S05017(2)	*Allium porrum*	Belgium	2005	3	0.139	0.005	0.275	0.002	2x + 4x
	S05007(1)	*Allium porrum*	Belgium	2005	2	0.139	0.010	0.268	0.004	2x + 4x
	B06011	*Allium porrum*	Belgium	2006	3	0.141	0.010	0.263	0.014	2x + 4x
	K05020(2)	*Allium porrum*	Belgium	2005	3	0.149	0.006	0.285	0.006	2x + 4x
	S05006(2)	*Allium porrum*	Belgium	2005	1	0.152		0.283		2x + 4x

	CBS 181.87	*Allium porrum*	Netherlands	1987	3	0.241	0.008			outlying
	S05031	*Allium porrum*	Belgium	2005	1	0.263				4x
	K06006(2)	*Allium porrum*	Belgium	2006	8	0.264	0.009			4x
	CBS 114100	*Allium porrum*	Denmark	1992	4	0.264	0.010			4x
	CBS 166662	*Allium porrum*	UK	1994	4	0.264	0.010			4x
	K12001	*Allium porrum*	Belgium	2012	1	0.265				4x
	S05029(1)	*Allium porrum*	Belgium	2005	5	0.268	0.012			4x
	S12001	*Allium porrum*	Belgium	2012	1	0.271				4x
	B06008	*Allium porrum*	Belgium	2006	2	0.272	0			4x
	K07015(4)	*Allium porrum*	Belgium	2007	2	0.273	0.001			4x
	K07015(5)	*Allium porrum*	Belgium	2007	1	0.273				4x
	K06007(2)	*Allium porrum*	Belgium	2006	1	0.273				4x
	K07015(2)	*Allium porrum*	Belgium	2007	2	0.273	0.014			4x
	K05025(1)	*Allium porrum*	Belgium	2005	2	0.274	0.011			4x
	CBS 802.95	*Allium porrum*	Netherlands	1992	3	0.274	0.018			4x
	S05011	*Allium porrum*	Belgium	2005	2	0.275	0.012			4x
	S05012(2)	*Allium porrum*	Belgium	2005	2	0.28	0.004			4x
	B06005(1)	*Allium porrum*	Belgium	2006	5	0.283	0.010			4x
	S05009	*Allium porrum*	Belgium	2005	2	0.283	0			4x
	CBS 673.95	*Allium porrum*	Netherlands	1995	1	0.298				4x
	K06005(1)	*Allium porrum*	Belgium	2006	2	0.324	0.019			5x
Phytophthora taxon parsley	BPIC 2584	*Petroselinum crispum*	Greece	2006	4	0.144	0.009	0.284	0.009	2x + 4x
	CBS 114156	*Petroselinum crispum*	Australia	2003	4	0.171	0.005	0.347	0.006	outlying
Phytophthora syringae	CBS 114110	*Almond*	Australia	2004	2	0.165	0.009			
	CBS 346.52	*Prunus armeniaca*	New Zealand	1952	2	0.166	0.005			
	CBS 110161	*Rhododendron*	Germany	1995	2	0.173	0.02			

6.3.2 Growth rate analysis

Mean growth rates (in mm d^{-1}) are shown in Table 6.2. Since only one diploid isolate (S05014(1)) was available, no grouping could be done and mean growth rates are shown for each isolate separately. For easy visual examination, in Figure 6.1, the distribution of mean growth rates of all isolates for each temperature is shown (green = 5x, blue = 4x, yellow = 2x + 4x, red = 2x). Isolate K06005(1), which had an inferred pentaploid (5x) DNA content, had a much slower growth rate than all other isolates at all temperatures. The diploid isolate S05014(1) showed a comparable mean growth rate to the other isolates at 15°C, while at lower temperatures, its growth rate clearly dropped relatively to the other isolates and was significantly lower than all other tetraploid and mixoploid isolates at 4°C. The mixoploid isolates showed comparable growth rates to the tetraploid isolates at all temperatures.

Table 6.2: Mean growth rates of 19 *P. porri* isolates representing different DNA ploidy levels.

Isolate	Inferred ploidy level	Growth rate (mm/day)					
		15°C		8°C		4°C	
		mean	SD	mean	SD	mean	SD
K05025(1)	4x	4.64	0.09	2.54	0.02	1.64	0.09
B06005(1)	4x	4.40	0.03	2.51	0.02	1.65	0.08
K07015(5)	4x	4.51	0.07	2.47	0.05	1.79	0.04
B06008	4x	4.43	0.01	2.47	0.09	1.72	0.09
K07015(2)	4x	4.15	0.09	2.44	0.07	1.73	0.00
K07015(4)	4x	4.31	0.21	2.40	0.05	1.73	0.05
B06011	2x + 4x	4.46	0.05	2.38	0.07	1.49	0.04
S05009	4x	3.83	0.02	2.18	0.06	1.50	0.04
S05012(2)	4x	3.77	0.03	2.17	0.02	1.48	0.05
K06007(2)	4x	4.64	0.23	2.16	0.12	1.30	0.08
S05010	4x	3.51	0.04	2.14	0.08	1.47	0.00
K05020(1)	2x + 4x	4.46	0.11	2.13	0.05	1.46	0.01
S05007(1)	2x + 4x	3.93	0.18	2.11	0.03	1.36	0.06
S05029(1)	4x	4.21	0.02	2.09	0.10	1.39	0.00
S05006(2)	2x + 4x	3.64	0.05	1.99	0.08	1.26	0.02
S05017(2)	2x + 4x	3.56	0.08	1.93	0.04	1.28	0.03
K06006(2)	4x	3.95	0.19	1.87	0.04	1.33	0.06
S05014(1)	2x	4.25	0.07	1.80	0.08	1.07	0.00
K06005(1)	>4x	2.54	0.06	1.17	0.05	0.77	0.02

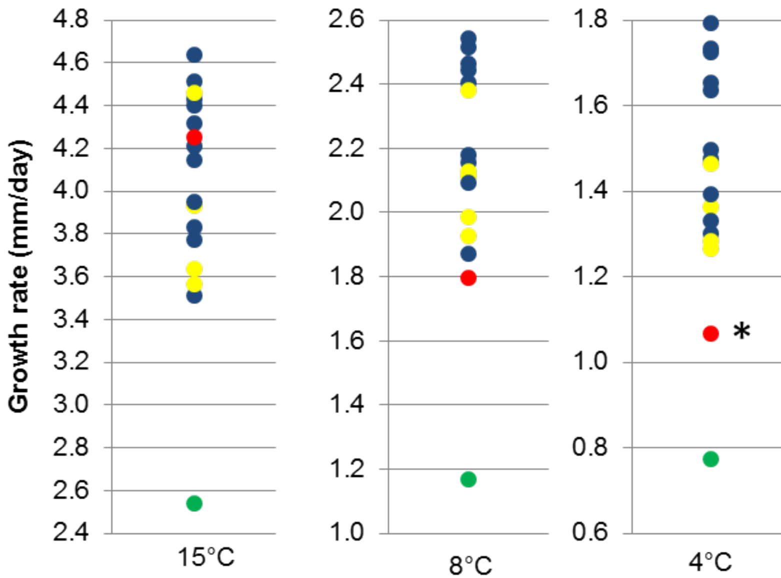

Figure 6.1: Distribution of growth rates for the 19 *P. porri* isolates at 3 temperatures (15, 8 and 4°C).
Blue dots represent tetraploid isolates, yellow dots represent mixoploid isolates, the red dot represents the diploid isolate S05014(1) and the green dot represents the pentaploid isolate K06005(1). Analysis of Variance was performed using SPSS to find significant differences between the growth rate means at a certain temperature. An asterisk* next to a dot means that the growth rate was significantly different from all other isolates at a confidence level of p = 0.05.

6.3.3 Survival after freezing treatment

Survival of 14 *P. porri* isolates representing different DNA ploidy levels was evaluated after a freezing treatment (8 hours at -20°C). The results are shown in Table 6.3.

All tetraploid and mixoploid isolates showed (nearly) complete regeneration by 27 days after the freezing treatment as determined by mycelial growth on V8 agar. A large variation in regeneration time (number of plugs showing growth) was detected with the fastest regeneration time being 3 days after plating for isolate B06011. The diploid isolate (S05014(1)) showed only 10% regeneration (1 of 10 plugs) after 14 days. The test was repeated with similar results for a subset of the isolates (here the survival percentage was determined 6 days after freezing).

Table 6.3: Survival of 14 *P. porri* isolates representing different DNA ploidy levels after 8h at -20°C. n.a.: not measured

isolate	DNA ploidy level	Number of surviving plugs after 27 days (Time in days by which max. survival was reached)	exp. 2 (number of surviving plugs after 6 days)	exp. 3 (number of surviving plugs after 6 days)
B06011	2x + 4x	10/10 (3)	4/5	n.a.
K06005(1)	5x	10/10 (6)	n.a.	n.a.
S05009	4x	10/10 (7)	5/5	n.a.
S05012(2)	4x	10/10 (7)	5/5	5/5
B06008	4x	10/10 (8)	5/5	5/5
K07015(4)	4x	10/10 (8)	5/5	5/5
B06005(1)	4x	10/10 (10)	5/5	n.a.
K07015(2)	4x	10/10 (10)	1/5	1/5
S05029(1)	4x	10/10 (13)	n.a.	n.a.
K05020(2)	2x + 4x	10/10 (13)	5/5	n.a.
K05025(1)	4x	10/10 (15)	n.a.	n.a.
S05017(2)	2x + 4x	10/10 (20)	4/5	n.a.
S05006(2)	2x + 4x	9/10 (13)	5/5	n.a.
S05014(1)	2x	1/10 (14)	0/5	0/5

In the first experiment, all isolates were subjected to DNA content measurement (using flow cytometry) before and after freezing treatment. No differences in DNA content were detected.

6.3.4 Fungicide resistance

Results were in perfect agreement for both formulations of the fungicide Metalaxyl-M (Ridomil 5G and Santhal®) and are presented in Table 6.4. There was no correlation between DNA ploidy level and Metalaxyl-M sensitivity. However, there was a correlation between isolation year and Metalaxyl resistance. Resistance was only found in isolates derived in and after 2005.

Table 6.4: Sensitivity to Metalaxyl-M of 18 *Phytophthora porri* isolates.
0 = sensitive, 1 = resistant. * at 100 mg/L Metalaxyl-M, isolate S05009 showed a growth reduction (only 1 cm growth had occurred, in comparison to about 4 cm in the control and in the lower Metalaxyl-M concentrations).

isolate	Year of isolation	DNA ploidy level	Metalaxyl-M		
			$2\,\mathrm{mg\,l^{-1}}$	$10\,\mathrm{mg\,l^{-1}}$	$100\,\mathrm{mg\,l^{-1}}$
CBS 181.87	1987	<4x	0	0	0
CBS 802.95	1992	4x	0	0	0
CBS 114100	1992	4x	0	0	0
CBS 673.95	1995	4x	0	0	0
CBS 116662	1994	4x	0	0	0
K05020(2)	2005	2x + 4x	0	0	0
S05007(2)	2005	2x + 4x	0	0	0
S05012(2)	2005	4x	0	0	0
S05017(2)	2005	2x + 4x	0	n.a.	0
S05029(1)	2005	4x	0	0	0
K05025(1)	2005	4x	0	0	0
K06006(2)	2006	4x	0	n.a.	n.a.
S05009	2005	4x	1	1	1*
K07015(4)	2007	4x	1	1	1
S05014(1)	2005	2x	1	1	1
B06011	2006	2x + 4x	1	1	1
B06005(1)	2006	4x	1	1	1
K07015(2)	2007	4x	1	1	1

6.3.5 Pathogenicity tests

The results of the pathogenicity tests with 11 *Phytophthora porri* isolates on leek are shown in Figure 6.2. The pathogenic potential was not correlated with ploidy level, and all isolates tested could invoke serious disease symptoms on leek, with varying disease index.

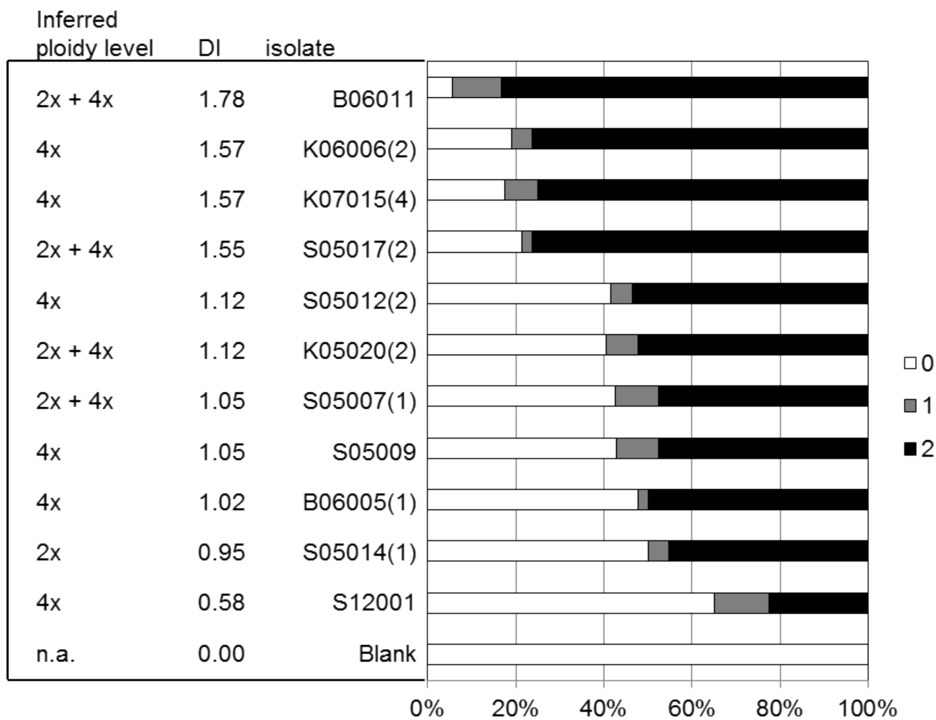

Figure 6.2: Disease severities of 11 *Phytophthora porri* isolates on leek. The disease index (DI) and the inferred ploidy level is indicated for each isolate.

6.4 Discussion

Phytophthora clade 8b is a distinctively cold tolerant clade: their optimum (18°C) and minimum (0°C) growth temperatures are among the lowest observed in the whole genus (see Chapter 3). Moreover, the diseases caused by these pathogens only occur during

winter seasons in regions with temperate climates. In this study, we have detected that polyploidy is a common feature of the species in this clade (see Chapter 4). Hence, the main hypothesis we wanted to test in this chapter was if polyploidy offers a fitness advantage at cold temperatures. This hypothesis has already been made a long time ago by Sansome (1977), who found that *P. infestans* isolates in temperate regions are mainly tetraploid, while isolates from Mexico (the species' center of origin) were found to be mainly diploid. Controlled experiments to test this hypothesis have never been done in *P. infestans*.
In plants, the occurrence of polyploid plants is significantly higher in arctic regions. Research on polyploidy in arctic plants is reviewed in Brochmann et al. (2004). The fact that polyploid genotypes originate more frequently in arctic regions might be due to cold-induced meiotic defects rendering unreduced gametes, as has been recently shown in different studies (De Storme et al., 2012; Mason et al., 2011). However, the *success* of polyploids in these regions must lie in an advantage over their diploid counterparts. Brochmann et al. (2004) conclude that the evolutionary success of polyploids in the Arctic may be based on their fixed-heterozygous genomes, which buffer against inbreeding and genetic drift through periods of dramatic climate change.

Evidently, when evaluating ecological differentiation between different ploidy levels of a species, each group should consist of a statistically relevant number of isolates. Otherwise, no significant conclusions can and should be made. In our case, only one diploid *P. porri* isolate was available for study. For this diploid isolate, we found a reduction in growth rate at low temperatures, which was not found for the tetraploid and mixoploid isolates. Moreover, the diploid *P. porri* isolate showed almost no survival after a freezing treatment of 8 hours at -20°C, while all other isolates showed an (almost) complete regeneration. More diploid isolates should be collected by field sampling to confirm these findings. No significant differences could be detected between diploid and polyploid isolates in Metalaxyl-M resistance or in pathogenic potential on their respective host plants. Resistance to Metalaxyl-M is most likely due to target site mutations in one or two proteins (Randall et al., 2014). Therefore, ploidy level differences are not expected to have a large effect on Metalaxyl-M resistance. Differences in baseline sensitivity to Metalaxyl-M have been described (Judelson and Senthil, 2006), and an effect of ploidy level on this

baseline sensitivity is more likely to be revealed in a quantitative test, such as the determination of the EC_{50} (Metalaxyl-M concentration at which the growth is reduced by 50% compared to the control) for each isolate.

A notable finding was that Metalaxyl-M resistance is occurs only in Belgian isolates isolated after 2005. Metalaxyl resistance has already been described for *P. porri* in the UK (Locke et al., 1997), where it arose shortly after the introduction of Metalaxyl-based fungicides in the control of white tip disease of leek. In Belgium, the resistance is probably also due to the introduction of this type of fungicides for white tip control. The Metalaxyl containing fungicide Folio Gold®(Syngenta) is used to combat this disease, although it was only officially registered in 2011 (Franky Vandenberghe, personal communication). Introduction of resistant isolates from outside Belgium, or spraying of Metalaxyl-M on other crops in close proximity to leek fields could also explain this observation.

Other factors could be involved in the dominance of polyploid isolates over diploid isolates that were not tested here. One plausible explanation is that, through polyploidy, the pathogen can expand its effector repertoires, which could be a selective advantage in the plant-pathogen co-evolutionary arms race. This hypothesis will be discussed in Chapter 7.

It is not the strongest of the species that survives, nor the most intelligent one. It is the one that is the most adaptable to change.
Charles Darwin

7

Why are there so many *Phytophthora* pathogens: does polyploidy play a role?

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Abstract

Phytophthora species are notorious for their genomic flexibility, enabling them to quickly overcome (acquired) host resistance or adapt to new hosts. The genomic flexibility is thought to be linked to changes in effector repertoire, which was found to be very dynamic. These changes include recombination or gene conversion among paralogs, gain or loss of repeated domains and selection on point mutations. In this chapter, we present an overview of the literature on polyploidy in *Phytophthora* and we postulate that polyploidy could be an important driver of effector evolution. Although it is unclear to what extent polyploidization events have occurred in the evolution of the genus, several extant species are polyploids, and genomic elements that are typically associated with polyploidy, such as repetitive sequences and transposons, are overrepresented in *Phytophthora* genomes.

7.1 Introduction

Polyploidy is very common among plants, where it is usually associated with increased vigour (Otto, 2007; Bennett, 2004; Ramsey and Schemske, 2002; Soltis and Soltis, 2009). Polyploidy has long since been associated with increased adaptive potential, although clear-cut examples are hard to come by (Ramsey, 2011). Polyploidy also occurs in some animal classes like fish and amphibians (Comai, 2005; Mable, 2003). To date, in mammals, polyploid species have not been detected. The only report of a tetraploid mammal, the South-American red vizcacha rat, was proven to be incorrect (Svartman et al., 2005). On the other hand, polyploidy is associated with some specific mammalian tissues, including liver cells and megakaryocytes. Moreover, polyploid and specifically aneuploid cells take up a large part of most malignant tumours in humans (Davoli and Lange, 2011). Evidence is emerging that polyploidy also played a role in the evolutionary history of other eukaryotic groups such as the Fungi and oomycetes (Albertin and Marullo, 2012; Martens and Van de Peer, 2010). However, studies on polyploidy in fungi/oomycetes are very limited.

Polyploids are divided into two main classes based on their mode of origin: allopolyploids contain two (slightly) divergent genomes and are formed as a consequence of interspecific hybridization, while autopolyploids originate through genome doubling within a single species, although it may involve crossing between genetically differentiated populations (Soltis and Soltis, 2009) and it is sometimes more appropriate to use the term 'allopolyploid' also for these intra-specific hybrids. Other types of polyploidy exist such as aneuploidy, endopolyploidy and mixoploidy. See Table 2.2 for a glossary of the key terminology in polyploidy research.

Phytophthora is a genus of plant pathogenic, filamentous oomycetes belonging to the eukaryotic lineage of Stramenopiles. *Phytophthora infestans*, the infamous potato late blight pathogen, not only gave birth to the discipline of plant pathology 150 years ago, but remains one of the most destructive plant diseases worldwide with the economic loss estimated at €5.2 billion per year (Haverkort et al., 2009). The genus *Phytophthora* contains more than one hundred species that are virtually all plant pathogens (Kroon et al., 2012). Within a single species, many different pathogenic lineages often exist, which are called 'races' in phytopathological jargon. During the last decade, the number of *Phytophthora* species has more than doubled and many new species remain to be described

(Brasier, 2009).

Recent leaps in the understanding of *Phytophthora* infection biology have been made due to advances in DNA sequencing technology which has caused a surge in genomic research ('the genomic era') (Jiang and Tyler, 2012). The most important notion that came out of this research is that oomycete genomes display a strong dual organization in which conserved housekeeping genes are concentrated in syntenic, gene-rich blocks that evolve slowly, whereas effector genes are predominantly located in highly dynamic, repeat-rich regions which show a fast evolution: the so-called '2-speed genome'. These effector genes include RXLR and CRN genes, which are very abundant in Phytophthora genomes and have been coined as the determinants of host specificity (Schulze-Lefert and Panstruga, 2011).

As mentioned, these effector genes are located in dynamic, gene-sparse regions in the genome that evolve at high speed. This probably provides the pathogen with the ability to quickly adapt to new host plants or acquired host resistance. But, exactly how this rapid evolution takes place remains largely unknown. A recent paper on the evolution of *P. ramorum* RXLR effectors (Goss et al., 2013) indicated loss or gain of repeated domains, recombination or gene conversion among paralogs, and selection on point mutations as likely evolutionary paths. Another recent paper describes host specialization in *P. mirabilis*, a close relative of *P. infestans* to be clearly linked to single amino acid polymorphisms in effector proteins and their associated host plant targets (Dong et al., 2014; Kå hrström, 2014).

In this chapter, we provide an overview of the available literature on polyploidy in *Phytophthora*, and we suggest that it could play an important role in adaptive evolution in *Phytophthora*. Integration of knowledge on polyploidy and hybridization from plant science into the field of (plant) pathology and vice versa could enhance the understanding of pathogen evolution on one hand and of adaptive evolution of polyploid species on the other hand. We strive to do this by giving a chronological overview of the literature on polyploidy in *Phytophthora*, followed by a discussion of how polyploidy could play a role in the flexibility of *Phytophthora* genomes. This last part is supported with parallel data from plant polyploidy research.

7.2 Techniques for *Phytophthora* karyotyping: old and new

Chromosome number is the most accurate measure of ploidy level and although plant chromosomes can easily be visualized by light microscopy, it proves to be problematic in Fungi and oomycetes due to the small size of the chromosomes (Wieloch, 2006). After the initial reports by Sansome and colleagues in the 1970s (Sansome and Brasier, 1973, 1974; Sansome, 1977), up until today, virtually no reports on *Phytophthora* karyotyping have been published. The lack of an easy karyotyping method has hampered the study of polyploidy in both Fungi and oomycetes. Alternative karyotyping methods include electrophoresis methods such as PFGE (pulsed field gel electrophoresis) and CHEF (contour-clamped homogeneous electric field) electrophorosis. These methods have been used successfully in Fungi (Mehrabi et al., 2007; Wieloch, 2006). For *Phytophthora*, these methods have been used as well, but they were not very successful. This is mainly due to the larger genomes (and thus larger chromosomes) of oomycetes compared to Fungi (Howlett, 1989; Tooley and Carras, 1992). Moreover, polyploidy is hard to detect by electrophoretic karyotyping since duplicated chromosomes will comigrate. Another method that has been used in the past is cytophotometry, in which nuclei are stained with a specific DNA stain, and then microscopically compared to nuclei with known DNA content. This technique can determine the total DNA content within a single nucleus, but gives no information on chromosome number and ploidy level. Recently, flow cytometric methods have been developed for *Phytophthora* DNA content estimation (see section 4.6 and D'hondt et al. (2011)). Flow cytometry is a high throughput alternative for cytophotometry that offers many advantages including reduced labor and increased resolution since thousands of nuclei can be measured per minute. The addition of DNA content measurements by flow cytometry to standard genetic diversity screenings of *Phytophthora* pathogens could be a valuable tool for detecting intra- or interspecific ploidy variation. Genome sequencing projects, which are very abundant nowadays, also deliver an estimate of the 1C (haploid) DNA content. Comparison of this 1C DNA content to the nuclear DNA content measured by flow cytometry can also give an indication of ploidy level.

7.3 Autopolyploidy: forming different pathogenic races of a species

The first report describing natural (auto)polyploidy in *Phytophthora* came from Dr. Eva Sansome. She was a pioneering fungal geneticist who also provided the evidence for vegetative diploidy in the oomycetes, in contrast to the long standing view that they were vegetatively haploid like the Fungi (Sansome and Brasier, 1973). Sansome observed by light microscopy of gametangial chromosomes, that British *P. infestans* isolates contained approximately twice the number of chromosomes compared to isolates from Mexico, which is the species' geographical center of origin (Sansome, 1977). She hypothesized that *P. infestans* may exist predominantly as a polyploid in temperate regions because it might be better adapted to cooler climates. Sansome's findings of polyploidy in *P. infestans* initiated a ploidy screening of *P. infestans* populations from many countries during the 1980s and early 1990s by cytophotometry of zoospore nuclei. These studies confirmed Sansome's findings, i.e. that isolates from Mexico are mainly diploid, while isolates from other countries are mainly polyploid (Tooley and Therrien, 1989; Whittaker et al., 1991). Different studies have reported on the formation of artificial autopolyploid and/or aneuploid genotypes when crossing strains of different mating types of *P. infestans* (Carter et al., 1999; Judelson and Yang, 1998; van der Lee et al., 2004), *P. cinnamomi* (Dobrowolski et al., 2002), *P. parasitica* (Förster and Coffey, 1990) and *P. ramorum* (Vercauteren et al., 2011). In a study by Judelson and Yang (1998), a cross between a Mexican and an American isolate resulted in 85% (58/68) polyploid progeny. The authors first transformed the parental species with antibiotic resistance genes and then used the appropriate double drug selective medium to recover doubly resistant progeny. The use of this method turned out to be essential for the recovery of the polyploid genotypes since the cross had a very low oospore germination rate ($\leq 0.1\%$), rendering the traditional method of micro-dissection of germinating oospores unsuitable. The most likely explanation for these abnormalities is meiotic irregularity in the parental gametangia, since polyploidy is already occurring in the F1 generation (see Figure 7.1, panel B). These meiotic irregularities are probably linked with heterothallism. When sexual reproduction of a heterothallic species is halted due to geographic isolation of mating types, such as has been historically confirmed for *P. infestans* and *P. ramorum*, a relaxation of selection pressure on

genes important in meiosis can occur, causing meiotic defects when sexual reproduction is restored. These meiotic defects can include non-disjunction creating unreduced (2n) or unbalanced gametes. Fusion of such gametes will deliver polyploid or aneuploid progeny. These progeny can be F1 polyploid hybrids (containing genetic material from both parents) or F1 autopolyploids, since heterothallic *Phytophthora* species can produce selfed progeny when induced by the mating type hormone of an isolate of the other mating type. With the advent of the genomic era around the year 2000, reports on (auto)polyploidy in *Phytophthora* diminished. However, over the past few years, a few new interesting studies were published. In one study, recent *P. infestans* populations were analyzed for DNA content using flow cytometry (Catal et al., 2010). Large variation in DNA content was detected, as well as the occurrence of heterokaryosis (see section 7.5). In this work, using flow cytometry, intra-specific ploidy variation was detected in several clade 8b species (see chapter 4).

Using advanced computational methods, Martens and Van de Peer (2010) revealed that the *Phytophthora* genome sequences of *P. infestans*, *P. sojae* and *P. ramorum* contain large numbers of small duplicated blocks of only two or three homologous genes, referred to as 2HOM and 3HOM blocks, respectively. This was interpreted as evidence for a whole genome duplication event in the ancestor of these species, after which the genomes were heavily rearranged. Although the authors state that many independant segmental duplications can not be excluded, the apparent similar timing of emergence of these duplicate blocks, led to the conclusion of a WGD event in the ancestor. Genes important in pathogenicity were found to have been retained in excess.

In a recent study, van Hooff et al. (2014) re-evaluated the WGD hypothesis of Martens and Van de Peer (2010) and came to a different conclusion. Namely that these duplications are more likely the result of multiple segmental duplications and transposition events. They make this assumption based on the fact that many pairs of duplicated blocks are present on the same scaffold. Moreover, two different timing methods indicate that the majority of the duplicated blocks arose after the divergence of the different *Phytophthora* lineages. They conclude that genome evolution of *Phytophthora* is most likely driven by other mechanisms, such as bursts of transposon activity. Although polyploidy might not have occurred in ancestral *Phytophthora* species, it is still possible that polyploidy plays an important role in adaptive evolution of current *Phytophthoras*. Polyploidy could

be driven by humans due to increased global trade in plant material (and associated pathogens), providing opportunities for closely related pathogens to come into contact. Yoshida et al. (2013) compared the genome sequences of 11 herbarium and 15 modern *P. infestans* strains. By estimating allele frequencies of the resequencing alignments, an inference of ploidy level could be made. The *P. infestans* strain that caused the 19th century epidemic (HERB-1) was identified as being diploid. HERB-1 was dominant for about 50 years after which it was replaced by another, closely related lineage (US-1). The two US-1 strains that were tested in the study were found to be triploid. The majority of the other modern strains tested also turned out to be triploid and one strain was found to be tetraploid. Only two of the 15 modern strains tested were diploid.

Most of these autopolyploids probably originated due to meiotic problems during crosses between genetically differentiated populations of the same species. Real autopolyploids, caused by the exact doubling of an organism's genome, are considered to be rare in plants (Soltis and Soltis, 2009). In *P. porri*, the causal agent of white tip disease on leek, a true autopolyploidization event could have occurred since AFLP profiles from diploid isolates are identical to those of polyploid (and mixoploid) isolates (Declercq et al. (2009) and Chapter 4).

7.4 Allopolyploidy: forming new species or lineages with a new or expanded host range

Allopolyploidy, resulting from interspecific hybridization between two biological species, had already been suggested for *P. meadii* early on (Sansome et al., 1991), but the first conclusive evidence for natural interspecific hybridization in *Phytophthora* came with the description of hybrids between *P. cactorum* and *P. nicotianae* (Man In 't Veld et al., 1998) by isozyme analysis and RAPD patterns. Another striking example is *Phytophthora alni* (Brasier et al., 1999). This new species was detected on alder trees in the 1990s and quickly became one of the most serious threats to riparian ecosystems. Molecular and cytological evidence showed that it evolved from a recent hybridization event between *P. cambivora* and *P. fragariae*, or closely related species (Brasier et al., 2004; Ioos et al., 2006), and consists of an evolving complex of allopolyploid genotypes. After this, a hand-

ful of studies were published, describing other cases of natural interspecific hybridization events in *Phytophthora* (Bertier et al., 2013; Donahoo et al., 2008; Goss et al., 2011; Nagel et al., 2013; Nirenberg et al., 2009). These studies suggest that interspecific hybridization is an important process in *Phytophthora* evolution, offering the possibility to create new strains and ultimately, new species (see Chapter 4). Hybridization in *Phytophthora* has already been reviewed multiple times elsewhere (Brasier, 2000; Érsek and Nagy, 2008; Olson and Stenlid, 2002; Schardl and Craven, 2003). It can be concluded that (allopolyploid) hybridization occurs mainly between very closely related species (mostly sister species in the phylogeny), between which reproductive barriers are incomplete. The increased occurrence of hybrid plant pathogens is thought to be linked with the increased global trade of (infected) plant material. In this way, species that are diverging in allopatry (different geographic locations) are brought together. Selection on the formation of reproductive barriers is known to be lower when species diverge in allopatry than when they diverge in sympatry. In Figure 7.1 panel A, the process of allopolyploid hybridization is explained. The distinction between autopolyploidy and allopolyploidy is based on the mode of formation, either by hybridization (allopolyploidy) or through genome duplication within a single species (autopolyploidy). Nonetheless, the underlying causes and molecular processes are essentially the same for both types. The mechanisms of polyploidization have been studied extensively in plants and mainly constitute a range of meiotic defects. For an extensive review, see De Storme and Geelen (2013). There are some interesting findings from the field of plant science that could be extrapolated to *Phytophthora* polyploidization events. First, recent evidence in plants shows that abiotic stresses like cold temperatures can stimulate the formation of unreduced gametes (De Storme et al., 2012; Mason et al., 2011). True autopolyploids, like *P. porri*, could have originated in this way. *Phytophthora porri* is a member of *Phytophthora* clade 8b, which is a distinctively cold tolerant clade (isolates still grow at 0°C) and species of this clade are associated with winter-grown crops in regions with temperate climates (see Chapter 3). The possible correlation of polyploidy and cold temperature habitats has already been made for *P. infestans* early on (Sansome, 1977). Experiments evaluating the formation of unreduced gametes in cold-treated *Phytophthora* isolates need to be done.

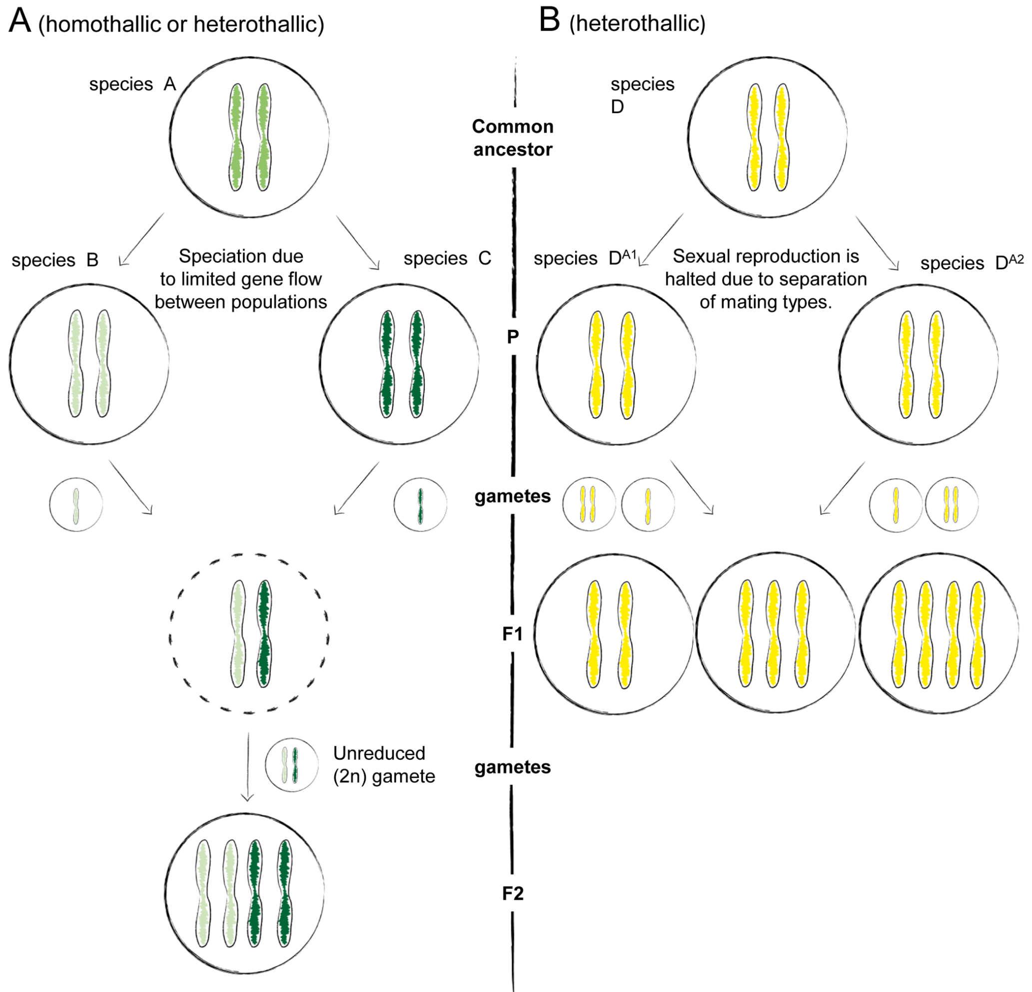

Figure 7.1: Possible ways of polyploidization in *Phytophthora*.
A, Formation of allopolyploid (interspecific) hybrids. Note that this could also occur between genetically differentiated populations of the same species (intraspecific hybridization). Speciation occurs due to limited gene flow between two populations of a species, which can be due to geographic isolation (allopatric speciation) or due to host adaptation (sympatric speciation). When two closely related species are brought together (most likely on a common host plant), but have not formed strong reproductive barriers, crossing is possible, forming a diploid zygote. Because of divergence between the different parental chromosomes (termed homeologs), the F1 generation will be meiotically unstable. A whole genome duplication (possibly through the formation of unreduced gametes) can restore fertility in the F2 generation. B, In heterothallic species, another type of polyploidization can occur when the two different mating types are geographically isolated, causing a halt to sexual reproduction. Over many (clonal) generations, this can cause a relaxation of selection pressure on genes important in meiosis. Therefore, unreduced or unbalanced gametes can be created at higher frequencies due to non-disjunction of chromosomes in meiosis. In this case, stable polyploids (triploid, tetraploid or aneuploid) can be created in the F1 generation.

7.5 Heterokaryosis and mixoploidy

Heterokaryotic strains (containing genetically different nuclei in the same mycelium) have been described multiple times in *Phytophthora* (Chapter 4; Catal et al. (2010); Gu and Ko (2000); Long and Keen (1976); Pipe et al. (2000); Sansome (1977); Tooley and Therrien (1987, 1989); Whittaker et al. (1991)). A few of these studies describe the occurrence of natural heterokaryons consisting of diploid and polyploid (mostly tetraploid) nuclei (mixoploidy). Mixoploidy has been detected in gametangial (Sansome, 1977), zoosporic (Tooley and Therrien, 1987; Whittaker et al., 1991) and hyphal nuclei (Chapter 4). The mononucleate zoospores produced from mixoploid lines were found to be either diploid or polyploid. Oddly, the single-zoospore progeny of these lines were again found to be mixoploid (section 4.3; Whittaker et al. (1991)). This points to a controlled process of polyploidization reminiscent of the process of endopolyploidy which is especially common in plants (De Veylder et al., 2011). However, endopolyploidy differs from true polyploidy in the fact that in endopolyploidy, doubled chromosomes are still attached by one chromocentre. This leaves the nucleus with a similar number of chromosome associations, but with a doubled DNA content (see box 2 in Comai (2005)). However, chromosome counts of gametangial and zoosporic nuclei in the mixoploid *Phytophthora* isolates showed a doubled number of chromosome associations, as in a true polyploid cell (Sansome, 1977; Whittaker et al., 1991). How these isolates originate and if mixoploidy has any biological significance, needs to be studied in more detail.

7.6 Is there a relationship between polyploidy and genomic flexibility in *Phytophthora*?

Polyploidy (either allo- or auto-) will bring together effector repertoires of both species or genotypes into one organism, which can lead to host range expansion and after selection on specific genotypes, it can lead to host specialization. While this can certainly be advantageous for pathogen evolution, the early generations after polyploidization are difficult for an organism (Soltis and Soltis, 2009). From plant research, it is known that the union of two genomes into one nucleus causes severe stress, the so-called 'genomic shock'. The

genomes of recent polyploids often undergo large-scale genetic and epigenetic changes. Genetic changes include movement of transposable elements and changes in genome size and structure (insertions, deletions, translocations) (Gaeta and Pires, 2010). Polyploidy consequently results in a dramatic reorganization of the transcriptome, metabolome and proteome (Jackson and Chen, 2010; Leitch and Leitch, 2008). These early generations after polyploidization have been described metaphorically as a 'polyploid ratchet' (Gaeta and Pires, 2010) since over the generations, many large-scale genetic changes occur, which cannot be undone and often lead to deterioration. However, when the the genetic changes occurring after polyploidization lead to diploidization before the occurrence of lethal genetic rearrangements, the intra-genomic recombinations can be reduced, causing genome stabilization.

In this work, a process of diploidization has been suggested for allopolyploid hybrids between *P. porri* and *P.* taxon parsley (Chapter 4). Homeologous recombination was shown by cloning rDNA internal transcribed spacer (ITS) sequences. In the hybrids, the ITS clones showed many different types of recombinations between the parental forms. Moreover, a continuous range in DNA content between the 2C and >4C values of the parents was found, and the hybrids' DNA content was positively correlated with the amount of recombination in the ITS region, suggesting a process of diploidization, keeping one of the two parental ITS states.

Homeologous recombination, as was detected in rDNA regions in clade 8b hybrids, can be due to either meiotic or mitotic recombination or gene conversion events. Mitotic gene conversion (often called 'loss of heterozygosity' or LOH) has been described for different species. Chamnanpunt (2001) detected large frequences of mitotic gene conversion in F1 crosses between different *P. sojae* genotypes. These gene conversion events lead to homozygosity at the target locus and were also shown to occur in effector gene regions (Whisson et al., 2004). In recent years, loss of heterozygosity has been described for several other *Phytophthora* species including *P. capsici* (Lamour et al., 2012; Hulvey et al., 2009; Hu et al., 2013) and *P. infestans* (Randall et al., 2014). Gene conversion tracts from 100bp up to >1Mbp have been detected. Mitotic recombination or gene conversion events in allopolyploid hybrids have not been described previously, but could be an important means of intragenomic genetic exchange. Mitotic recombination events can also be inherited by the progeny, since there is no predestined germline in *Phytophthora*. Therefore, mitotic

recombination events could contribute greatly to genome evolution after polyploidization.

Considering the above, an important question for plant pathologists is the fate of genes involved in plant-pathogen interaction (e.g. RXLR effector genes) after polyploidization. In recently formed allopolyploid hybrids, but also in autopolyploid species arising from crosses between genetically differentiated populations, the complete effectorome of both parental species or genotypes will be present. This might enable the pathogen to invade new hosts or environments. Transposon-mediated recombination, mitotic recombination or gene conversion and sexual reproduction, which is especially easy in homothallic species can deliver a range of different progeny. Thousands of oospores can be produced within days after plant infection and although many of these oospores will abort (see section 4.3 and Brasier et al. (1999)) due to lethal gene combinations or chromosome imbalances, a small percentage can be viable and might constitute new advantageous gene combinations that can enable the pathogen to adapt to a new host or environment. Mitotic recombination events are thought to have less lethal effects in asexual progeny, so they could also be an important driver of adaptive evolution.
After polyploidization, both genome upsizing and downsizing (compared to the newly formed polyploid) can take place (Leitch et al., 2008). In angiosperms, a trend towards genome downsizing has been found (Renny-Byfield et al., 2013). Genome upsizing in angiosperms has been associated with the expansion of repetitive DNA families, such as the Ty3/Gypsy class retrotransposons (Renny-Byfield et al., 2013). It has been argued that transposon activity in general plays an important role in (plant) polyploid evolution, and particularly on the diploidization process (Parisod et al., 2010). Interestingly, the *P. infestans* genome consists of large numbers of transposable elements and it has been stated that a recent proliferation of Gypsy class retrotransposons underlies its genomic expansion (Haas et al., 2009).
In plants, epigenetic processes such as DNA methylation and histone modifications are thought to play an important role in the phenomenon of heterosis (hybrid vigor) in allopolyploid hybrids (Chen, 2013). In comparison, genes involved in epigenetic processes, such as histone methyltransferases, are also enriched in the dynamic regions of *Phytophthora* genomes (Raffaele et al., 2010b). After polyploidization, epigenetic modifications could cause differential expression of the effector repertoire, possibly causing changes in

host range.

7.7 Conclusion

In this chapter, we brought together available literature on polyploidy and hybridization in *Phytophthora*. We argue that allopolyploids and most autopolyploids are formed by the same process, i.e. the crossing of genotypes that have diverged genetically (by geographic isolation or host adaptation), but have not formed solid reproductive barriers. However, it must be noted that there are slight differences in polyploidization processes between homothallic and heterothallic species (see Figure 7.1). It is clear that humans can play an important role in bringing together these genotypes by increased global trade. In our opinion, polyploidy deserves to be studied in more detail as it could play an important role in host specialization. Polyploidy has been described for several species, either intraspecific or as a consequence of hybridization, and large scale screenings for the presence of polyploidy (for example by flow cytometry) could shed more light on the occurrence and importance of the phenomenon in *Phytophthora*.

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The important thing is not to stop questioning. Curiosity has its own reason for existing.

Albert Einstein

8 General discussion and future perspectives

8.1 General Discussion

As mentioned in the problem statement (Chapter 1), this project initiated from five hypotheses. Only the first three hypothesis were investigated in this project. Each of those hypotheses will be restated below, followed by a discussion of the data gathered in this work that either confirmed or rejected it.

Hypothesis 1 and 2: *Phytophthora* clade 8b contains a group of cold tolerant, homothallic, soil-borne species that are specialized in causing disease in winter-grown vegetables and host adaptation to specific plant families and species is going on.

This project started with a genetic diversity study of *Phytophthora* clade 8b isolates morphologically similar to *P. porri*, *P. primulae* or *P. brassicae* (Chapter 3). Many reports had been published describing diseases caused by such isolates on host plants different from leek, cabbages or *Primula* spp. and a detailed study was needed. One hundred and six isolates were collected from culture collections around the world and through personal contacts or were freshly isolated from diseased plants. A first characterization of these isolates was done using rDNA ITS sequencing. ITS sequencing is cheap, fast and reliable and is now the method of choice for identifying unknown *Phytophthoras* to the species level (Robideau et al., 2011). However, our results raised some concern on the reliability of ITS sequencing for species identification, as will be discussed below.
A clear correlation between ITS sequence and host plant was detected. All isolates derived from the same hosts (leek (48 isolates), *Primula* spp. (12 isolates) and *Brassica* spp. (16 isolates)) showed identical or near-identical ITS sequences and could hence be assigned to the species *P. porri*, *P. primulae* and *P. brassicae*, respectively. All isolates derived from other hosts (30 isolates in total) had ITS sequences markedly different from *P. porri*, *P. primulae* and *P. brassicae* (96 to 99% homology in DNA sequence), except for the two isolates from parsley that had an ITS sequence identical to that of *P. primulae*. Sequencing of a second barcoding gene, the mtDNA *Cox1* gene, supported the ITS data and even provided more phylogenetic signal compared to ITS. To verify whether these groups of isolates could be assigned to separate species, the isolates were subjected to a detailed morphological and physiological study. The combined data led us to officially

describe three new species in *Phytophthora* clade 8b: *Phytophthora cichorii* sp. nov., *Phytophthora dauci* sp. nov and *Phytophthora lactucae* sp. nov., expanding the clade to a group of six species. With these descriptions, a few important common ecological features which confirmed our first two hypotheses became clear. One is their apparent host-specificity: all species described have only been found on their respective host plants (mostly vegetables) and were named accordingly. Second is their prevalence at low temperatures. All *Phytophthora* clade 8b isolates isolates were found to cause disease during winter seasons in regions with temperate climates. Moreover, it was shown that their optimal and minimal temperature for growth are among the lowest observed for the whole genus (almost all clade 8b isolates still grow at 0°C) making them well adapted to attack plants at cold temperatures.
All new species were homothallic, except for *P. cichorii* which was shown to be heterothallic. The four *P. cichorii* isolates tested were all of the A1 mating type, however, two of them produced either no oospores or only aborted oospores when combined with the A2 tester isolate.

Hypothesis 3: Interspecific hybridization is possible in *Phytophthora* clade 8b, possibly causing shifts in host range.

In a previous study done by Declercq et al. (2009), three isolates (isolated from onion and garlic) were found to have intra-specific polymorphisms in the ITS region. These polymorphisms were exactly at those positions where the ITS sequences of *P. porri* and *P. primulae* were different. Therefore, it was suggested that these isolates could have arisen by an interspecific hybridization event between *P. porri* and *P. primulae.*
Intra-isolate polymorphisms in the ITS region were found in another 10 isolates in our collection. Five of these were isolated from *Allium cepa*, two from the related wild onion species *Allium victorialis* and the other three isolates were derived from *Chrysanthemum sp.*, *Pastinaca sativa* and *Parthenium argentatum.* In Chapter 4, we showed that these 13 isolates originated from at least three independent interspecific hybridization events between different parental species belonging to clade 8b. This was done by sequencing and cloning different nuclear (ITS and *Ypt1*) and mitochondrial (*Cox1* and *Nadh1*) loci. The largest group (hybrid type 1; 11 isolates) most likely originated through hybridiza-

tion between *P. porri* and *P. primulae* or *P.* taxon parsley. Sequencing and cloning of the single copy nuclear gene *Ypt1* showed unambiguously that two different haplotypes were present within the same organism. One corresponded to that of *P. porri* and the other to that of *P. primulae* and *P.* taxon parsley. Sequencing of the mitochondrial genes could identify the species that acted as the maternal parent, since mitochondria are inherited through the oogonia. In this way, *P.* taxon parsley was identified as the maternal parent in the interspecific cross giving rise to hybrid type 1. Alternatively, it is possible that both hybrid type 1 and *P. taxon parsley* originated in a hybridization event between *P. porri* and *P. primulae*, and have accumulated mutations in their mtDNA over time. Hybrid type 2 (1 isolate) was shown to have a different maternal origin, namely a species closely related to *P. primulae*, while *P. porri* also acted as the paternal parent. The third hybrid type was shown to be unrelated to the first two types and originated through a hybridization event between two unknown taxa that are closely related to *P. cichorii*.

Also in Chapter 4, we measured nuclear DNA contents of over 50 *Phytophthora* clade 8b isolates. In contrast to the non-hybrid species, the hybrid type 1 isolates showed a large variation in DNA content. The hybrid isolates with the highest DNA contents were consistent with (allo)hexaploidy. The hybrid isolates with the lowest DNA contents corresponded to the diploid DNA content of *P. porri* and *P.* taxon parsley.
From the literature it is known that, after polyploidization (either through interspecific hybridization or through genome duplication), an organism generally enters the gradual process of diploidization. Diploidization is defined as the transition of a polyploid back to the more stable diploid cytogenetic state. However, diploidization is not necessarily associated with chromosome (and thus DNA content) loss. Differentiation between the different pairs of homeologous chromosomes in a polyploid, minimizes the amount of multivalent formation in meiosis and hence, causes the organism to behave as a diploid, cytogenetically (with only bivalent formation in meiosis). In the case of the *Phytophthora* clade 8b hybrids, chromosome (and DNA content) reduction seems to be part of the diploidization process, as will be explained below.
By cloning and sequencing the ITS region of the hybrid isolates, we found that a single ITS copy (the ITS region is repeated in tandem arrays of hundreds of copies) often contained parts of the sequence of both parents. This observation suggests that homeologous

recombination has taken place between the different parental genomes in the allopolyploid hybrids. Moreover, the amount of intra-isolate ITS polymorphism and the amount of homeologous recombination, was positively correlated with DNA content in the hybrids. This indicates that during the process of diploidization, the *Phytophthora* isolates tend to keep one of the two parental ITS states (instead of both ITS types or a new ITS type which is a mixture of the parental ones). The observation could also indicate that, in the hybrid isolates with low DNA contents, chromosome loss has occurred in the early generations after interspecific hybridization, offering no opportunity for homeologous recombination to take place.
In any case, the possibility of homeologous recombination in interspecific *Phytophthora* hybrids offers a means of adaptation to new hosts or environments. It must be noted that we are still unsure about the amount of homeologous recombination in other parts of the genome. However, it can be stated that this phenomenon could be of great importance in genomic regions containing genes important in plant-pathogen interaction, such as effectors. Homeologous recombination in these regions could cause a process that can be compared to the shuffling of a deck of cards (the combined effector repertoire of both parental organisms) which provides the hybrid progeny with new, unique combinations to play with.

Next to this, the DNA content measurements indicated that polyploidy is also ubiquitous among the non-hybrid clade 8b species (see section 4.3). Intra-specific DNA ploidy variation was detected in *P. porri* and in *P. brassicae*. In *P. porri*, three different DNA ploidy levels were detected, most likely representing diploidy, tetraploidy and mixoploidy (the presence of both diploid and tetraploid nuclei in the same mycelium). In a previous study by Declercq et al. (2009), AFLP fingerprinting had been done on isolates belonging to these three different ploidy levels. These AFLP profiles were identical for all three ploidy levels, indicating autopolyploidy in this species. In *P. brassicae*, three different DNA ploidy levels were detected, probably representing triploidy, tetraploidy and hexaploidy. In the other clade 8b species, no intra-specific DNA ploidy variation was detected. However, since most species had a DNA content in the range of the polyploid *P. porri* and *P. brassicae* species, we assume that also these species are polyploids. Evidence for this assumption in the form of chromosome counts is needed, since interspecific (and even in-

traspecific) DNA content variation does not always correlate with ploidy variation (Suda et al., 2006), i.e. two different organisms with very different DNA contents might have the same chromosome number, even when they are closely related.

As noted above, evidence in the form of chromosome counts is needed to explicitly prove these hypotheses. Unfortunately, due to their small size and difficulties in obtaining clear metaphase preparations, karyotyping is not well studied in *Phytophthora*, as is discussed in section 7.2. During this work, attempts were made to visualize *Phytophthora* chromosomes using the Germ Tube Burst Method described by Mehrabi et al. (2012), but these were not satisfactory (results not shown). Further improvements in this area could greatly facilitate hybridization and polyploidy research in *Phytophthora*.

As already touched upon above, the use of ITS sequencing for identifying unknown *Phytophthora* cultures to the species level is not ideal. The data presented in Chapter 4 indicates that hybridization events can be easily overlooked in this way. The ITS region is located in the rDNA genes that are repeated in tandem arrays of several hundreds of copies. Because of this organization, the ITS region is sensitive to concerted evolution, i.e. the homogenization of the DNA sequence of the different repeats. In the clade 8b hybrids, we detected unidirectional concerted evolution, most likely caused by large scale genomic changes in the early generations after hybridization. In this way, only one of the two parental haplotypes prevails, thus erasing the footprints of hybridization. Hence, the presence of interspecific hybridization is easily overlooked when sequencing only the ITS region. The addition of a second barcoding gene, the mtDNA *Cox1* which is already in use as a 'barcode of life' in the animal kingdom (see `http://www.barcodeoflife.org/`) has been suggested by (Robideau et al., 2011). However, the addition of this locus will not identify hybrids either since the mitochondria are inherited maternally. Hence, hybrids will show the same *Cox1* sequence as the maternal parent.
For these reasons, it is possible that the occurrence of interspecific hybridization in *Phytophthora* is highly underestimated. The sequencing and cloning of single copy nuclear genes, like *Ypt1* or others (see Goss et al. (2011)) will most likely detect a recent hybridization event. The addition of DNA content measurements, especially in large scale sampling and genetic diversity studies, will offer even more insights in the occurrence of

interspecific hybridization and polyploidy in *Phytophthora*.

In conclusion, in Chapter 4 we have shown that interspecific hybridization is a common event in *Phytophthora* clade 8b. We have speculated that hybridization and polyploidy could be an important way of adaptation and speciation in this clade and in the genus *Phytophthora* in general. Studies evaluating the pathogenic potential of the clade 8b hybrids were needed to test this hypothesis. These experiments were performed in this project and are described in Chapter 5.
The pathogenic potential of the three hybrid types was tested on four different hosts (leek, onion, lettuce and Chinese cabbage) and compared to that of the non-hybrid clade 8b species and taxa. The data showed that all three hybrid types generally displayed higher virulence levels than the non-hybrid species. From Chapters 3 and 4, the question arose how and where the different clade 8b species can meet intimately, which is a requirement for interspecific hybridization to occur. The question is noteworthy, since the clade 8b species were shown to be very host-specific. However, all three hybrid types have been detected on onion (*Allium cepa*), which led us to hypothesize that onion could be a common susceptible host to different clade 8b species, offering a hot spot for interspecific hybridization to occur in nature. Also, since the clade 8b species show a clear preference for winter-grown field vegetables that are commonly used in crop rotation systems, it is likely that the different species can be present in the same field. The pathogenicity tests conducted in Chapter 5 show that onion is indeed a common susceptible host for many of the clade 8b species and hence can function as a hot spot for interspecific hybridization between different clade 8b species.

Based on the findings in Chapters 3 and 4, an important new research question arose, namely: 'Does polyploidy offer an ecological advantage to the pathogen?'. In the last two chapters, we investigated this question.

In Chapter 6, we tried to link the prevalence of polyploid *P. porri* isolates with an ecological fitness advantage. Specifically, we evaluated the cold, frost and fungicide (Metalaxyl-M) tolerance of the different ploidy levels in these species. We also evaluated the pathogenic potential of isolates with different ploidy levels on leek.

A main obstacle in these experiments is that only one diploid *P. porri* isolate was available for study. Therefore, valuable comparisons between groups could not be made. Nonetheless, there were clear differences in cold and frost tolerance of the diploid isolate compared to the mixoploid and tetraploid isolates. The diploid *P. porri* isolate showed a clear reduction in growth rate compared to the polyploid isolates at 8°and 4°C, while its growth rate was not clearly different from the polyploid isolates at 15°C. Moreover, when mycelium plugs underwent a freezing treatment of 8 hours at -20°C, the diploid isolate almost never survived, while the polyploid isolates almost always survived the treatment.

No correlation could be found between ploidy level and pathogenic potential on leek or Metalaxyl-M resistance. All *P. porri* isolate tested could invoke serious disease symptoms on leek, with varying disease severity. Resistance to Metalaxyl-M was either absent (complete growth inhibition) or complete (no growth inhibition compared to the control). However, in our tests we used a qualitative test (growth or no growth at 2, 10 and 100 mg/L Metalaxyl-M amended to V8 medium), while a quantitative test (f.e determination of the EC50 for each isolate) might have been more informative to detect adaptation due to polyploidy. Metalaxyl-M resistance was only detected in Belgian strains isolated in and after 2005, indicating a build-up of resistance against Metalaxyl-M in the *P. porri* population, which is probably related to the introduction of Metalaxyl based fungicides in the control of white tip disease in leek. However, Metalaxyl-M was only registered for use in leek production in 2011. This discrepancy could be explained by introduction of resistant strains from outside Belgium, or by contact with Metalaxyl based products used in other crops.

The data gathered on interspecific hybridization and polyploidy in this work, together with a literature survey on the role of polyploidy in plants, led us to conclude that the advantage of polyploidy in *Phytophthora* probably should be sought in a broader evolutionary context.
In Chapter 7, we brought together available literature on polyploidy and hybridization in *Phytophthora* and suggest that polyploidy could be an important mechanism contributing to the genomic flexibility found in *Phytophthora* pathogens. *Phytophthora* species are notorious for their genomic flexibility, enabling them to quickly overcome (acquired) host

resistance. Reports of polyploidy (either auto- or allo-) have been published scattered over the past four decades. The last few years, more and more reports are emerging on polyploidy in *Phytophthora* thanks to technological advances (flow cytometry, genomics, etc.), indicating that it could play an important role in the evolution of the genus. Next to adaptation to new ecological niches, polyploidy could enhance host adaptation by expanding and recombining the pathogen's effector repertoire through different genetic processes that can be associated with either vegetative or sexual reproduction. The formation of polyploids could be mainly due to crossing of genotypes that have diverged genetically (by geographic isolation or by host adaptation), but have not formed solid reproductive barriers. The occurrence of autopolyploidy in *Phytophthora* (such as in *P. infestans*) could be linked with heterothallism (see Figure 7.1). When the two different mating types of a heterothallic species are geographically isolated, sexual reproduction is halted. Over many (clonal) generations, this can cause a relaxation of selection pressure on genes important in meiosis. When sexual reproduction is restored, unreduced or unbalanced gametes can be created at higher frequencies due to non-disjunction of chromosomes in meiosis. In this case, (auto)polyploids can be formed in the F1 generation.

8.2 Conclusion and future perspectives

Our findings of polyploidy and hybridization and the roles that these processes play in adaptive evolution of this pathogen are probably not limited to *Phytophthora* clade 8b only. Interspecific hybridization has been described at least six times in different clades of the genus *Phytophthora* and new reports are emerging at an increasing rate. The addition of DNA measurements by flow cytometry in genetic diversity studies will deliver more information on the extent of polyploidy in the genus *Phytophthora*.

Intergenomic exchange between the different parental genomes in *Phytophthora* hybrids, creating novel genetic diversity, can take place during either vegetative growth (mitotic recombination) or sexual reproduction (meiotic recombination). Sexual reproduction is very common in clade 8b since most of the species are homothallic and readily form oospores after infecting their hosts. In heterothallics, like *P. infestans*, sexual reproduc-

tion is less common in nature. Clonal reproduction is so common in this species that even the popular plant pathology textbook Agrios (2005) states that sexual reproduction is *extremely* rare in nature. However, recent reports deliver evidence that sexual reproduction in *P. infestans* might be more common than once believed, or alternatively it might be on the rise due to the introduction of the second mating type in many regions (Fry, 2008). The first introduction of the A2 mating type in Europe was most likely sometime in the 1970s, the first report came from Switzerland (Hohl and Iselin, 1984). Nowadays, in northern Europe (Scandinavia) there seems to have formed a second center of diversity where sexual reproduction is common (Yuen and Andersson, 2013). Moreover, there is a clear link between the introduction of sexual reproduction and the rising problems with *P. infestans* since the 1980s (Fry, 2008; McDonald and Linde, 2002; Yoshida et al., 2013).

Hence, the phenomena studied in this work most likely play a role in many *Phytophthora* species, and maybe even in other genera of plant pathogenic eukaryotes. Interspecific hybridization has already been described for *Verticillium* (Clewes et al., 2008; Inderbitzin et al., 2011), *Botrytis* (Staats et al., 2005) and many other genera (Olson and Stenlid, 2002).

Still, *Phytophthora* clade 8b offers a good model system for studying adaptive evolution in polyploid species. Some suggestions for future work are given below.

8.2.1 Comparative genomics of hybrids and parental species

Next generation sequencing (NGS) technology could be very useful for evaluating some of the hypotheses formulated in this thesis. Whole genome sequencing of the different hybrid type 1 isolates and comparison with the parental species, combined with a study of the effector repertoires in these isolates compared to the parental species could provide answers to the following questions: does intergenomic recombination occur in effector gene regions and if yes, to what extent? A possible confounding factor is that we do not have the specific isolates available that acted as parents in the interspecific cross giving rise to hybrid type 1. This is a problem because effector repertoires might be different between isolates of the same species, and hence, an absolute comparison can not be done.

Another possibility is to compare one of the hybrid isolates with its F1 progeny and check the amount of genomic recombination between two generations by using NGS. An obstacle here are the meoitic problems in the hybrid isolates, causing very low numbers of viable F1 progeny. However, we have already successfully derived seven single oospore descendants of isolate CBS 126737, which showed the lowest amounts of aborted oospores and the highest amounts of homeologous recombination in the ITS region (see section 4.3), using micro-dissection of germinating oospores (results not shown).
Another possibility is to compare the effector regions of diploid and polyploid isolates of the same species, to assess the amount of neo- and/or subfunctionalization going on in these regions in polyploids. Another confounding factor in the use of NGS for studying the clade 8b hybrids is their polyploidy. Polyploidy causes difficulties in sequence assembly. However, recent advancements in the technology (longer read lengths and lower prices), make the technology more available for the study of polyploid genomes (Buggs et al., 2012).

8.2.2 Karyotyping

Karyotyping (chromosome counts) are missing to provide conclusive proof for the polyploidy in *Phytophthora* clade 8b. Further efforts in this area could greatly enhance the study of hybridization and polyploidy in *Phytophthora*.

Summary

Plants are constantly threatened by a variety of harmful organisms such as insects, bacteria, Fungi and oomycetes. *Phytophthora* is an important genus of plant pathogenic oomycetes that cause many devastating plant diseases such as potato late blight caused by *P. infestans*. The oomycetes are very similar to the Fungi in their lifestyle and morphology, but have evolved from an independent origin. Oomycetes differ from Fungi in some important morphological and biochemical aspects and in the fact that they are diploid in their vegetative lifestyle, whereas most Fungi are monoploid. We studied a group of related *Phytophthora* species belonging to the phylogenetic clade 8b. The species in this group are cold-tolerant, slow growing and host-specific, and are known to cause disease in a range of field grown vegetables during winter seasons. Before this work, the clade contained only three species: *P. porri*, *P. brassicae* and *P. primulae*, respectively causing disease in leek, cabbages and *Primula* spp. Several reports had been published during the last few decades describing diseases caused by similar, but unknown *Phytophthora* organisms. Hence, a detailed study of the genetic diversity in this group was needed.

We collected one hundred and six *Phytophthora* isolates from the previously recognized hosts leek, cabbages and *Primula* spp., and from new hosts such as onion, carrot, lettuce, chicory, parsley, etc. Two barcoding genes (ITS and *CoxI*) were used to identify the unknown isolates to the species level. In the phylogenetic analysis based on these two loci, we found several taxonomic entities that differed from the known species. Moreover, there was a clear correlation with the host plant: almost all taxonomic entities (or clades) could be correlated to a specific host plant or plant family. To verify whether these groups represented separate species, a detailed morphological and physiological study was performed. Based on these results we described three new species in clade 8b, *P. cichorii* sp. nov., *P. dauci* sp. nov. and *P. lactucae* sp. nov. Two other taxonomic novelties were found that possibly also represent new species, but could not be officially described at this time due to the low numbers of isolates available and because these isolates did not form sexual structures.

Several isolates were found to show intra-isolate polymorphism in the rDNA ITS region, a distinctive mark of interspecific hybridization. Further characterization of these isolates by sequencing and cloning of three more loci (*Ypt1*, *CoxI* and *Nadh1*) and measuring DNA

contents using flow cytometry, confirmed their hybrid origin. Three hybrid types could be distinguished, originating from interspecific crosses between different clade 8b species. The combination of multilocus sequencing and cloning and DNA content estimation provided valuable insights in the processes involved in and after interspecific hybridization in *Phytophthora*. A recently formed hybrid will contain the complete complement of both parental genomes (allopolyploidy). Novel gene combinations such as a combined effector repertoire can enable the pathogen to invade new habitats or hosts. Using in vitro pathogenicity tests on four different hosts, we found that all three hybrid types can infect more hosts than their respective parents. Next to this, the hybrids generally show higher virulence levels compared to the non-hybrid species. After allopolyploidization, different genetic processes such as chromosome loss and chromosomal rearrangements (which can be between the different parental genomes through homeologous recombination) can enable the pathogen to further adapt to a new habitat or host. The progeny of a single hybridization event can follow different evolutionary paths, which ultimately can result in the formation of new species. It is clear from our data that the footprints of hybridization can be easily overlooked when using only a few barcoding genes for identification. Therefore, it is possible that the occurrence and importance of hybridization in *Phytophthora* is highly underestimated.
While most *Phytophthora* species are host-specific to one or a few host plant species, we found that onion (*Allium cepa*) is a common susceptible host to many of the clade 8b species. Therefore, onion might function as a hot spot for interspecific hybridization between the clade 8b species in the field. Indeed, since most *Phytophthora* clade 8b species attack winter-grown vegetable crops that are commonly used in crop rotation systems, the different species can be present in the same field. When onion is cultivated on those fields, it can be simultaneously infected by different species, providing the intimate contact that is needed for an interspecific cross.

DNA content estimates of the non-hybrid clade 8b species indicated that polyploidy is a general feature of the complete clade. Both in *P. porri* and in *P. brassicae*, three different DNA ploidy levels were detected. In *P. porri*, the different DNA ploidy levels correspond to diploidy, tetraploidy and mixoploidy (diploid and tetraploid nuclei in the same mycelium) and in *P. brassicae*, the different DNA ploidy levels correspond to triploidy, tetraploidy and hexaploidy. However, chromosome counts that would provide conclusive proof for the inferred ploidy levels, are still lacking. The DNA contents of the other clade 8b species did not show intraspecific variation. However, their DNA contents were in the same range as that of the polyploid *P. porri* and *P. brassicae* isolates, indicating that

these species are polyploids too.

The main research question that we wanted to answer after these findings of hybridization and polyploidy was: ‘Does polyploidy offer an ecological advantage to the pathogen?’

Phytophthora porri isolates of the three different ploidy levels were compared in their tolerance to cold and frost and in their sensitivity to the fungicide Metalaxyl-M. Moreover, the pathogenic potential on leek was investigated for the different *P. porri* ploidy levels. Significant differences in cold and frost tolerance could be detected between diploid and polyploid isolates. However, since only one diploid isolate was available for study, no strong conclusions could be drawn. No apparent differences were observed in Metalaxyl-M sensitivity or pathogenic potential on leek.

Our results combined with an extensive literature study of polyploidy and hybridization in the genus *Phytophthora* and in plants led us to conclude that the advantage of polyploidy probably lies in a broader evolutionary context. *Phytophthora* species are notorious for their genomic flexibility, enabling them to overcome (acquired) host resistance very quickly. *Phytophthora* genomes show a typical dual organization, with conserved housekeeping genes concentrated in syntenic, gene-rich blocks that evolve slowly, whereas virulence genes are dispersed into highly dynamic, repeat-rich regions which evolve rapidly: the so-called ‘2-speed genome’. Intra- and intergenomic recombination between effector regions in (allo)polyploids during sexual reproduction or even during vegetative growth, can provide the progeny with new and unique effector combinations. Further study of hybridization and polyploidy in *Phytophthora* clade 8b could provide valuable insights in the significance of these processes in the evolution of virulence in this important family of plant pathogens. Polyploidy and hybridization could give a pathogen the plasticity it needs to stay one step ahead of the plant in their co-evolutionary arms race.

Samenvatting

Planten worden continu bedreigd door verschillende types schadelijke organismen, zoals insecten, bacteriën, schimmels en oömyceten. *Phytophthora* (oömycota) is een belangrijke familie plantenziekteverwekkers die vele schade berokkenen in de landbouw, maar ook in natuurlijke omgevingen. De aardappelplaag, veroorzaakt door *Phytophthora infestans* is erg bekend. Oömyceten zijn lijken morfologisch sterk op schimmels (rijk Fungi) en vertonen ook vaak een gelijkaardige levensstijl. Nochtans zijn de oömyceten taxonomisch niet verwant met de schimmels en zijn hun overeenkomsten het gevolg van convergente evolutie. Oömyceten verschillen van schimmels in enkele belangrijke morfologische en fysiologische eigenschappen en in het feit dat ze een diploïde vegetatieve levensstijl vertonen, terwijl schimmels meestal een haploïde vegetatieve levensstijl vertonen.
In dit werk hebben we een groep van verwante *Phytophthora* soorten bestudeerd die behoren tot de fylogenetische 'clade 8b'. De soorten in deze groep zijn koudetolerante, traaggroeiende en waardplantspecifieke pathogenen van verschillende volleveldsgroenten in winterteelten. Voor dit werk bestond de groep uit slechts drie soorten: *P. porri*, *P. brassicae* en *P. primulae*, die respectievelijk ziektes veroorzaken in prei, kolen en *Primula* spp. Gedurende de voorbije decennia zijn er meerdere meldingen gemaakt van gerelateerde *Phytophthora* soorten die ziekte veroorzaakten in andere waardplanten. Een gedetailleerde studie van de genetische diversiteit in deze groep van weinig bestudeerde *Phytophthora* soorten drong zich dus aan.

Honderd en zes *Phytophthora* isolaten werden verzameld, zowel van de gekende waardplanten prei, kolen en *Primula* spp. als van andere waardplanten zoals ui, wortel, sla, witloof, peterselie, ... Deze isolaten werden gekarakteriseerd door sequentiebepaling van twee genen (ITS en *CoxI*) die als 'barcode' worden gebruikt voor de identificatie van ongekende *Phytophthora* isolaten. Op de verkregen sequenties werd een fylogenetische analyse uitgevoerd waaruit verschillende nieuwe taxonomische eenheden konden worden ontwaard. Er was ook een duidelijke correlatie met de waardplant: bijna alle taxonomische

eenheden (of clades) waren geassocieerd met een specifieke waardplant of plantenfamilie. Om na te gaan of deze groepen isolaten konden worden aanzien als afzonderlijke soorten, werd een gedetailleerde morfologische en fysiologische studie uitgevoerd. Op basis van deze gegevens konden drie nieuwe soorten officiëel worden beschreven: *P. cichorii* sp. nov., *P. dauci* sp. nov. en *P. lactucae* sp. nov. Hiernaast vonden we nog twee andere taxonomische eenheden die vermoedelijk ook als nieuwe soorten kunnen worden aanzien. Een officiële beschrijving bleek echter prematuur, omwille van het lage aantal beschikbare isolaten, en omdat de soorten geen of nauwelijks seksuele structuren vormden.

Verschillende isolaten vertoonden intra-isolaat variatie in de ITS regio, wat een kenmerk kan zijn van interspecifieke hybridisatie. Verdere karakterisatie door sequentiebepaling en klonering van drie extra loci (*Ypt1*, *CoxI* en *Nadh1*) en genoomgroottebepalingen met behulp van flow cytometrie bevestigden de hybride oorsprong van de isolaten. Drie verschillende types, ontstaan uit interspecifieke kruisingen tussen verschillende clade 8b soorten, konden worden ontwaard. Hybride type 1 bevatte 11 isolaten en types 2 en 3 bevatten elk een isolaat. De sequentiebepaling en klonering van verschillende loci gecombineerd met genoomgroottebepalingen leverde interessante inzichten in de processen tijdens en na interspecifieke hybridisatie in *Phytophthora*. Een recent gevormde hybride zal het volledige genoomcomplement van de twee ouders bevatten (allopolyploïdie). Nieuwe gencombinaties zoals de gecombineerde effector-repertoires kunnen de pathogeen de kans geven zich te adapteren aan een nieuwe habitat of waardplant. In vitro infectietesten op vier verschillende waardplanten wezen uit dat de drie types hybriden meer waardplanten kunnen infecteren dan hun respectievelijke ouders. Bovendien toonden de hybriden over het algemeen hogere virulentieniveau's vergeleken met de oudersoorten. Na hybridisatie en polyploïdisatie kunnen verschillende genetische processen zoals chromosoomverlies en chromosoomherschikkingen (die ook tussen de verschillende ouderlijke genoomcomponenten kunnen plaatsvinden onder de vorm van homeologe recombinatie) ervoor zorgen dat de pathogeen zich verder kan aanpassen aan specifieke habitats of waardplanten. De nakomelingen van een enkele interspecifieke kruising kunnen een sterk verschillende evolutie vertonen, wat uiteindelijk kan leiden tot het ontstaan van nieuwe soorten. Uit de data verkregen in dit werk werd ook duidelijk dat de voetafdrukken van interspecifieke hybridisatie snel vervagen. Daarom wordt een hybride oorsprong dikwijl niet ontdekt indien enkel een aantal specifieke 'barcode' genen gebruikt worden voor identificatie. Het is daarom mogelijk dat de frequentie en het belang van hybridisatie in de evolutionaire geschiedenis van *Phytophthora* sterk wordt onderschat.
Hoewel de meeste clade 8b soorten een sterke mate van waardplantadaptatie vertonen,

konden we aantonen dat ui (*Allium cepa*) een gemeenschappelijke waardplant is van verschillende clade 8b soorten. De waardplant ui zou dus kunnen functioneren als een 'hot-spot' voor interspecieke hybridisatie in het veld. Gezien de meeste *Phytophthora* clade 8b soorten volleveldsgroenten in winterteelten aanvallen die dikwijls in vruchtwisselingschema's worden geteeld, is het erg plausibel dat verschillende clade 8b soorten aanwezig kunnen zijn op hetzelfde veld. Wanneer ui wordt gecultiveerd op deze velden kan het gelijktijdig worden geïnfecteerd door deze verschillende soorten. Zo kunnen deze *Phytophthora* soorten in nauw contact met elkaar komen, wat een voorwaarde is voor interspecifieke hybridisatie.

Genoomgroottebepalingen van de andere (niet hybride) clade 8b soorten wezen uit dat polyploïdie een gemeenschappelijk kenmerk is van de clade. Zowel in *P. porri* als in *P. brassicae* werden drie verschillende DNA ploïdieniveau's gedetecteerd. In *P. porri* kwamen deze DNA ploïdieniveau's overeen met diploïdie, tetraploïdie en mixoploïdie (een combinatie van diploïde en tetraploïde kernen binnen een mycelium). In *P. brassicae* kwamen de verschillende genoomgroottes overeen met triploïdie, tetraploïdie en hexaploïdie. Om deze inferenties te bevestigen zijn chromosoomtellingen nodig, die in dit werk niet konden worden gedaan. De genoomgroottes van de andere clade 8b soorten toonden geen verschillende ploïdieniveau's, maar de genoomgrootte's waren steeds in dezelfde grootteorde als die van de polyploïde *P. porri* en *P. brassicae* isolaten, wat erop wijst dat ook de andere soorten polyploïden zijn.

De hamvraag die bleef na deze bevindingen was de volgende: "Kan polyploïdie een ecologisch voordeel opleveren voor de pathogeen?"
Om deze vraag te beantwoorden werden de verschillende *P. porri* ploïdieniveau's met elkaar vergeleken in hun koude- en vriestolerantie. Daarnaast werd ook de gevoeligheid aan het fungicide Metalaxyl-M en hun virulentie op prei nagegaan. Significante verschillen tussen diploïde en polyploïde isolaten in hun koude- en vriestolerantie konden worden aangetoond. Ondanks deze verschillen kunnen we hieruit geen betrouwbare conclusies trekken aangezien slechts een diploïd isolaat beschikbaar was. Verschillen in Metalaxyl-M en virulentie op prei werden niet gedetecteerd tussen de verschillende *P. porri* ploïdieniveau's.

Onze resultaten, gecombineerd met een uitgebreide literatuurstudie van het fenomeen polyploïdie in *Phytophthora* en in planten, wezen erop dat het voordeel van polyploïdie moet worden bekeken in een bredere evolutionaire context. *Phytophthora* pathogenen

zijn berucht voor hun ongeëvenaarde genomische flexibiliteit, waardoor ze zich zeer snel kunnen aanpassen aan (verworven) resistentie van planten. *Phytophthora* genomen vertonen een typische tweeledige opbouw: belangrijke huishoudgenen bevinden zich in traag-evoluerende, genendense gebieden, terwijl genen belangrijk in de interactie met planten (zoals de zogenaamde effectorgenen) zich bevinden in regio's die een snelle evolutie bevatten en over het algemeen weinig genen bevatten. Intra- of intergenomische recombinatie tussen effectorregio's in (allo)polyploiden tijdens seksuele voorplanting of zelfs tijdens vegetatieve groei, kan nieuwe en unieke effectorcombinaties opleveren.

Verdere studie van hybridisatie en polyploïdie in *Phytophthora* clade 8b kan belangrijke inzichten leveren in het belang van deze processen in de evolutie van virulentie in deze belangrijke familie van plantenziekten. Polyploïdie en hybridisatie kunnen pathogenen de flexibiliteit leveren die ze nodig hebben om telkens een stapje voor te zijn op de plant in hun co-evolutionaire wapenwedloop.

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Curriculum vitae

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Place of birth: Roeselare
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Education

2008: Master in Bioscience Engineering, University of Leuven (KULeuven)
Master thesis: "Testing of a site-specific recombination system for the removal of the selection marker in transgenic banana cultures"
Promotor: Prof. Dr. Rony Swennen

2003: High school diploma (Science and Mathematics), St. Janscollege, Poperinge

Additional training

Introduction to Python programming (2 days), 2014
Basic Bio-informatics Concepts and Tools (3 days), 2013
Introduction to R (certified; 12 contact hours), 2013
Building Bio-Informatics work flows (1 day), 2013
Comparative Genomics in Eukaryotes (1 day), 2013
Plant Breeding and Plant Sexual Reproduction (certified; 1 week summer school), 2012
Practical Plant Flow Cytometry Workshop (1 day), 2012
Advanced Academic English: Writing Skills (certified; 20 contact hours), 2011
Effective Scientific Communication (certified; 20 contact hours), 2010
Personal Effectiveness (certified; 20 contact hours), 2010

Professional Record

October 2013 - March 2014: PhD scholarship UGent
+ **October 2009 - September 2013**: IWT personal fellowship

PhD candidate at the Department of Crop Protection, Faculty of Bioscience Engineering, Ghent University (UGent)
PhD thesis: "The expansion of *Phytophthora* clade 8b: host adaptation and speciation through hybridization and polyploidy"
Promotor: Prof. Dr. Monica Höfte

September 2008 - July 2009: Research scholar at the Florida Medical Entomology Department, Institute of Food and Agricultural Sciences, University of Florida (UF)
Topic: "Development of a biological insecticide against mosquito larvae using genetically modified *Pichia pastoris*"
Promotor: Prof. Dr. Dov Borovsky

Publications

Peer reviewed:

Bertier L, Brouwer H, de Cock AWAM, Cooke DEL, Olsson CHB, Höfte M (2013) The expansion of *Phytophthora* clade 8b: three new species associated with winter grown vegetable crops. Persoonia, 31, 63-76

Bertier L, Leus L, D'Hondt L, De Cock AWAM, Höfte M (2013) Host adaptation and speciation through hybridization and polyploidy in *Phytophthora*. PLOS One, 8, e85385

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Hua GKH, Bertier L, Soltaninejad S, Höfte M (2014) Cropping systems and cultural practices determine the *Rhizoctonia* anastomosis groups associated with *Brassica* spp. in Vietnam. Submitted to PLOS One

Conference Proceedings

Bertier L, Brouwer H, D'hondt L, Leus L, De Cock AWAM, Höfte M. (2012) Polyploidy in *Phytophthora porri*, the causal agent of white tip disease in leek. Communications in Agricultural and Applied Biological Sciences. 2012;77:27-31

Participation to conferences and symposia and invited seminars

Bertier L, D'hondt L, Brouwer H, de Cock AWAM, Leus L, Höfte M (2011) Evolutie van het *Phytophthora porri* complex door waardplantadaptatie, hybridisatie en (allo)polyploïdisatie. 20e Bijeenkomst van de KNPV werkgroep *Phytophthora* & *Pythium*, April 28 , 2011, Ghent, Belgium. Oral presentation by L Bertier

Bertier L, D'hondt L, Brouwer H, de Cock AWAM, Leus L, Höfte M (2011) Occurrence, genetic diversity and host adaptation of *Phytophthora* clade 8b species. 63rd International Symposium on Crop Protection, May 24, 2011, Ghent, Belgium. Poster presentation by L Bertier

Bertier L, Brouwer H, D'hondt L, Leus L, De Cock AWAM, Höfte M. (2012) Polyploidy in *Phytophthora porri*, the causal agent of white tip disease in leek. 17th Symposium on Applied Biological Sciences, February 10, 2012, Leuven, Belgium. Oral presentation by L Bertier

Bertier L, D'hondt L, Leus L, Höfte M (2012) Variatie in ploïdieniveau bij *Phytophthora porri*. 21e Bijeenkomst van de KNPV werkgroep *Phytophthora* & *Pythium*, April 17, 2012, De Lier, The Netherlands. Oral presentation by L Bertier

Bertier L, Brouwer H, D'hondt L, Leus L, de Cock AWAM, Höfte M (2012) Evolution of *Phytophthora* clade 8b plant pathogens: does polyploidy play a role? International Conference on Polyploidy, Hybridization and Biodiversity, May 7-10, 2012, Průhonice, Czech Republic. Poster presentation by L Bertier

Bertier L, Brouwer H, D'hondt L, Leus L, de Cock AWAM, Höfte M (2013) Why are there so many *Phytophthora* species: does polyploidy play a role? A case study of *Phytophthora* clade 8b. Oomycete Molecular Genetics Network Meeting, March 9-12, 2013, Asilomar, California. Oral presentation by L Bertier

Bertier L, Brouwer H, D'hondt L, Leus L, de Cock AWAM, Höfte M (2013) Why are there so many *Phytophthora* species: does polyploidy play a role? A case study of *Phytophthora* clade 8b. March 15, 2013, UC Davis Genome Center, Davis, California. Invited seminar presented by L Bertier

Bertier L, D'hondt L, Brouwer H, de Cock AWAM, Leus L, Höfte M (2013) The expansion of *Phytophthora* clade 8b: three new species associated with the winter grown vegetable crops carrot, lettuce and chicory. 65thInternational symposium on Crop Protection, May 21, 2013, Ghent, Belgium. Oral presentation by L Bertier

Hua GKK, Bertier L, Soltaninejad S, Höfte M (2013) Characterization and pathogenicity of *Rhizoctonia* isolates associated with cruciferous vegetables in Vietnam. 65thInternational symposium on Crop Protection, May 21, 2013, Ghent, Belgium. Oral presentation by GKK Hua

Attended conferences and symposia without participation

19[e]Bijeenkomst van de KNPV werkgroep *Phytophthora* & *Pythium*, April 8, 2010, Roelofarendsveen, The Netherlands

62[nd]International Symposium on Crop Protection, May 18, 2010, Ghent, Belgium

64[th]International Symposium on Crop Protection, May 22, 2012, Ghent, Belgium

KNPV Symposium on 'Intraspecific Pathogen Variation: implications and opportunities', January 22, 2013, Wageningen, The Netherlands

Training@VIB symposium 'New Paradigms in Plant Epigenetics, Heterosis, Stress and Evolution', November 18, 2013, Zwijnaarde, Belgium

Awards

Poster award at the 63[rd]International Symposium on Crop Protection, May 24, 2011

Bertier L, D'hondt L, Brouwer H, de Cock AWAM, Leus L, Höfte M (2011) Occurrence, genetic diversity and host adaptation of *Phytophthora* clade 8b species