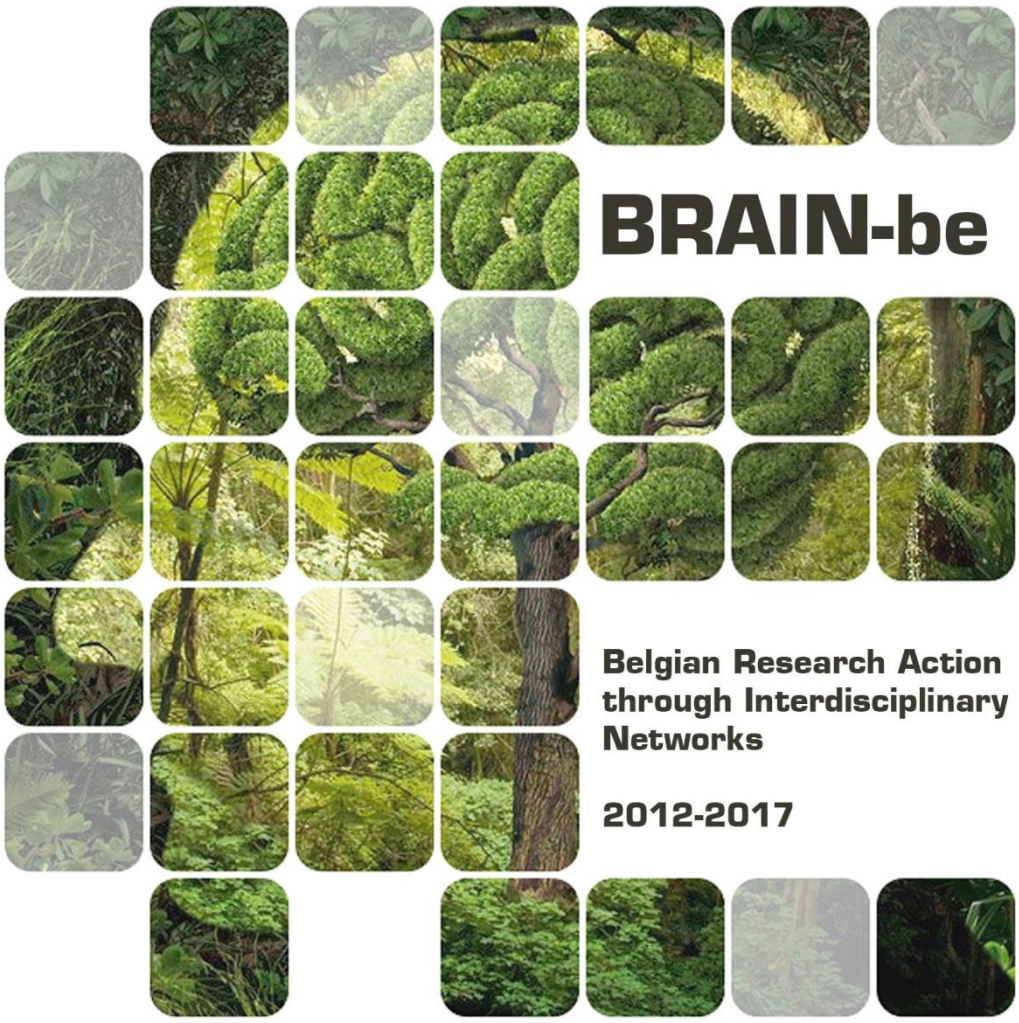


# RESIPATH

## Response of European Forests and Society to Invasive Pathogens

Dr Kurt Heungens (ILVO)

Dr Anne Chandelier (CRAW)

**Axis 1: Ecosystems, biodiversity and evolution**

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NETWORK PROJECT

# RESIPATH

## Response of European Forests and Society to Invasive Pathogens

**Contract - BR/132/A1/RESIPATH-BE**

## FINAL REPORT

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## ABSTRACT

### Context

Trees are increasingly threatened by invasive pathogens that have a significant impact on their health. The RESIPATH project aims to study how European forest communities have been affected by and responded to invasive fungal pathogens in order to develop means to mitigate their impact. The Belgian partners in this project were involved in two (out of five) work packages (WP3 and WP4).

### Objectives

The objective of WP3 is to develop sensitive methods to detect *Phytophthora* hybrids and to use these methods to determine the frequency of hybridization between *Phytophthora* species in different environments and across European climatic regions. The WP4 focuses on validating a method combining a spore trapping system adapted to the forest environment with high-throughput amplicon sequencing for the early detection of aerial spores of potentially invasive forest pathogens.

### Conclusions

WP3: Two methods, GBS (Genotyping By Sequencing) and flow cytometry, have been optimized, validated and used on a collection of 836 *Phytophthora* isolates. With GBS, we could confirm the hybrid nature of known hybrid species but we could also identify several other isolates or species as hybrids, in several cases for the first time. We could often also determine which groups of isolates of the parental species in our database were related to the observed hybrids. The second technique, flow cytometry, helped in the identification and characterization of allopolyploid hybrids. When the results of both techniques were put together, hybrids were detected in *Phytophthora* clades 1, 6, 7 and 9.

WP4: Three spore trapping systems (filter paper, Burkard volumetric sampler and rotating-arm spore traps (rotorod-style)) have been compared for the detection by real-time PCR of 3 forest pathogens displaying different spore sizes and belonging to different taxa. Based on these preliminary results, the Rotorod samplers were selected for the trapping of spores in the forest. An amplicon sequencing protocol and a bioinformatic pipeline for the analysis of the data (NGS protocol) were optimized on mock communities of fungi. By applying this protocol to DNA samples extracted from Rotorod samplers put in different forests stands, the results were compared to those obtained with real-time PCR for the three forest pathogens used as fungal markers. The new NGS was less sensitive than real-time PCR. However, it allowed the detection of numerous other fungi, among which tree pathogens.



### Keywords

Flow cytometry, Genotyping by sequencing, High throughput sequencing, Invasive fungal pathogens, Phytophthora hybrids, spore trapping.

# 1. INTRODUCTION

Trees are increasingly threatened by invasive pathogens that have a significant impact on their health. Several factors including the international trade of plant material (wood, wood packaging, nursery plants), climate changes making trees more susceptible to infections and allowing invasive pathogens to extend their geographic range, or tourists that bring back plants, fruits, seeds from distant countries contribute to the increased risk of introduction of new pathogens in Europe.

The RESIPATH project aims to study how European forest communities have been affected by and responded to invasive fungal pathogens in order to develop means to mitigate their impact. The Belgian partners in this project were involved in two (out of five) work packages.

The first work (WP3) relates to mechanisms of hybridization in *Phytophthora* species. Such hybrids are potentially invasive forest pathogens of which we hypothesize that their occurrence is increasing.

The second work package (WP4) relates to methods for the rapid detection of invasive fungi and oomycetes in water and air.

# 2. STATE OF THE ART AND OBJECTIVES

## 2.1. Hybridization in *Phytophthora* species

*Phytophthora* hybrids such as *P.* x *cambivora* have been described a long time before being recognized as hybrids. In other cases, such as *Phytophthora* x *serendipita* (hybrids of *P. cactorum* and *P. hedraiandra*), the hybrids seem to have emerged recently, and are displacing the parental species in some of the nursery environments. The seeming increase in the emergence of *Phytophthora* hybrids may be the result of increased international trade of plants among nurseries. With plant displacements, latently present *Phytophthora*'s are also displaced and previously separated species may come into contact and in some cases hybridize. Such hybrids, receiving and combining an arsenal of enzymes from each parent, may have an increased pathogenicity and host range and may hence become invasive and pose a serious risk to plant health. As several *Phytophthora*'s are also tree pathogens, this may include new threats to forest health. In contrast, maybe hybrids have been around much more than we think, even in natural forest and river environments, and have not caused more damage in those environments than non-hybrid species. Finding out the relative presence of hybrids in these different environments was an objective of our research. The Resipath partners had to collect *Phytophthora* isolates from these different environments during the project. This includes isolates from the north to the south range of Europe in order to include a climate factor to the study.

The current techniques to detect hybrids are mostly based on the cloning and sequencing of reference genes and looking for DNA sequence polymorphisms that extend beyond regular heterozygosity. Although this technique will be used in part of this study, it is relatively labour intensive and not always reliable as they are limited to only one or a few genes, in which (by chance) there may be no polymorphisms. Hence our first objective was to optimize and validate techniques for the detection of hybrids that are less labour intensive and that are more reliable, so they could be applied to a range of isolates from the different environments (nurseries, forests, rivers). We chose to focus on "Genotyping by Sequencing" (GBS), a genome complexity reduction technique, in combination with Illuminia high throughput sequencing. A second technique was flow cytometry, a technique that allows a direct measure of nuclear size. Both techniques are not novel but have not previously been used or optimized for the detection and characterization of hybrids. In the case of GBS we also needed to develop a data handling pipeline and methods to identify the hybrids.

## 2.2. Detection of invasive fungi with an aerial dispersal

When they are introduced in a new environment, invasive (or exotic) fungal pathogens are present in very low concentration in the air. The monitoring of these specific organisms requires spore trapping systems that are very efficient. As invasive fungal pathogens can belong to different taxa, the spore traps must be capable to collect a wide range of fungal species differing by their spore sizes/shapes, or their daily patterns of discharge. The type of environment studied in this project is also important to take into account. Forest sites are not always accessible with motorized vehicles. They are also often open to the public. For these reasons, the spore traps should be inexpensive, portable and easy to install.

Once collected on the surface of the spore trap, the spores must be analysed to determine the fungal species to which they belong. Identification based on morphological and cultural characteristics is time consuming, and requires someone with a high experience in fungal taxonomy. In addition, the number and the diversity of spores present on a spore trap is often too important to allow a correct identification based on morphology, or isolation on culture media. The combination of spore trapping and PCR for the specific detection of airborne spores of interest has greatly enhanced the efficiency of this technique. However, detection of fungal spores in a context of surveillance requires a non-specific approach. High throughput sequencing offers a solution to this issue. Such techniques have already been applied to the study of fungal communities in natural ecosystems. However, these techniques are not often used for a diagnostic purpose, where the identification at the species level is crucial. Furthermore, reference fungal databases (required to assign a DNA sequence to a taxonomic unit) are not updated frequently enough, which is a problem for the detection of invasive pathogens capable to spread rapidly in a new environment. In this project, different spore trapping systems will be evaluated in forests stands, and techniques based on specific qPCR primers will be compared with generic detection methods based on high throughput sequencing (HTS) for the detection of fungal pathogens present in European forests and belonging to different taxa. The information gathered will be compiled in a detailed protocol available for laboratories involved in surveillance of the airborne inoculum of fungal species.

# 3. METHODOLOGY

## 3.1. Hybridization in Phytophthora species

### 3.1.1. Establishment of a *Phytophthora* collection

#### 3.1.1.1. Isolates available at ILVO at the beginning of the project

For the establishment of the collection, a total of 324 *Phytophthora* isolates from at least 24 species (or hybrids) were transferred to fresh medium, cleaned from contaminants, and hyphal-tipped. A working collection was established on V8 medium with isolates from *Phytophthora cryptogea, P. cambivora, P. kernoviae, P. ramorum,* and *P. syringae, P. citrophthora* and *P. cactorum*. An overview of the number species and isolates per species are presented in appendix 1. As some of the older identifications were based on the PCR-RFLP method of Cooke and Duncan (1997), in some cases only a group of species can be listed.

#### 3.1.1.2. Collection of *Phytophthora* from Belgian beech forests (CRAW)

Due to the importance of beech (*Fagus sylvatica*) in Belgian forests, and to the potential infection of this tree species by different *Phytophthora* species (Jung et al. 2005), notably in Belgium (Schmitz et al. 2007), a survey was conducted in beech forests in Wallonia in 2014 and 2015. Fourteen stands located near a stream (< 1 m width), containing beech (pure or mixed forest except for two stands) and evenly distributed in the area with beech in the Southern part of Belgium were selected (Figure 1, Table I). In 2014, all stands were visited at two periods of the year, from March to July (period 1, spring) and in September (period 2, summer), to take into account of the variable efficiency of isolation according to the season and *Phytophthora* species. In 2015, ten of the 14 stands were visited only in summer.

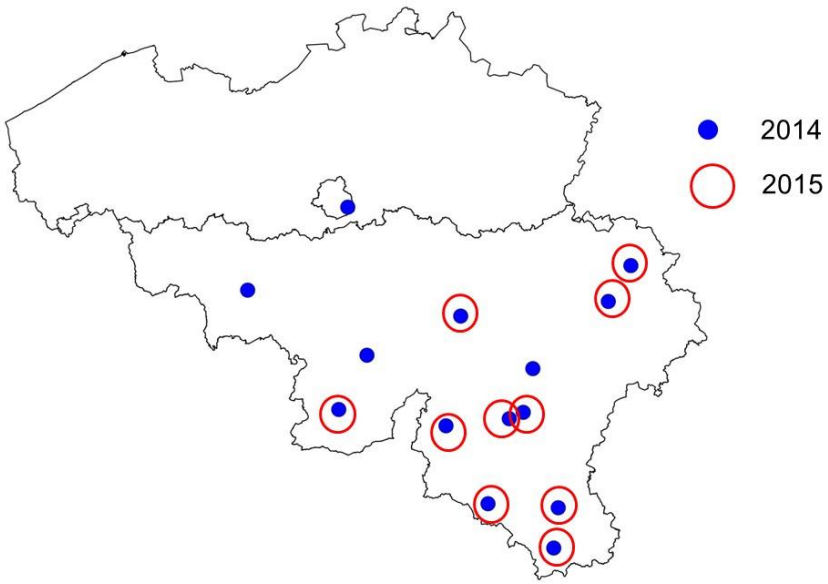


Figure 1 - Location of the beech stands for the survey of *Phytophthora*

Table I - Characteristics of the 14 forest stands selected for the baiting of *Phytophthora* in beech forests

| | | Beech | | | | | water | | soil | | | Lambert coordinates | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| # | Location | % | Mean circumference[1] | regeneration[2] | bleeding canker[3] | Other tree species | pH | velocity | pH KCL | ecological group[4] | Altitude | X | Y |
| 1 | Ethe | <10 | 60 | 0 | 0 | larch, oak, ash, maple | 5,7 | low | 4,5 | 5,8,10,11 | 282 | 235593 | 33436 |
| 2 | Marenne | 90 | 229 | 3 | 0 | maple, ash | 4,7 | medium | 3,4 | 10 | 342 | 227458 | 103307 |
| 3 | Goé | 95 | 110 | 1 | 1 | oak | 5 | low | 3,9 | 10,3 | 340 | 265593 | 143439 |
| 4 | Aublain | 10 | 66 | 1 | 1 | birch, maple, ash | 6,5 | low | 3,8 | 8 | 275 | 151962 | 87382 |
| 5 | Erbisoeul | 0 | | | | birch, alder, maple | 5,3 | low | 3,3 | 3 | 86 | 116521 | 133878 |
| 6 | Mortehan | 95 | 36 | 3 | 1 | spruce, oak | 4,7 | low | 4,0 | 5,9 | 449 | 210020 | 50689 |
| 7 | Vonêche | 30 | 122 | 3 | 0 | spruce, oak, alder | 4,7 | medium | 3,8 | 4, 11 | 388 | 193681 | 81038 |
| 8 | Gesves | 20 | 117 | 1 | 1 | maple, oak,birch, wild cherry | 5 | low | 4,2 | 5,10,8 | 276 | 199401 | 123770 |
| 9 | Mochamps | 90 | 154 | 2 | 0 | oak | 4,7 | medium | | 4, 11 | 515 | 223737 | 86286 |
| 10 | Rulles | 95 | 80 | 2 | 0 | oak, alder | 4,7 | low | 3,7 | 4 | 440 | 237340 | 49124 |
| 11 | Awenne | 30 | 87 | 2 | 1 | oak, spruce, douglasfir | 4,7 | medium | 3,3 | 10 ,4 | 417 | 218288 | 83797 |
| 12 | Morialmé | 0 | | | | maple, alder, ash, blackthorn | 7 | high | 3,4 | 5,8 | 230 | 162921 | 108525 |
| 13 | Spa | 95 | 95 | 2 | 0 | oak, spruce, holly | 4,5 | medium | 3,3 | 4,10, 11 | 449 | 256823 | 129467 |
| 14 | Brussels | 95 | 194 | 1 | 1 | maple, ash | 7 | medium | 5,9 | 11 | 85 | 155472 | 166202 |

[1]: mean circumference of the trunk (in cm) measured at 1.5 m from the ground on a sample of 10-15 trees in the stand

[2]: Importance of beech regeneration in the stand (0=nil ; 1= low ; 2= medium ; 3=high)

[3]: Occurrence of trees with bleeding cankers in the stand (0= no tree with bleeding canker, 1= tree with bleeding canker)

[4]: according to "Le fichier écologique des essences forestières", Région wallonne (Anonymous, 1991).

Note: pH of water and pH of soil (pH KCl) were measured in September 2014.

*Phytophthora* isolates were collected by baiting in water with the method described by Thomas Jung (partner 13 of the project). This method consists of floating leaves of plants susceptible to *Phytophthora* in a mesh bag (kept in place by a styrofoam block) during several days (Figure 2). The zoospores of *Phytophthora,* which are motile in water, infect the leaves. In 2014, we used as baits mature leaves of *Rhododendron* (this species being susceptible to a large number of *Phytophthora* species), young leaves of beech (*Fagus sylvatica*) and young leaves of oak (*Quercus petraea*) collected on one-month old seedlings produced in the greenhouse. In 2015, we used only *Rhododendron* leaves. The mesh bags were maintained during 7 days in water before isolation on PARP-V8 from leaves displaying necrotic spots.

Figure 2 - Baiting of *Phytophthora i*n water

The *Phytophthora* isolates visualized under the microscope have been subcultured onto CMA (Corn Meal Agar). Hyphal tip cultures have been produced and stored in sterile water at 13°C. *Phytophthora* were identified by sequencing the ITS with primers ITS2/ITS5. A BLAST analysis was carried out to compare the sequence to ITS sequences available in the GenBank/EMBL databases.

#### 3.1.1.3. Collection of *Phytophthora* used for flow cytometry and GBS analysis

The collection (836 samples from 121 (putative) species) used for flow cytometry and GBS analysis consisted in isolates from ILVO (see subsection 3.1.1.1), a subsamples of *Phytophthora* isolates collected in Belgian beech forests (see subsection 3.1.1.2) during the project and *Phytophthora* isolates sent by international Resipath partners (Table II). This also included reference isolates, necessary to establish our reference data (flow cytometry and GBS) for species throughout the different *Phytophthora* clades. In most cases we single-spored or hyphal tipped the isolates and made sure they were pure before placing them in long term storage until needed for either DNA extraction (GBS) or preparation of nuclei (flow cytometry). We also contacted Arthur de Cock (affiliated with CBS = Centraalbureau voor schimmelcultures, Utrecht, Nederland) and were allowed to use his collection of frozen DNA samples from *Phytophthora* (reference) species. Those were also used for GBS analysis and are especially valuable as they are made from isolates in a respected culture collection and include several reference isolates of the species. His collection contained 126 samples from 81 species. Details on the origins of the isolates, the species, the number and nature of the samples for each species are provided in Tables II and III. Identity is based on preliminary identification (e.g. rDNA ITS sequencing), and may in some cases change based on GBS analysis, as it is a more powerful method for species identification.

Table II - Origin of *Phytophthora* samples and number of samples and species that are maintained in collection at ILVO for use in flow cytometry and GBS analyses. This number does not include 123 additional ILVO isolates that were not yet fully identified. Samples from SLU and CBS are DNA only.

| contact person | institute | country | Number of *Phytophthora* species | Number of isolates |
|---|---|---|---|---|
| Anne Chandelier | CRA | BE | 6 | 13 |
| Claude Husson | INRA | FR | 2 | 46 |
| Sabine Werres | JKI | DE | 21 | 48 |
| Thomas Jung | UALG | PT | 46 | 204 |
| Jaime Aguayo | ANSES | FR | 4 | 5 |
| Miguel Redondo | SLU | SE | 19 | 72 |
| Tamara Corcobado | BFW | AU | 2 | 3 |
| Kurt Heungens | ILVO | BE | 31 | 309 |
| Arthur Decock | CBS | NL | 81 | 126 |
| Slavtcho Slavov | ABI | BG | 7 | 10 |
| total | | | | 836 |

Table III - *Phytophthora* species of which cultures and/or DNA are maintained in collection at ILVO for use in flow cytometry and/or GBS analyses. Numbers represent the numbers of samples (DNA and/or cultures) for each species (or hybrid). This list excludes 123 isolates with incomplete identification.

| Species name | DNA | cultures | total |
|---|---|---|---|
| unknown species | | 2 | 2 |
| alticola | 1 | | 1 |
| amnicola | | 11 | 11 |
| andina | 1 | | 1 |
| arecae | 1 | | 1 |
| austrocedri (austrocedrae) | 1 | 2 | 3 |
| avicenniae | 1 | | 1 |
| bahamensis | 1 | | 1 |
| batemanensis | 1 | | 1 |
| bilorbang | 1 | 2 | 3 |
| bisheria | 1 | | 1 |
| boehmeriae | 2 | | 2 |
| boodjera | | 1 | 1 |
| botryosa | 2 | 2 | 4 |
| brassicae | 2 | | 2 |
| cactorum | 22 | 33 | 55 |
| capsici | 3 | 2 | 5 |
| captiosa | 1 | | 1 |
| castaneae | | 3 | 3 |
| chlamydospora | | 10 | 10 |
| cinnamomi | 3 | 30 | 33 |
| citricola? | 3 | 11 | 14 |
| citricola E | | 1 | 1 |
| citrophthora | 2 | 5 | 7 |
| clandestina | 1 | | 1 |
| colocasiae | 2 | | 2 |
| constricta | 1 | | 1 |
| crassamura? | | 2 | 2 |
| cryptogea | 7 | 7 | 14 |
| drechsleri | 3 | 7 | 10 |
| elongata | 1 | | 1 |
| epistomium | 2 | | 2 |
| erythroseptica | 4 | | 4 |
| europaea | 1 | 1 | 2 |
| fallax | 1 | | 1 |
| foliorum | 1 | | 1 |
| fragariae | 1 | 5 | 6 |
| frigida | 1 | | 1 |
| gallica | 2 | 1 | 3 |
| gemini | | 3 | 3 |
| gonapodyides | 11 | 20 | 31 |
| gonapodyides variant | | 4 | 4 |
| gregata | 2 | | 2 |
| hedraiandra | | 9 | 9 |
| heveae | 3 | 4 | 7 |
| hibernalis | 3 | 3 | 6 |
| humicola | 1 | | 1 |
| hydropathica | | 7 | 7 |
| idaei | 1 | | 1 |
| infestans | | 4 | 4 |
| Ilicis (nemerosa/pseudosyringae) | | 2 | 2 |
| inundata | 1 | 2 | 3 |
| ipomoeae | 1 | | 1 |
| iranica | 1 | | 1 |
| katsurae | 1 | | 1 |
| kernoviae | | 4 | 4 |
| lacustris | 9 | 46 | 55 |
| lateralis | 2 | 5 | 7 |
| meadii | 1 | 5 | 6 |
| meadii-like (A1) | | 5 | 5 |
| megakarya | 2 | | 2 |
| megasperma | 5 | 15 | 20 |

| Species name (continued) | DNA | cultures | total |
|---|---|---|---|
| megasperma? | | 1 | 1 |
| mirabilis | 1 | | 1 |
| morindae | 1 | | 1 |
| multivesiculata | 2 | | 2 |
| multivora | 1 | 11 | 12 |
| nicotianae | 4 | 15 | 19 |
| niederhauserii | 1 | 5 | 6 |
| occultans | | 4 | 4 |
| palmivora | | 1 | 1 |
| parvispora | 2 | 1 | 3 |
| pini | 1 | 4 | 5 |
| plurivora | 10 | 47 | 57 |
| polonica | 1 | 2 | 3 |
| polymorphica | 1 | | 1 |
| porri | 1 | 2 | 3 |
| primulae | 1 | 2 | 3 |
| pseudocryptogea | 1 | | 1 |
| pseudocryptogea_X_kelmania | | 4 | 4 |
| pseudosyringae | 3 | 10 | 13 |
| pseudotsugae | | 1 | 1 |
| psychrophila | 1 | 2 | 3 |
| PT_sp1_X_pseudocryptogea | | 6 | 6 |
| PT_sp3_thermophila-like | | 1 | 1 |
| PT_sp5_Peru4-like | | 4 | 4 |
| PT_sp6_xHennops-like | | 6 | 6 |
| quercetorum | 1 | 4 | 5 |
| quercina | 4 | 8 | 12 |
| quininea | 1 | | 1 |
| ramorum | 2 | 62 | 64 |
| richardiae | 1 | 1 | 2 |
| riparia | 1 | | 1 |
| rosacearum | 2 | | 2 |
| rubi | 1 | | 1 |
| siskiyouensis | 1 | | 1 |
| sojae | 1 | | 1 |
| sp kelmania | | 1 | 1 |
| spinosa var. lobata | 1 | | 1 |
| X attenuata-like | | 1 | 1 |
| X botryosa-like (A1) | | 3 | 3 |
| X Hennops | | 1 | 1 |
| X WS (Clade 9 hybrid) | | 1 | 1 |
| syringae | 3 | 8 | 11 |
| taxon parsley | 1 | | 1 |
| tentaculata | 2 | 3 | 5 |
| terminalis | | 1 | 1 |
| thermophila | | 6 | 6 |
| trifolii | 1 | | 1 |
| tropicalis | 2 | | 2 |
| uliginosa | 2 | 1 | 3 |
| uniformis | 5 | 9 | 14 |
| vesicula | 2 | | 2 |
| vignae | | 1 | 1 |
| x alni | 3 | 15 | 18 |
| x cambivora | 6 | 35 | 41 |
| x heterohybrida | | 28 | 28 |
| x incrassata | | 7 | 7 |
| x multiformis | | 10 | 10 |
| x pelgrandis | 2 | 5 | 7 |
| x serendipita | | 22 | 22 |
| Total number of species | 85 | 79 | 121 |
| Total number of samples | 198 | 638 | 836 |

### 3.1.2. Characterisation of *Phytophthora* isolates (Hybrid detection)

#### 3.1.2.1. Evaluation of the hybrid status of *Phytophthora cambivora* isolates from beech forests by sequencing of single copy genes

A *Phytophthora cambivora* isolate was collected in winter 2013/2014 in a beech stand located in Mortehan (Luxembourg province) displaying serious problems of dieback of trees. By using a PCR method specific to *Phytophthora* sp. targeting a single copy gene (Ypt gene, ras-related protein gene, Schena et al. 2008) and performing a sequencing of the amplification product, the blast analysis revealed a 98% identity with *P. cambivora* and *P. alni uniformis*, two closely related species. An analysis of the chromatogram revealed multiple peaks, a phenomenon which might indicate that the isolate corresponds to a hybrid (Figure 3).

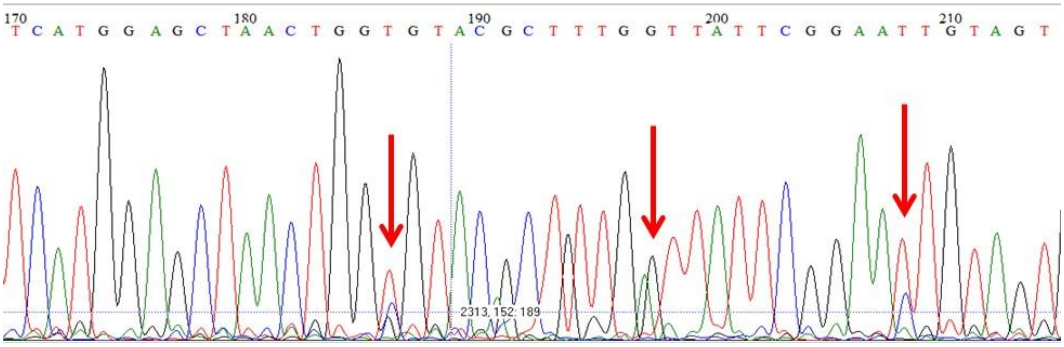


Figure 3 - Chromatogram of the Ypt gene in isolate 4557H. The arrows indicate multiple peaks

In order to confirm this result, two single copy genes (HSP90 and Beta-tubulin genes) have been amplified and cloned in plasmid vectors for this isolate (code 4557H) and for another *P. cambivora* isolate available in the CRAW collection and isolated from beech in 2005 (code 3399H). The protocol used for the amplification and the cloning of amplification products was provided by T. Jung (partner 13 of the project). We used a high fidelity Taq DNA polymerase (Pfu, Promega) for the amplification. The consensus sequences of the different clones were aligned with the BioEdit software and analyzed for the presence of SNPs (Single-Nucleotide Polymorphism). The number of haplotypes has been determined for both isolates based on the different combinations of SNPs.

An inoculation test was conducted in August 2015 to compare the aggressiveness of this isolate with other *Phytophthora* pathogenic on beech. The roots of beech seedlings (9 replicates/treatment) were immersed in river water supplemented with zoospores (1000 zoospores.ml$^{-1}$) of *Phytophthora cambivora* (isolate 4557H & isolate 3399H), *P. gonapodyides, P. plurivora* and *P. pseudosyringae* (resi045, 054 and 069 respectively, appendix 2) during 48h. As a negative control, beech seedlings were also immersed in non-inoculated river water. The plants were then potted in loam soil and maintained in the greenhouse at 20°C with daylight. After one month, the number of wilted plants was determined.

### 3.1.2.2. Flow cytometry

A. Introduction

The first method to detect the hybrids is flow cytometry, in which the genome size of the potential hybrids is determined and compared to the genome sizes of non-hybrid species. As some hybrids are allopolyploids, they can have large genome sizes. This method should also allow detection of genome size fluctuations in unstable hybrids, in which parts of the parental genomes are lost. The flow cytometry method is based on the individual measurement of fluorescence from a large number of nuclei that were stained using a fluorescing DNA-intercalating dye. To adopt flow cytometry for this purpose, an existing protocol that was applied to *Phytophthora ramorum* in Vercauteren *et al.* (2010) was optimized. One of the necessary improvements was to increase the resolution of the measurements. The fluorescence peaks should be sharp and reproducible, allowing measurements on a linear instead of on a logarithmic scale. The ultimate goal was to obtain a protocol that results in sufficient precision and in limited variation (sufficient accuracy). To obtain these goals we optimized the growing conditions of the *Phytophthora* cultures and we also optimized the choice and ratio of reference material.

B. Optimization of the protocol

Optimizations were evaluated using species of *Phytophthora* from clades 7, 8 and 10 (according to Blair et al. 2008) with an expected variation in genome size (sizes determined in public genome sequencing projects): *Phytopthora cambivora* (clade 7a; 432 Mb), *P. cryptogea* (clade 8a; 198 Mb), *P. syringae* (clade 8b; size unknown), *P. ramorum* (clade 8c; 108 Mb) and *P. kernoviae* (clade 10; 86 Mb). Working cultures of these species were actively maintained. One of the first optimizations consisted of testing different ages of the *Phytophthora* cultures that were used to prepare the nuclei. This age can affect the homogeneity of the nuclear sizes throughout the mycelium. Specifically, we tested nuclei derived from 7, 8, 9 or 10-day old cultures on V8 agar (3 replicates). A second optimization consisted of testing the type of nutrient medium (V8 versus C&N (Chee and Newhook,1965)) and the concentration of the nutrient medium (V8 medium with 20%, 10%, 5% or 2.5% V8 juice). These may affect the amount of interfering fluorescent particles that may reduce the sharpness of the peaks. A third optimization involved the use of *Arabidopsis* (2C= 0.32 pg or 313 Mb) instead of *Raphanus* (2C= 1.11 pg or 1076 Mb) as the reference genome for *Phytophthora* species with a relatively small genome. Measurement on a linear scale requires that the difference in genome size between the sample and the reference be limited. Lastly, we tested different ratios of reference to sample material.

C. Flow cytometry on *Phytophthora* isolates

The first set of isolates consisted of (potential) hybrids in clade 7a that were provided by the Portuguese partner. This set consisted of 16 isolates of *P. x heterohybrida* (proposed as a new species; origin = Taiwan), seven isolates of *P. x incrassate* (proposed as a new species; origin = Taiwan) and 24 isolates of *P. cambivora* (now also recognized as a hybrid species; origins = Australia, Belgium, Chile, Germany, Spain, Italy, Japan, Korea, Portugal,

Slovakia, USA). Our analysis consisted of determining the genome sizes, to confirm the potential allopolyploid and hybrid nature of these isolates.
The second set was a selection of isolates of *P. cambivora, P. alni*, *P.uniformis*, *P. multiformis* and *P. pseudosyringae,* collected by our Belgian project partner (CRA-W). Among the *P. cambivora* isolates are the interesting one in terms of number of haplotypes of specific genes – see subsection 3.1.2.1). One of the goals was to obtain reference data across the different *Phytophthora* species. Therefore, the selection of isolates from our collection was based on representation of species from the different phylogenetic clades. It also included known hybrids from clade 1 isolates. The isolates used are listed in Table XII (in the section where the results are presented).
The third set concerned the following species: *P. castanea, P. hevea, P. hydropathica, P. lateralis, P. polonica, P. pseudosyringae, P. quercetorum, P. syringae, P. tentaculata*. Isolates of the following species are still under flow cytometry analysis: *P. drechsleri, P. europae, P. fragariae, P. gallica, P. hibernalis, P. infestans, P. lacustris, P. megasperma, P. niederhauserii, P. parvispora, P. primulae, P. quercina, P. richardiae, P. uliginosa, P. vignae, P.* x *alni and P.* x *multiformis.*

All isolates were grown and analyzed two or three times, on separate days. Within each day, the isolates were also measured three times. The averages of a day's measurement are taken as replicates.

#### 3.1.2.3. Genotyping by sequencing (GBS)

A. Introduction

The second method to detect the hybrids is genotyping by sequencing (GBS). This is a "complexity reduction" technique in which the sequence of DNA fragments that flank restriction sites is determined using a next generation sequencing (NGS) technique (Illumina platform). The technique (in the version that we use it, with two enzymes) is explained in Figure 4. We use the two enzyme version as it has downstream advantages in data analysis. GBS data of unknown candidate *Phytophthora* hybrids, in combination with data from non-hybrid reference species must allow us to identify hybrids and their parental species. GBS is technically more advanced than flow cytometry. From the data it is possible to not only determine whether the isolate is a hybrid (even if the hybridization event took place a long time ago and a large proportion of the DNA of one of the parents has been eliminated) but also to determine the nature of the parental species and whether different hybrid isolates are likely derived from the same hybridization event or not.

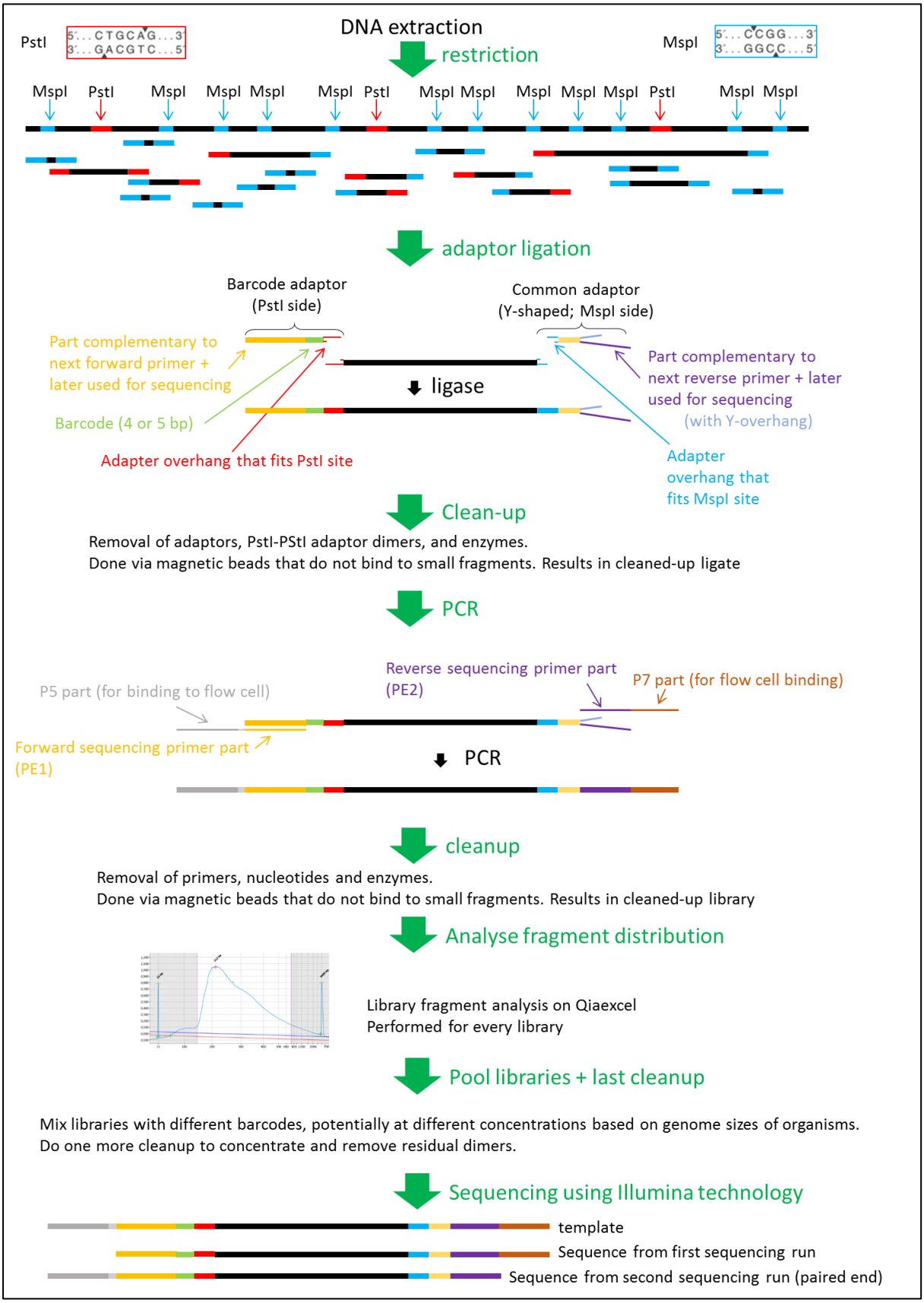


Figure 4 - Schematic of the GBS protocol

B. Optimizations for the GBS technique

Different parameters have been optimized for applying the GBS method on *Phytophthora* isolates.

Mycelial growth in mineral media or on more standard organic media such as V8. The motivation for wanting to work with a mineral medium is to avoid contaminating DNA from the plant. Media that were evaluated were C&N (see section flow cytometry), E&K (Erwin and Katznelson, 1961) and P-4 (Hohl, 1983). The weight of mycelial mats of *P. cambivora, P. ramorum, P. citrophthora, P. cactorum,* and *P. kernoviae* was determined 10 days post inoculation on these media and compared to the weight on the reference medium (2% V8).
Tissue maceration techniques for the level of shearing and for their contribution to the enhancement of DNA yield. This was conducted with *P. cambivora* only, which was chosen because of the large size of its genome. The maceration techniques were beadbeating, manual crushing of the mycelium in N2, and just a freeze-thaw cycle. Beadbeating was performed with or without buffer, with 0.5 mm versus 3 mm beads, and with one beadbeating cycle of 30 s versus two such cycles). DNA was extracted using the Quickpick method, which itself creates only little additional shearing. The DNA yield and the level of shearing was evaluated using gel electrophoresis (after an RNAse treatment).

The DNA extraction. Quality of grinding in liquid $N_2$ with either plastic or metal micropestles was compared. Several DNA extraction methods were evaluated using this macerated mycelium, included a standard CTAB technique and commercial DNA extraction kits: Nucleospin, Quickpick, invisorb, DNeasy, Puregene. DNA amount was evaluated using nanodrop and Quantus readings. DNA quality (shearing) was evaluated using electrophoresis. Most tests were conducted using DNA from *Phytophthora ramorum* and *P. cambivora*. Extracted DNA was also cut with restriction enzymes *Hind*III and *Eco*RI, to check the absence of inhibitors for such enzymes in the DNA extract.

The restriction enzymes and conditions for the restriction digest that would work well on DNA of all *Phytophthora* species were determined. We were interested in identifying a 4-cutter and a 6-cutter enzyme that would fulfill this criterion. We evaluated the enzymes using gel electrophoresis: clear and uniform smears needed to be present without the presence of bands and without residual uncut DNA. In a first step, we tested the enzymes with DNA of *P. ramorum* and *P. cambivora* (small and large genome, respectively) using 100 ng genomic DNA that was extracted with the Nucleospin kit (considered the best extraction method, see the results section). The enzymes tested were *Eco*RI, *Hind*III, *Not*I, *Xba*I, *Sal*I, *Pst*I, *Hpa*II/*Msp*I, *Pst*I and *Pae*I (FastDigest version from Thermofisher). We also tested whether additional digest time (90 min versus 15 min) resulted in improved activity and whether the amount of enzyme (recommended amount versus half concentration) could be reduced. The best two enzymes were tested using a larger number of *Phytophthora* species (*P. megasperma*, *P. cinnamomi*, *P. hedraiandra*, *P. inundata*, and *P. citrophthora* on top of *P. ramorum* and *P. cambivora*).

The amount of adaptors. Too many adaptors can lead to adaptor dimers, who would be preferentially sequenced due to their smaller size. Not enough adaptors could result in a loss of the number of restriction fragments that would receive the adaptors, leading to a loss of information. Amounts tested included 0.1, 0.25 and 0.5 pmol.

The number of PCR cycles. Too few cycles results in insufficient amplification of all restriction fragments. Too many cycles will reduce the number of restriction fragments that will be amplified to a sufficiently large number of copies, while some restriction fragments will be amplified to an unnecessary large number of copies, resulting in a reduction of desired information. The number of cycles tested was 12, 14, 16, 18, and 20. One of the criteria in this evaluation is the amount of DNA (ng) in the 100 to 1000 bp fragment range (after cleanup), as evaluated with the Qiaxcel machine (automated electrophoresis).
The amount of primers should be large enough so as to avoid shortage of primers, but not too large so that primers peaks would become a problem in the profile. The difference between 1 or 2 µL of 10 µM primer solutions was tested.

The PCR annealing temperature. We tested if the annealing temperature could be lowered from 65°C to 62°C, in order to increase the amount of product (size of the library) obtained.
The PCR elongation time. We tested if the elongation time cold be shortened, in order to have an additional selection for the short fragments. These short fragments are the only ones that can/will be sequenced and therefore their enrichment might be beneficial (signal focused on the relevant fragments). We tested elongation times of 10, 20 and 30 seconds.

C. First library preparation and submission for sequencing

The final, optimized protocol was applied to 96 DNA samples from 75 isolates. These isolates were from 29 *Phytophthora* species. The number of DNA samples is larger than the number of isolates because several isolates were analyzed in triplicate, including independent DNA extraction and ligation steps, to determine the reproducibility of the protocol. Most isolates were grown in the mineral C&N medium, to exclude the introduction of non-target DNA via a nutrient medium that would be derived from plant substrates (such as PDA and V8) and which thus might contain contaminating plant DNA. However, because some isolates did not grow well on the C&N medium, they were grown on V8. To evaluate the effect of V8 on the final sequence results, some isolates were included after growth on C&N as well as after growth on V8 medium. Each sample was run with a different barcode (ligated via the *Pst* adaptor), allowing multiplexing of 96 isolates in a single sequencing run. DNA amounts were adjusted as a function as the (estimated) genome size of the species, to avoid that isolates with relatively large nuclear sizes would have fewer copies of individual genes in the sample. The species used were *cactorum, hedraiandra, x serendipita, pelgrandis, nicotianae, citrophthora, multivora, pini, plurivora, inundata, lacustris/riparia, megasperma, PgChlamydo, gonapodyides, cambivora, camb/alni, cinnamomi, cryptogea/drechsleri, erythroseptica, lateralis, ramorum, kernoviae, syringae, occultans, x alni, x multiformis, uniformis, heveae, hydropathica, pseudosyringae, quercetorum,* and

*quercina*. As with flow cytometry, they were chosen to represent the different *Phytophthora* clades. The merged libraries were sent in for Illumina Hiseq sequencing via Genohub.

## 3.2. Detection of invasive fungi with an aerial dispersal

### 3.2.1. Comparison between different spore trapping systems

#### 3.2.1.1. Selection of fungal pathogens

In order to fulfill the objectives of the study, different conditions had to be met in the selection of fungal pathogens to study. The pathogenic fungi have to
- have an aerial dispersal of spores
- produce spores of different sizes (as trapping efficiency is linked to the spore size)
- release spores at different periods of the year (to scale the experiments over an extended period as the equipment and technical staff are limited)
- belong to ascomycetes and basidiomycetes (as PCR primers used for Next Generation Sequencing approach do not necessary display the same efficiency on both groups);
- concern forest species present in Belgium.

Based on these criteria, three fungal pathogens were chosen (Table IV).

Table IV - Characteristics of the fungal pathogens selected for the study

| | | | Spore | | |
|---|---|---|---|---|---|
| Fungal pathogen | Fungal division | Host plant | Type | Mean size | Period of release |
| *Heterobasidion annosum* | Basidiomycete | *Picea sp., Abies sp., Pinus sp.* | Basidiospore | 3,5 x 2,5 µm | Spring/Fall |
| *Erysiphe alphitoïdes* | Ascomycete | *Quercus robur, Q. petraea* | Conidia | 30 x 18 µm | End of spring/Summer |
| *Hymenoscyphus fraxineus* | Ascomycete | *Fraxinus excelsior* | Ascospore | 17 x 4 µm | Summer |

#### 3.2.1.2. Selection of the (forests) stands

There were two types of forest plots. One plot located at Floriffoux is a mixed forest where the three fungal pathogens are present (=non-specific site). Three other plots correspond to forests where one of the three pathogens selected in the study is present at a high inoculum level (=specific sites). These were:
- Maissin = douglas fir plantation with lots of spruce stumps on which fruiting bodies of *Heterobasidion annosum* are frequent
- Bièvre = pure oak forest highly affected by *Erisiphe alphitoïdes* in 2013
- Morialmé = ash stand with all trees infected by *Hymenoscyphus fraxineus*

An additional plot was established by ILVO at the Proefcentrum voor Sierteelt in Destelbergen (PCS), in a mock nursery tree stand that is used to monitor pests and diseases (e.g. powdery mildew). The purpose of adding this site to our study was to see if the results (e.g. regarding the type of rods in the RST samplers) can be confirmed at an

independent institute but also to determine if the aerial spore trapping methods can be used in a nursery setting, which may be very relevant when trying to detect introduced exotic pathogens.

### 3.2.1.3. Selection of spore trapping systems

Two types of filter papers (Whatman) displaying different porosity (n°1 and n°3) have been tested. They are soaked with TE4x as recommended by Garbelotto et al. (2008) prior to trapping to avoid germination of spores. An “in house” rotating-arm spore trap system (called Rotorod thereafter) already used for the collection of ascospores of *Hymenoscyphus fraxineus* (Chandelier et al. 2014) has also been evaluated. Two types of arms (different widths) have been tested. Type 1 is composed of matches as rods (width=2.5 mm) while type 2 is composed of flat nails as rods (width=1.25 mm). The arms are covered with double side tape (Tesa, double side universal). A Burkard 7-day volumetric sampler has also been tested as reference in one forest stand (Floriffoux) in 2014. The capturing surface of the Burkard sampler is made of Melinex tape covered with vaseline (manual application).

All spore traps were put at ~ 1 m from the ground in close proximity to each other. The different spore trapping systems are illustrated in Figure 5.


Figure 5 – Spore samplers evaluated in the project.

### 3.2.1.4. Experimental design (2014 & 2015)

Non-specific forest site (Floriffoux): an experimental design comprising the three types of spore traps was installed in 2014 in a privately owned mixed forest. The site is mainly composed of oaks but the other tree species under study were also present in the vicinity. From April to November 2014, 8 tests were carried out for a period of 14 days (~one experiment/month). The Burkard sampler was in use from day 1 to day 15 as two filters n°1 and two filters n°3. The Rotorods (two of type 1 and two of type 2) and additional filters (two filters n°1 and two filters n°3) were used during a 2-day period (corresponding to the maximum period of collection for the Rotorod - from day 1 to day 3, and from day 13 to day 15).
In 2015, eleven experiments were conducted from June to September considering two types of Rotorods with the same width (1.25 mm) but differing in length (2.5 or 4 cm), and filter papers (Whatman No. 3) (the Burkard sampler was not used in 2015). Three replicates were considered for each type of trap. All spore traps were in use for 2 days.

Specific forest sites (Maissin, Bièvre, Morialmé): These stands were surveyed in 2014. We used Rotorods with nails and matches and filter papers (Whtaman 1 and 3). The Burkard sampler was not evaluated in public forests due to the risk of vandalism. The periods of spore collection within a test were the same as those established for the non-specific plot (Floriffoux). Three experiments targeting *H. annosum* were carried out in Maissin (one in April, one in September and one in October 2014). One experiment was conducted in the two other locations (in June/July 2014 in Bièvre for *E. alphitoïdes* and in July/August 2014 in Morialmé for *H. fraxineus*).

Nursery tree site (PCS): At this site ILVO used two Burkard spore samplers, four RST samplers (two of type 1 and two of type 2), and four filter samplers (filters n°1 and n°3). Sampling was conducted during 6 weeks, starting July 28, 2014.The samples from the nursery site were going to be processed by ILVO with the assays for *Hymenoscyphus fraxineus* and *Erysiphe alphitoïdes*, as those are the pathogens than may be present at this site. However, a first comparison between the samplers was conducted with primers that were designed at ILVO for the specific detection of *Fusarium oxysporum*. This organism is ubiquitous in air and allows comparison between the samplers in case the volume of the spores of *Hymenoschyphus* and *Erysiphe* is low. Because the results with *F. oxysporum* were clear and because the levels of even *F. oxysporum* were low, detection of the other pathogens in the samples was no longer conducted.

### 3.2.1.5. Efficiency of two types of rods used with Rotorod device

Two types of rods (matches or nails) were compared for the collection of spores using Rotorod devices. The surface of the impaction area (double side tape) was the same for both types of rods (50 $mm^2$). However, the width was different (match, 2 mm; nail, 1.25 mm).

The rods were soaked in cotton blue for 30 s in order to stain the spores (at least the hyaline ones). The impaction surface were then mounted on microscopic slides and spores were counted with an inverted microscope Olympus IX83 (magnification 400x) in two randomly selected areas of 1 x 2 mm on each impaction surface (= 4 replicates/ Rotorod type) (Figure 6). Two categories of spores were counted: those of less than 10 µm ("small spores"), and those of more than 20 µm ("large spores"). The test was done on impaction surfaces from Rotorods put in two forest stands (Maissin and Bièvre) at two different periods of the year 2014 corresponding to periods conducive to the release of specific spores (basidiospores of *Heterobasidion annosum* in Maissin, "small spores", and conidia of *Erysiphe alphytoides* in Bièvre, "large spores"). Both types of Rotorods (match/nail) were in use during 2 days in the forest stands. Statistical analyses (linear regression) were carried out on log-transformed counts using the R software.

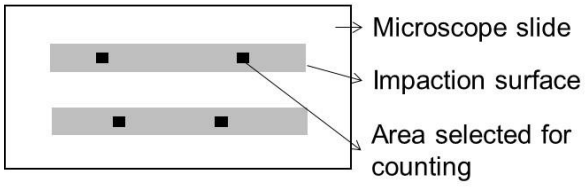


Figure 6 – schematic of the microscope slide with the counting areas on the impaction surface.

At ILVO, DNA extraction from nails as rotorod rods was not successful, likely due to an interaction between the nails and the DNA extraction method used. An alternative type of rod, consisting of plastic, was evaluated as an alternative.

### 3.2.1.6. Optimization of DNA extraction

- Test 1: Comparison of DNA extraction kits: spores of *Erysiphe alphitoides* were spiked on filter paper (porosity 1) at two concentrations (52000 and 5200 spores) with two replicates per level. Two kits were evaluated for the recovering of DNA: the Nucleospin Plant II kit from Macherey Nagel, and the High Pure Template Preparation kit from Roche Diagnostic with a lysis buffer developed at CRAW. Both DNA kits were also compared on Rotorod samplers. In this case, spores of *Erysiphe alphitoides* were spiked on matches and nails covered with double site tape at two concentrations (800 and 80 spores) with two replicates per level.
- Test 2: Collection of spores from filter paper: spores of *Erysiphe alphitoides* were spiked on filter paper (porosity 1) at two concentrations (16000 and 4000 spores) with two replicates per level. Two methods were evaluated for the recovering of spores: the method described by Garbelotto et al. (2008) (filters put in Petri dishes with 20 ml TE4X at 65°C and mechanic shaking during 1h prior to centrifugation at 4000 *g*) and an alternative method (filters put in sealed plastic bags with 20 ml TE 4X at room temperature and manual shaking during 10 min prior to centrifugation at 4000 *g*). The DNA were extracted with the High Pure Template preparation kit.
- Test 3: Collection of spores from Burkard and Rotorod samplers: matches covered with double site tape (Rotorod) and Melinex covered with vaseline (Burkard) were spiked with a

suspension of spores of *Erysiphe alphitoides*. There were three replicates for each type of substrate. Four treatments of spore recoveries were evaluated (Table V) prior to DNA extraction with the High Pure Template Preparation kit.

Table V. Description of the different treatments evaluated for the recovery of spores from Rotorod and Burkard.

| Treatment | Details of the protocol |
|---|---|
| T1 | Beat beater during 30 s at 30 Hz |
| T2 | Modality 1 with zirconium beads (200 mg) |
| T3 | Two period of stirring with a Vortex during 30 s separated by a period of 5 min at -20°C |
| T4 | Modality 3 with zirconium beads (200 mg) |

For the three tests, the comparison was based on the Ct values obtained with the real-time PCR developed in this project for the detection of *Erysiphe alphitoides.* The best conditions were those providing the lowest Ct values.

In order to determine the efficiency of the DNA extraction method for Whatman filter (No. 3), double-side tape (on nail or match, Rotorod) and Melinex covered with Vaseline (Burkard sampler), serial dilutions of known concentrations of *E. alphitoides* conidia (3 concentration levels and two replicates per level) were spiked on the different adhering surfaces. DNA extractions were then carried out with the methods optimized for each trapping system. As a control, DNA extractions were carried out on the same spore concentrations suspended in sterile water. The DNA samples were then analyzed with the real-time PCR method targeting *E. alphitoïdes* developed in this project.
In order to calculate the percent recovery, the Ct values were transformed in log of spore equivalents using the equation of the standard curve generated for spores in suspension. Then, the calculated log-transformed spore concentrations were compared to the theoretical log-transformed spore concentrations (ratio of both values).

### 3.2.1.7. Real-time PCR for the specific detection of the selected fungal pathogens

- *Heterobasidion annosum*: we used a Taqman assay described in the literature (Bodles et al. 2006) for the detection of this pathogen.

- *Hymenoscyphus fraxineus*: we used a Taqman assay developed at CRAW for the quantification of ascospores of the pathogen in the air (Chandelier et al. 2014).

- *Erysiphe alphitoïdes*: as the sole test available in the literature was a real-time PCR based on SYBR Green detection (Heuser & Zimmer, 2002), we developed a Taqman assay. Sequences specific to *E. alphitoïdes* were selected after alignment of ITS sequences available in the databases (using Clustal Omega software). Primers and a Taqman probe were designed using the Primer Express Software (Life Technologies). A standard curve

was established on DNA extracted from serial dilutions of conidia. The test proved to be sensitive by detecting variation in the concentration of DNA in the sample. The test was also specific (no cross-reaction with *E. quercicola*, a fungal pathogen genetically close to *E. alphitoïdes*, and occurring mainly on flag shoots in May/June) (Feau et al. 2012).

Plasmid clones containing the amplification product generated with PCR primers targeting the three pathogens were used as positive controls in the qPCR tests carried out in the study.

#### 3.2.1.8. Analysis of forest samples

The DNA were extracted from the different substrates (double-side tape on nail or on match, filter paper No. 1 and 3, Melinex tape + Vaseline) collected on spore traps put in the forest in 2014 and 2015 using the methods optimized for the different spore traps. They were then analysed with the three qPCR methods selected for the detection of *H. annosum, H. fraxineus* and *E. alphitoides* using a StepOne PCR thermocycler and a manually set threshold to enable the comparison between different PCR runs.

### 3.2.2. Optimization of the NGS protocol (High throughput sequencing and bioinformatics analysis)

#### 3.2.2.1. Optimization of DNA quantification by fluorescence

In order to guarantee reproducible results with the Next Generation Sequencing method (hereafter called “NGS”), it is essential to quantify accurately the DNA extracts that will be used as starting material for the analysis. We tested the two most frequently used methods of DNA quantification: UV spectrophotometry and ds-DNA specific fluorometric quantification. Both measures were carried out with an Eppendorf Biospectrometer fluorescence device. For the UV measurements, we used the micro-volume cuvette, Eppendorf µCuvette® G1.0 which allows direct measurement (without dilution) on a limited volume (5 µl) of DNA extract. For the fluorescence measurements, we used the Quant-iT PicoGreen dsDNA Assay (Invitrogen) with disposable UV-cuvettes micro (Brand) which allow the measurement on a limited volume (100 µl) of diluted DNA extracts (corresponding to 5 µl of pure DNA). The instrument was calibrated according to the manufacturer's instructions.
Both methods were evaluated on serial dilutions (from 10 ng/µl to 1 pg/µl) of a commercial solution of pure DNA at 10 mg/ml. The DNAs extracted from spore trap (RST-nails, collection 2014) were then quantified with the optimized protocol.

### 3.2.2.2. First PCR of the NGS protocol

One of the most critical steps in the application of high throughput sequencing techniques is the choice of PCR primers for amplicon sequencing. In this project, primers had to meet different requirements:

- the absence of cross-reaction with DNA from plant (as pollen can be collected on spore traps)
- their capacity to detect a broad taxonomic range of fungi (Basidiomycota, Ascomycota)
- their capacity to detect a low concentration of a fungal species (in this context, primers targeting multi-copy genes should be preferred)
- their capacity to give amplification products whose sequences
    - are available in the reference databases
    - are sufficiently polymorphic to distinguish two different fungal species

Based on these constraints, we have used primers targeting the ITS regions of the ribosomal RNA gene. Three primer pairs have been selected: two described by Toju et al. (2012) to carry out NGS analyses on fungi (one amplifying the ITS1 – set 1, and the other one amplifying the ITS2 – set 2), and one pair (set 3) frequently used for the study of fungal communities (Op de Beeck et al. 2014) and amplifying the ITS1. The sequence of the primers used for the first PCR of the NGs protocol is provided in Table VI.

Table VI - List of modified primers (ITS primers + overhang adapters) used for the first PCR

| set | Name | Sequence (5'-3') | Targeted region |
|---|---|---|---|
| 1 | NGSITS1KYO2F | TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG TAG AGG AAG TAA AAG TCG TAA | ITS 1 |
| | NGSITS2KYO2R | GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GTT YRC TRC GTT CTT CAT C | |
| 2 | NGSITS3KYO2F | TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG GAT GAA GAA CGY AGY RAA | ITS 2 |
| | NGSITS4R | GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GTC CTC CGC TTA TTG ATA TGC | |
| 3 | NGSITS1-F | TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CTT GGT CAT TTA GAG GAA GTA A | ITS 1 |
| | NGSITS2-2 | GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGC TGC GTT CTT CAT CGA TGC | |

The NGS technology technique (Illumina platform) in the version that we have used is explained in Figure 7. The first PCR (with HPLC-purified ITS primers tailed to overhang adapters – Table VI) was carried out at CRAW while the other steps of the procedure including the second PCR (adding the Illumina dual indices and the sequencing adapters), the library quantification and normalization, and the pooling were carried out by the private company carrying out the sequencing (DNA Vision, Belgium).

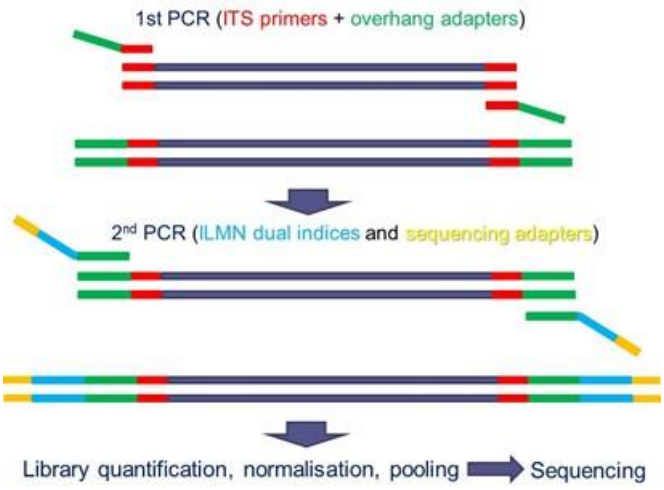


Figure 7 - ITS amplicon workflow for the NGS technique (Illumina MiSeq)

### 3.2.2.3. Preparation of the mock communities

In order to validate the NGS protocol, it is essential to generate sequence data of a defined community composed of organisms with known sequence. It is notably needed to assess the capacity of the method to correctly assign the fungi present in a complex sample at the species level, or to evaluate the influence of the amplicon size or concentration on the reliability of the method. To this end, we have constituted “mock communities” (MC) of 10 fungal species using isolates available in the CRAW collection. These species were selected on four criteria (Tables VII & VIII):

- Their potential presence in the forest stands under study
- Their belonging in one of the two fungal divisions (Ascomycota, Basidiomycota)
- Their belonging to variable genera/species
- The size of the amplification product (variable) with the three primer sets selected for the sequencing.

Table VII - Taxonomic position of the 10 fungal species used in the mock community

| Division | Class | Order | Family | Genus | Species |
|---|---|---|---|---|---|
| Ascomytoca | Leotiomycetes | Helotiales | Heotiaceae | *Hymenoscyphus* | *fraxineus* |
| Ascomytoca | Ascomycetes | Xylariales | Diatrypaceae | *Eutypa* | *lata* |
| Ascomytoca | Dothideomycetes | Pleosporales | Venturiaceae | *Phaeocryptopus* | *gauemannii* |
| Ascomytoca | Sordaryomycetes | Ophiostomales | Ophiostomataceae | *Ophiostoma* | *novo-ulmi* |
| Ascomytoca | Sordaryomycetes | Hypocreales | Nectriaceae | *Gibberella* | *lateritium* |
| Ascomytoca | Sordaryomycetes | incertae sedis | Plectosphaerellaceae | *Verticillium* | *dahliae* |
| Basidiomycota | Agaricomycetes | Agaricales | Physalacriaceae | *Armillaria* | *gallica* |
| Basidiomycota | Agaricomycetes | Polyporales | Polyporaceae | *Trametes* | *versicolor* |
| Basidiomycota | Agaricomycetes | Russulales | Bondarzewiaceae | *Heterobasidion* | *annosum* |
| Basidiomycota | Agaricomycetes | Polyporales | Ganodermataceae | *Ganoderma* | *adspersum* |

The amplicon size with the three sets of primers has been determined *in silico* based on reference sequences available in the NCBI database, and the 10 fungal species have been clustered into three groups based on this parameter (Table VIII).

Table VIII - Amplicon size (in bp) with the three sets of PCR primers for the 10 fungal species of the mock community. Each color represents a group of species based on the amplicon size (yellow= small, group 1; orange=medium, group 2; green=large amplicon size, group 3).

| | | Amplicon size (bp) | | |
|---|---|---|---|---|
| Strain # | Species | Primer Set 1 | Primer Set 2 | Primer Set 3 |
| 3732 | *Verticillium dahliae* | 238 | 347 | 245 |
| 3817 | *Hymenoscyphus fraxineus* | 593 | 328 | 596 |
| 3821 | *Fusarium lateritium* | 260 | 345 | 268 |
| 3841 | *Eutypa lata* | 298 | 338 | 306 |
| 4547 | *Ophiostoma novo-ulmi* | 320 | 395 | 317 |
| 4855 | *Phaeocryptopus gaeumannii* | 254 | 321 | 261 |
| 3343 | *Armillaria gallica* | 346 | 565 | 357 |
| 3561 | *Trametes versicolor* | 294 | 372 | 303 |
| 4137 | *Ganoderma adspersum* | 311 | 375 | 308 |
| 14/0011 | *Heterobasidion annosum* | 297 | 382 | 294 |

In order to evaluate the capacity of the method to detect variation in the concentration of target sequences, the different fungal species were used at 100 pg/PCR (MC1) or at different concentrations (100, 10, 1 pg/PCR) according to the size of the amplicon (MC2 to MC7) following the organization provided in Table IX. All MC were prepared in duplicate.

Table IX - Concentration (in pg/PCR) of the different fungal species clustered into 3 groups (according to the amplicon size with the three sets of primers – see table 3) in the different mock communities (MC).

| MC | Group 1 | Group 2 | Group 3 |
|---|---|---|---|
| MC1 | 100 pg | 100 pg | 100 pg |
| MC2 | 10 pg | 100 pg | 100 pg |
| MC3 | 100 pg | 10 pg | 100 pg |
| MC4 | 100 pg | 100 pg | 10 pg |
| MC5 | 1 pg | 100 pg | 100 pg |
| MC6 | 100 pg | 1 pg | 100 pg |
| MC7 | 100 pg | 100 pg | 1 pg |

### 3.2.2.4. Bioinformatic analysis of the data

A. Analysis carried out with published pipeline/reference database

The OTUs (Operational Taxonomic Units) were determined considering a 97% similarity cut off and the reference UNITE database (Version no. 7.1, release date 2016-08-22, https://unite.ut.ee/repository.php; Abarenkov et al. 2010). Taxonomy assignment (OTUs tables) were carried out on sequences from the mock communities, and from the forest samples using the QIIME pipeline (version 1.9.0, http://qiime.org/). The analysis has been carried out by the company DNA Vision.

B. Analysis carried out with a new pipeline/reference database

The fungal species were determined considering a 98.7% similarity cut off and a new reference ITS database developed at CRAW (prepared from sequences available on ncbi website, https://www.ncbi.nlm.nih.gov/ ). An original pipeline has been developed using BioPerl and R languages. The analysis has been carried out by Thibaut Olivier, CRAW, virology laboratory.

C. Analysis of forest samples

DNA samples extracted from Rotorods put in the forest in 2014 and 2015 were selected for high throughput sequencing (list of samples in appendix 3, two NGS runs). The bio-informatic analysis has been carried out with the pipeline/reference fungal database developed at CRAW.

# 4. SCIENTIFIC RESULTS AND RECOMMENDATIONS

## 4.1. Detection of Phytophthora hybrids

### 4.1.1. Survey of *Phytophthora* in Belgian beech stands

There were three objectives for this task: (1) the constitution of a recent collection of *Phytophthora* from forest environment for hybrid detection (by flow cytometry and GBS analysis), (2) the optimization of a protocol for the baiting of *Phytophthora* from streams in forest stands, and (3) the evaluation of *Phytophthora* species found in Belgian beech stands. This last task was relevant as beech is an important tree species in Belgian forest, and *Phytophthora* have often been associated with decline of this tree species in Europe (Jung et al. 2003; Jung et al. 2005), and notably in Belgium (Schmitz et al. 2007).

#### 4.1.1.1. Diversity of *Phytophthora* species in Belgian beech stands

A total of 98 isolates have been collected in streams of beech stands during the project. There were 65 isolates collected in 2014 (31 in spring, and 34 in summer), and 33 isolates collected in summer 2015. Six *Phytophthora* species were identified among which *P. gonapodyides* and *P. cambivora* were the most represented in the collection (Figure 8). Four of these six species are pathogenic on beech: *P. cambivora, P. plurivora*, *P. pseudosyringae* and *P. gonapodyides* (Cleary et al. 2016; Jung et al. 2003; Jung et al. 2005; Jung & Burgess, 2009). *P. chlamydospora* is widespread in wet soils and riparian environments (Hansen et al. 2015). *P. lacustris* is also a species of riparian habitats. Recently, it has been found associated with alder decline in Portugal (Kanoun-Boulé et al. 2016).

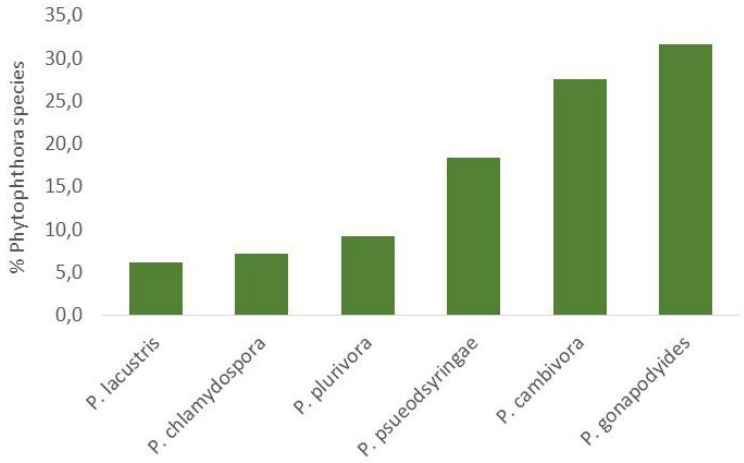


Figure 8 - Percentage of *Phytophthora* species isolated from streams in Belgian beech stands (Survey in 2014 and 2015) in the CRAW collection

In parallel to the survey in streams, *Phytophthora* were also isolated in 2014 from beech bark with bleeding cankers (Figure 9) when such symptoms were observed in the forest stands under study.


Figure 9 - Bleeding cankers on beech in forest stands
"Mortehan" (**A**, *P. cambivora*) and "Brussels" (**B**, *P. pseudosyringae*)

A total of 18 isolates were recovered from beech bark. The four *Phytophthora* species pathogenic on beech according to the literature were identified (Figure 10), with *P. cambivora* being the species most frequently isolated (recovered in 7 of the 14 stands under study). *P. pseudosyringae* was also frequent (recovered in 6 of the 14 stands) while this *Phytophthora* species had (so far) never been described causing bleeding canker in beech in Belgium.

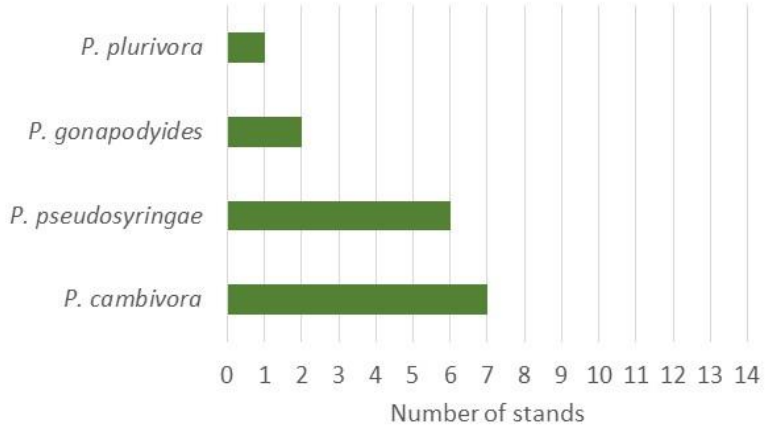


Figure 10 – Number of stands (total=14) in which *Phytophthora* species were isolated from beech bark in 2014

Hyphal tip cultures were produced for the 116 *Phytophthora* isolates. A permanent collection ("resi collection") describing these *Phytophthora* isolates has been created at CRAW (appendix 2).

#### 4.1.1.2. Influence of the baiting leaves on the baiting efficiency

In 2014, 3 types of baiting leaves were compared for the collection of *Phytophthora* from streams in beech forests (Rhododendron mature leaves, oak and beech leaflets). As shown in Figure 11, most of the *Phytophthora* isolates were recovered from rhododendron leaves. Beech leaf was the less efficient material, except for P*. cambivora*. Very often, beech leaflets disappeared after the baiting (probably eaten by insect larvae) or they were infected with *Pythium* species.

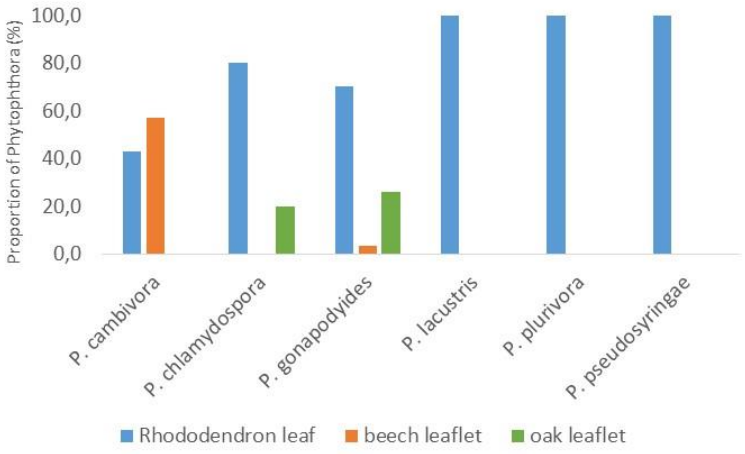


Figure 11 - Percentage of *Phytophthora* isolated from three baiting materials (beech leaflet, oak leaflet, rhododendron mature leaf)

Based on this result, it was decided to conduct the survey 2015 with rhododendron leaves only.

#### 4.1.1.3. Influence of the season on the baiting efficiency

All six *Phytophthora* species identified in Belgian beech forests were baited in summer. In contrast, only three species (*P. cambivora, P. gonapodyides* and *P. pseudos*yringae) were baited in spring (Figure 12). The number of stands in which *P. pseudosyringae* was recovered was higher in spring than in summer. In contrast, three *Phytophthora* species were only isolated in summer.

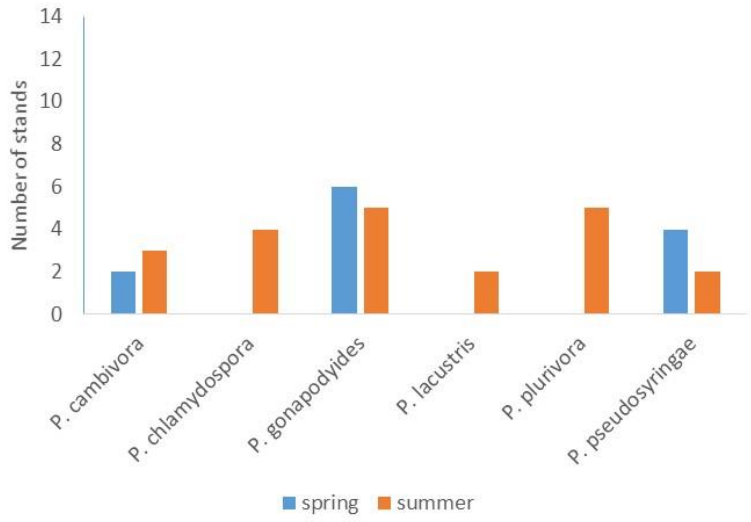


Figure 12 - Number of stands (total=14) from which *Phytophthora* species were isolated in spring and in summer.

Based on this result, it was decided to conduct the survey 2015 in summer only.

#### 4.1.1.4. Comparison between two years for the collection of Phytophthora

Ten of the fourteen forest stands surveyed in 2014 were surveyed in 2015. The number of these stands in which *Phytophthora* were baited on *Rhododendron* leaves (the baiting material used in both years) was higher in 2014 than in 2015 except for *P. cambivora* (Figure 13). This might be due to climatic conditions more conducive to *Phytophthora* sporulation in summer 2014 than in summer 2015.

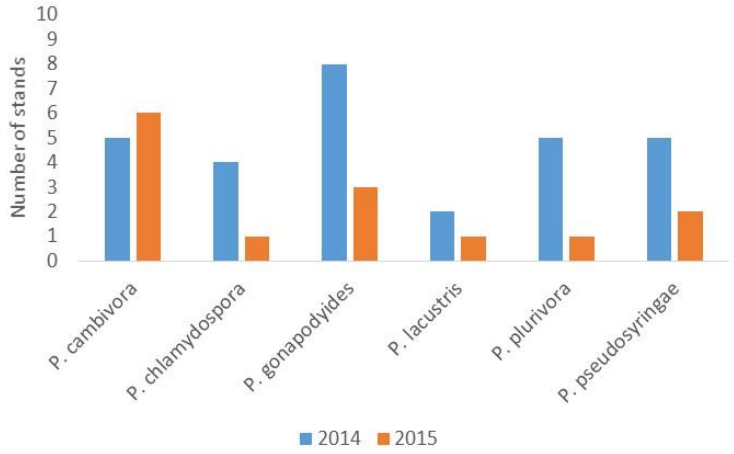


Figure 13 – Number of stands (total=10) in which *Phytophthora* isolates were recovered in 2014 and 2015 (survey in summer, rhododendron leaves used as bait in both years)

This result suggests that at least two years are necessary to evaluate the diversity of *Phytophthora* species in forest environments.

### 4.1.2. Hybrid status of *Phytophthora cambivora*

The objective if this task was to know whether a *Phytophthora* isolate recovered from a beech stand with numerous trees displaying bleeding cankers and tree mortality (Mortehan, Province of Luxembourg) could be a hybrid, more aggressive than a "typical" *P. cambivora* isolate. To this end, this isolate (4557H) was compared to another isolate available in the CRAW collection (isolate 3399H) recovered in 2005 from a beech stand in Aublain (Province of Namur) where only one tree displayed bleeding canker. The comparison was made at the genetic level, and at the phenotypical level.

#### 4.1.2.1. At the genetic level

Two single copy genes, HSP90 and Beta-tubulin genes, were amplified and cloned in plasmid vectors. A total of 89 plasmid clones were produced for the comparison.
The alignment of the consensus sequence of isolates 3399H (19 clones) and 4557H (27 clones) for HSP90 gene was 894 bp in length. A total of 17 SNPs have been identified, among which one SNP with 3 possibilities (position 214, C, G or T according to the clone). Only two SNPs were common in both isolates (positions 670 and 742). The other SNPs were observed in only one isolate, either 3399H or 4557H (Table X). As shown in Figure 14, the SNPs did not appear at random, but at specific positions in the gene.

Table X - Polymorphisms in HSP90 gene of two *Phytophthora cambivora* isolates

| | SNP position in HSP90 (894 bp) | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Isolates | 70 | 115 | 160 | 202 | 214 | 235 | 313 | 384 | 427 | 460 | 514 | 621 | 655 | 670 | 730 | 742 | 790 |
| | C | G | T | T | C | T | C | T | G | G | G | G | G | G | G | C | C |
| 3399H | C | A | C | C | G | C | C | T | C | G | C | G | G | T | G | T | C |
| | T | G | C | C | C | C | T | T | C | T | C | A | G | T | G | C | C |
| | C | G | C | C | G | C | C | A | C | G | C | G | A | G | C | T | T |
| 4557 | | | | | T | | | | | | | | | | | | |

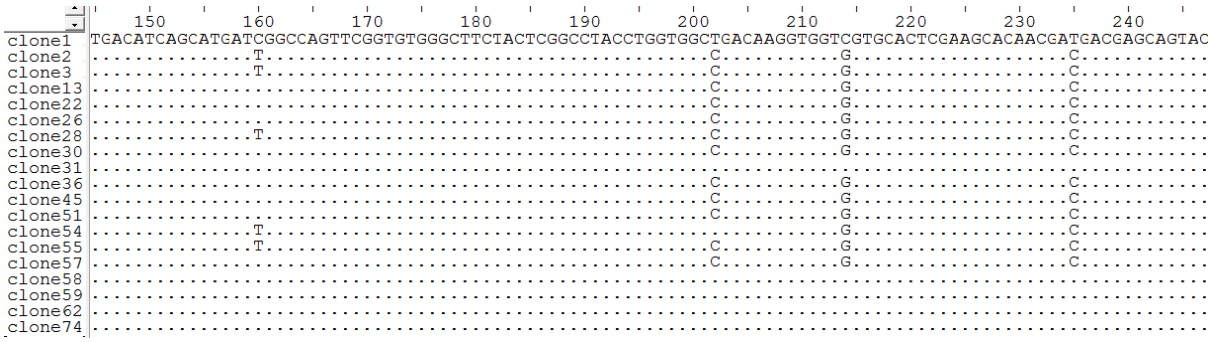


Figure 14 - Alignment with Bioedit for HSP90 gene in isolate 3399H
(19 plasmid clones for the comparison)

The alignment of the consensus sequence of isolates 3399H (19 clones) and 4557H (24 clones) for Beta-tubulin gene was 989 bp in length. A total of 18 SNPs were identified and 8 were common in both isolates (positions 73, 175, 250, 274, 349, 394, 406, 421) (Table XI).

Table XI - Polymorphisms in Beta-tubulin gene of two *Phytophthora cambivora* isolates

| | SNP position in Beta-tubulin gene (989 bp) | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Isolates | 43 | 49 | 73 | 133 | 175 | 250 | 274 | 349 | 394 | 401 | 406 | 421 | 439 | 517 | 598 | 612 | 868 | 913 |
| | G | C | T | C | T | T | T | T | C | A | C | C | C | T | T | G | G | A |
| 3399H | G | C | C | C | C | C | C | C | G | G | T | T | C | T | C | G | G | G |
| | G | C | T | T | T | T | T | T | C | G | C | C | C | T | C | G | G | G |
| 4557H | A | T | C | C | C | C | C | C | G | G | T | T | T | C | C | A | T | G |

The sequences of all clones for both genes have been grouped into 12 different haplotypes for isolate 3399H, and into 18 haplotypes for isolate 4557H. These results demonstrate that both isolates have a ploidy level higher than the “normal” one (diploid) and result probably from hybridization with more than one species.

#### 4.1.2.2. At the phenotypical level

As shown in Figure 15, inoculation of beech seedlings with *Phytophthora cambivora* lead to wilted plants.

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Figure 15 – Inoculation test.
On the left, negative control (roots in river water); on the right, inoculated plant
(roots immerged in river water supplemented with a suspension of *P. cambivora* zoospores)

Inoculation of beech seedlings with *Phytophthora cambivora* and *P. plurivora* lead to high percentage of wilted plants while *P. gonapodyides* acted as a weak pathogen and caused wilt in only one plant (~10%). We did not observe any wilting of seedlings inoculated with *P. pseudosyringae* (Figure 16). These results are consistent with those of Fleischmann et al. (2004) who showed that roots of beech saplings infected with *P. pseudosyringae* did not differ from those of the control plants while *P. cambivora* and *P. plurivora* (previous name=*P. citricola*) were very aggressive. As *Phytophthora pseudosyringae* displays an aerial dispersal

of spores (Scanu and Webber, 2015) and was identified as the causal agent of bleeding cankers in beech in Belgium (this project, survey carried out in 2014), our results suggest that infections by this *Phytophthora* species in beech are caused by an airborne inoculum. The number of beech seedlings displaying wilting differed for both *P. cambivora* isolates, with the proportion of wilted plants being higher for the isolate 4557H.

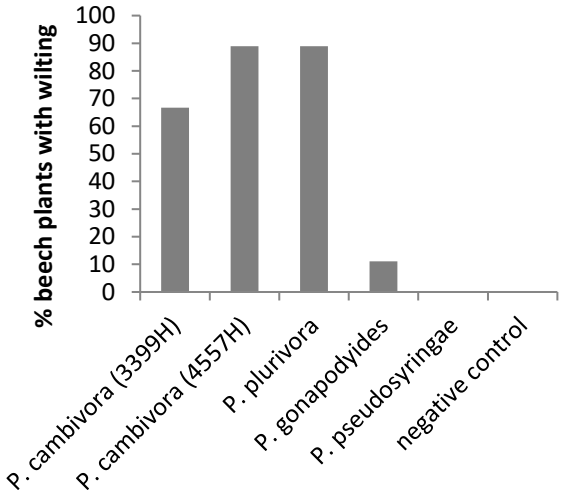



Figure 16 - Percentage of beech seedlings with wilting after root inoculation with different Phytophthora species

### 4.1.3. Flow cytometry

#### 4.1.3.1. Optimization

Testing the age of the *Phytophthora* cultures (7 to 10 days) showed that this factor did not have a significant effect on the flow cytometry measurements. However, the variation was somewhat lower when using younger cultures. The concentration of the nutrient medium (2.5 to 20% V8 juice) did play a big role: sharper peaks were observed when using more dilute medium (best = 2.5% V8 juice). The type of nutrient medium (2.5% V8 versus C&N) did not further influence the quality of the peaks. The use of *Arabidopsis* as the reference genome was another adjustment that allowed reliable measurement on a linear scale. The optimum ratio of sample to reference consisted of half an *Arabidopsis* flower stalk combined with approximately 1.5 mg filter-dried mycelium, which is processed and stained together before analysis. When combining these results in an optimized protocol, preliminary data indicate that relatively precise and repeatable measurements can be obtained, with an average coefficient of variation of 4.0%. An example of a measurement is shown in Figure 17. The large peak (P1) around the FL4 fluorescence intensity of 79 corresponds to the non-dividing nuclei of *Phytophthora ramorum*. The peak A1 around 157 is similarly derived from the non-dividing nuclei of the *Arabidopsis* reference. The peak A2 is caused by the nuclei with double DNA content (due to the nuclear division process in some cells). The peak P2 is hidden behind A1. The apparent peak at the left side of the graph is from background fluorescence and should not be taken into account. By using the ratio of 79/157 and the genome size of

the *Arabidopsis* reference genome (313 Mb), the genome size of *P. ramorum* is here estimated at 157.5 Mb.

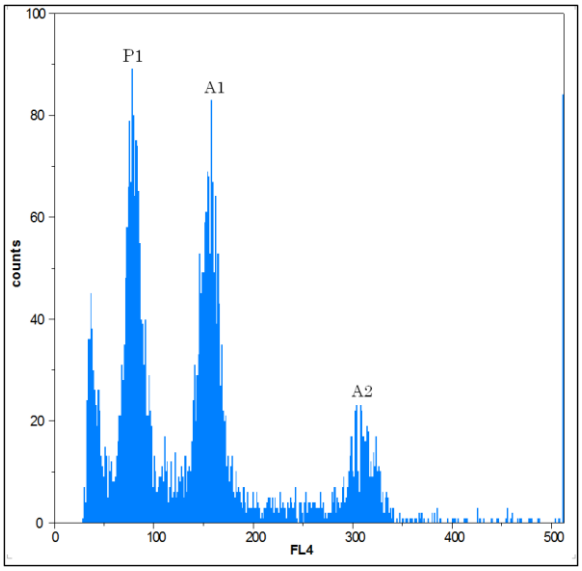



Figure 17 - Example of a flow cytometry-based determination of the nuclear size of *Phytophthora ramorum* (peak P1). The reference is *Arabidopsis* (peaks labeled A1 and A2). Further details are provided in the text.

### 4.1.3.2. Results from isolate set 1

The results of the flow cytometry-based quantification of the nuclear content of the *Phytophthora* isolate set 1 are shown in Figure 18. Between the three species there were clear differences in average nuclear (x-ploid) genome size: *P. x heterohybrida* around 325 Mbp, *P. x incrassate* around 625 Mbp and *P. x cambivora* around 460 Mbp. These genome sizes are large, reflecting their multiploid genomes. The hybrid nature of *P. x cambivora*, demonstrated by the cloning of single copy genes (see subsection 4.1.2.1) was supported by our flow cytometry data. The relative differences in genome size are correlated with the number of haplotypes of specific genes (data from project partner).

Within *P. x incrassate* and *P. cambivora* we observed a few isolates with either a deviating smaller genome size (P8-04), or with two peaks (e.g. TW269 and P7-04), the latter reflecting the presence of two kinds of nuclei in the mycelium. We hypothesize that in these less stable hybrids, the large nuclei can return to a reduced size after removal of some of the chromosomes. Preliminary data would indicate that fresh single zoospore cultures tend to have the full genome size, after which this phenomenon of nuclear reduction starts in part of the derived mycelium, after a few weeks of culture, explaining the two types of nuclei.

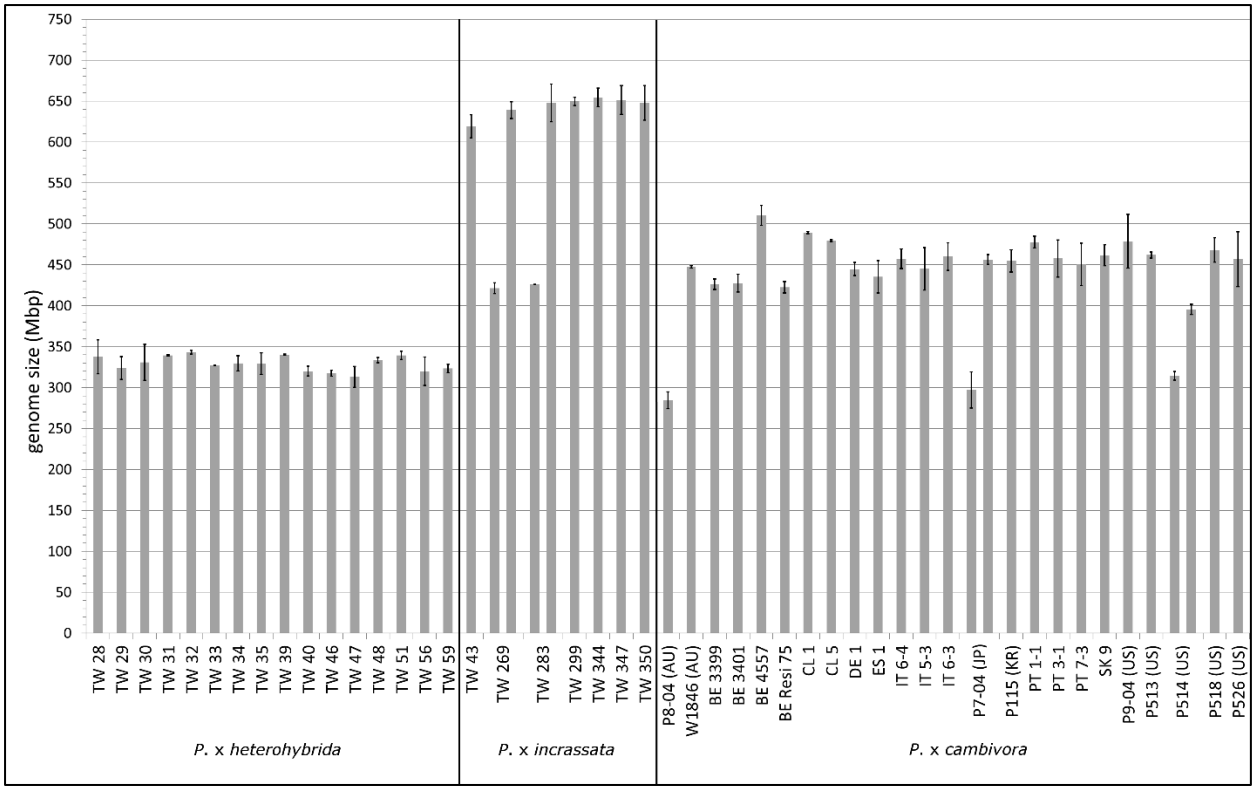



Figure 18 - Nuclear genome size of the Phytophthora isolates in set 1 (= selected hybrid isolates from Phytophthora clade 7a), as measured by flow cytometry

### 4.1.3.3. Results from isolate set 2

The results of the flow cytometry-based quantification of the nuclear content of the *Phytophthora* isolate set 2 are shown in Table XII. As with the first set of *Phytophthora* isolates, the results are quite consistent within the species, taking into account that for some isolates, full identification (using GBS) is still in progress. The range of the nuclear sizes is from 112 to 843 Mbp, the latter most likely due to an allopolyploid status after a (or several) hybridization event(s). The species average was 246 Mbp.

Some clades contain several hybrids, such as clades 1 and 7a. The hybrids from clade 1 however do not have a genome size that is larger than their parental species. *P. x serendipita* is a hybrid of *P. cactorum* and *P. hedraiandra*. *P. x pelgrandis* is a hybrid of *P. nicotianae* and *P. cactorum*. These all have similar nuclear sizes. This would indicate that they are true sexual hybrids and not polyploids. Isolates belonging to the *P. x alni* (*sensu lato*) group in clade 7 (where also the hybrids from set 1 belong to) on the other hand all have large genomes, correlated with their proposed multiploid hybrid nature. Within *P. alni*, a single isolate (2280) was identified with a very large genome size. This isolate also contains a larger number of haplotypes of specific genes (data CRA-W), consistent with its polyploid nature.

Table XII - Flow cytometry-based quantification of the nuclear content (in pg and in Mbp) of the *Phytophthora* isolate set 2 (= representative species selected throughout 7 of the 10 *Phytophthora* clades). Reference (Ref) is either *Arabidopsis* (A) or *Raphanus* (R). Species identification for some isolates is still limited to RFLP group.

| clade | species / group | isolate code | ref | avg pg | sd pg | avg Mbp | sd Mbp | CV | species avg | species stdev |
|---|---|---|---|---|---|---|---|---|---|---|
| **1 a** | **cactorum** | 05/005 | A | 0,192 | 0,009 | 187,5 | 8,6 | 4,6 | | |
| | | 05/008 | A | 0,186 | 0,005 | 181,6 | 4,8 | 2,6 | | |
| | | 06/002 | A | 0,182 | 0,003 | 178,2 | 3,2 | 1,8 | 182,4 | 4,7 |
| | **hedraiandra** | 06/018 | A | 0,193 | 0,005 | 188,8 | 4,6 | 2,4 | | |
| | | 06/019 | A | 0,190 | 0,007 | 186,3 | 6,7 | 3,6 | | |
| | | 10/013 | A | 0,184 | 0,003 | 179,7 | 2,8 | 1,6 | 184,9 | 4,7 |
| | **x serendipita** | ph48 | A | 0,188 | 0,009 | 183,8 | 9,0 | 4,9 | | |
| | | HC21 | A | 0,179 | 0,005 | 175,0 | 4,5 | 2,6 | | |
| | | HC25 | A | 0,196 | 0,002 | 191,8 | 1,9 | 1,0 | | |
| | | HC32 | A | 0,190 | 0,003 | 185,7 | 2,4 | 1,3 | | |
| | | HC42 | A | 0,183 | 0,006 | 178,7 | 5,6 | 3,1 | | |
| | | HC27 | A | 0,184 | 0,006 | 180,0 | 5,7 | 3,2 | | |
| | | HC39 | A | 0,181 | 0,010 | 177,5 | 10,2 | 5,7 | | |
| | | HC50 | A | 0,188 | 0,003 | 183,8 | 2,7 | 1,5 | | |
| | | ph41 | A | 0,189 | 0,002 | 185,3 | 2,2 | 1,2 | | |
| | | W54 | A | 0,189 | 0,004 | 185,2 | 4,3 | 2,3 | | |
| | | W70 | A | 0,182 | 0,005 | 178,3 | 5,1 | 2,9 | | |
| | | PI80 | A | 0,184 | 0,004 | 180,4 | 3,5 | 1,9 | | |
| | | PD11 | A | 0,185 | 0,001 | 180,7 | 1,1 | 0,6 | 182,0 | 4,5 |
| **1** | **x pelgrandis** | 15/006 | A | 0,190 | 0,002 | 185,9 | 1,9 | 1,0 | | |
| | | 15/008 | A | 0,191 | 0,007 | 186,9 | 7,0 | 3,7 | | |
| | | 15/009 | A | 0,184 | 0,002 | 179,8 | 2,0 | 1,1 | 184,2 | 3,8 |
| | **nicotianae** | 13/036 | A | 0,189 | 0,007 | 184,9 | 6,8 | 3,7 | | |
| | | 13/048 | A | 0,180 | 0,002 | 176,1 | 2,3 | 1,3 | 180,5 | 6,3 |
| **2 a** | **citrophthora** | 10/083 | A | 0,134 | 0,006 | 131,0 | 5,7 | 4,4 | | |
| | | 10/087 | A | 0,128 | 0,003 | 124,8 | 3,2 | 2,6 | | |
| | | 10/099 | A | 0,134 | 0,003 | 131,3 | 2,8 | 2,2 | | |
| | | 13/037 | A | 0,126 | 0,005 | 122,9 | 4,5 | 3,7 | 127,5 | 4,3 |
| | **occultans** | 12/021 | A | 0,135 | 0,006 | 131,7 | 5,8 | 4,4 | | |
| | | 05/034 | A | 0,132 | 0,004 | 129,2 | 3,6 | 2,8 | 130,5 | 1,7 |
| **2 c** | **citri/multi/pluri/pini** | 11/010 | A | 0,125 | 0,003 | 122,1 | 3,2 | 2,6 | | |
| | | 11/014 | A | 0,128 | 0,003 | 124,8 | 3,1 | 2,5 | | |
| | | 12/023 | A | 0,122 | 0,003 | 119,4 | 2,6 | 2,2 | | |
| | | 14/013 | A | 0,123 | 0,000 | 120,2 | 0,4 | 0,3 | 121,6 | 2,4 |
| | **multivora** | 10/014 | A | 0,127 | 0,001 | 124,0 | 0,7 | 0,5 | | |
| | | 10/055 | A | 0,121 | 0,004 | 118,7 | 3,5 | 2,9 | 121,4 | 3,8 |
| | **pini** | 10/030 | A | 0,125 | 0,003 | 122,2 | 2,4 | 2,0 | | |
| | | 14/002 | A | 0,122 | 0,008 | 119,1 | 7,6 | 6,4 | 120,7 | 2,2 |
| | **plurivora** | 10/033 | A | 0,127 | 0,001 | 124,3 | 0,7 | 0,6 | | |
| | | 10/034 | A | 0,125 | 0,004 | 122,7 | 4,2 | 3,4 | 123,5 | 1,1 |
| **3** | **pseudosyringae** | resi13 | R | 0,152 | 0,005 | 147,4 | 3,7 | 2,5 | | |
| | | resi50 | R | 0,151 | 0,002 | 147,8 | 1,5 | 1,0 | 147,6 | 0,3 |
| **6 a** | **inundata** | 09/001 | R | 0,310 | 0,004 | 303,2 | 3,5 | 1,1 | 303,2 | |
| | **inundata /humicola** | 09/003 | R | 0,302 | 0,009 | 295,2 | 8,7 | 2,9 | 295,2 | |
| | **lacustris/riparia** | 07/002 | A | 0,157 | 0,017 | 153,4 | 16,6 | 10,8 | | |
| | **lacustris/riparia?** | 13/005 | A | 0,163 | 0,009 | 159,4 | 9,2 | 5,8 | 156,4 | 4,3 |
| | **megasperma** | 05/043 | R | 0,297 | 0,003 | 290,1 | 2,5 | 0,9 | | |
| | | 09/002 | R | 0,289 | 0,006 | 282,6 | 5,9 | 2,1 | | |
| | | 13/013 | R | 0,292 | 0,017 | 285,5 | 17,0 | 6,0 | | |
| | | 14/079 | R | 0,270 | 0,008 | 263,8 | 7,8 | 3,0 | | |
| | | 14/083 | R | 0,289 | 0,014 | 283,2 | 13,3 | 4,7 | | |
| | | 14/085 | R | 0,289 | 0,007 | 283,2 | 7,3 | 2,6 | 281,4 | 9,1 |
| | **chlamydospora** | 07/003 | A | 0,155 | 0,013 | 151,7 | 12,6 | 8,3 | | |
| | | 12/040 | A | 0,145 | 0,018 | 141,5 | 17,1 | 12,1 | | |
| **6 b** | **gonapodyides group 1** | 04/001 | A | 0,153 | 0,008 | 149,8 | 8,1 | 5,4 | | |
| | | 04/004 | A | 0,155 | 0,010 | 151,6 | 9,8 | 6,5 | 150,7 | 1,3 |
| | **gonapodyides group 2** | 06/001 | R | 0,272 | 0,006 | 266,2 | 6,1 | 2,3 | | |
| | | 07/004 | R | 0,278 | 0,011 | 271,9 | 10,5 | 3,9 | | |
| | | 07/007 | R | 0,280 | 0,009 | 274,0 | 8,5 | 3,1 | 270,7 | 4,0 |
| | **gonapodyides group 3** | 14/049 | A | 0,180 | 0,007 | 176,3 | 6,9 | 3,9 | 176,3 | |
| | **gonapodyides group 4** | 05/004 | A | 0,175 | 0,011 | 170,9 | 10,8 | 6,3 | | |
| | | 05/015 | A | 0,182 | 0,010 | 177,7 | 9,5 | 5,4 | | |
| | | 05/017 | A | 0,167 | 0,006 | 163,8 | 6,2 | 3,8 | 170,8 | 7,0 |
| | **gonapodyides group 5** | 14/057 | A | 0,115 | 0,011 | 112,2 | 11,2 | 10,0 | | |
| **7 a** | **cambivora** | 05/027 | A | 0,482 | 0,006 | 471,9 | 6,0 | 1,3 | | |
| | | 05/006 | A | 0,469 | 0,009 | 458,3 | 8,8 | 1,9 | 465,1 | 9,6 |
| | **x alni** | 2199 | R | 0,544 | 0,006 | 532,1 | 6,2 | 1,2 | | |
| | | 2201 | R | 0,524 | 0,030 | 513,0 | 29,6 | 5,8 | 522,6 | 13,5 |
| | **x alni?** | 2280 | R | 0,862 | 0,033 | 843,7 | 32,0 | 3,8 | 843,7 | |
| | **uniformis** | 2271 | R | 0,356 | 0,009 | 348,4 | 9,2 | 2,7 | | |
| | | 2276 | R | 0,324 | 0,005 | 316,8 | 4,7 | 1,5 | | |
| | | 2277 | R | 0,334 | 0,003 | 327,2 | 2,7 | 0,8 | 330,8 | 16,1 |
| | **x multiformis** | 2274 | R | 0,667 | 0,013 | 653,0 | 12,6 | 1,9 | 653,0 | |
| **7 b** | **cinnamomi** | 10/009 | A | 0,415 | 0,020 | 406,4 | 19,4 | 4,8 | | |
| | | 14/003 | A | 0,409 | 0,016 | 400,1 | 15,9 | 4,0 | 403,3 | 4,5 |
| **8 a** | **cryptogea/andina/boehmeria** | 13/006 | A | 0,193 | 0,018 | 188,9 | 17,2 | 9,1 | | |
| | **cryptogea/drechsleri** | 12/016 | A | 0,224 | 0,007 | 219,0 | 6,7 | 3,1 | | |
| | | 12/025 | A | 0,225 | 0,003 | 220,0 | 3,2 | 1,5 | | |
| | | 14/011 | A | 0,229 | 0,008 | 223,8 | 7,4 | 3,3 | 220,9 | 2,6 |
| | **erythroseptica** | 05/022 | A | 0,221 | 0,008 | 216,1 | 7,6 | 3,5 | 216,1 | |
| **8 b** | **lateralis** | 13/001 | A | 0,159 | 0,001 | 155,9 | 1,3 | 0,8 | 155,9 | |
| | **ramorum** | 14/004 | A | 0,137 | 0,010 | 134,2 | 10,1 | 7,5 | | |
| | | 02/001 | A | 0,144 | 0,006 | 141,3 | 5,9 | 4,1 | | |
| | | 02/002 | A | 0,137 | 0,007 | 134,4 | 7,1 | 5,3 | | |
| | | 11/038 | A | 0,144 | 0,009 | 140,4 | 8,6 | 6,1 | | |
| **10** | **kernoviae** | 06/003 | A | 0,140 | 0,019 | 137,0 | 18,8 | 13,7 | 137,6 | 3,8 |
| | | 10/022 | A | 0,131 | 0,003 | 131,5 | 6,3 | 4,8 | 134,3 | 3,9 |
| | **syringae** | 04/00 | A | 0,176 | 0,023 | 172,6 | 22,8 | 13,2 | 172,6 | |
| | | | average | 0,220 | | **215,4** | **7,4** | **3,7** | **245,9** | **5,0** |
| | | | minimum | 0,115 | | 112,2 | | | | |
| | | | maximum | 0,862 | | 843,7 | | | | |
| | | | median | 0,184 | | 179,7 | | | | |

#### 4.1.4.4. Results from isolate set 3

The results of the flow cytometry-based quantification of the nuclear content of the *Phytophthora* isolates of set 3 are shown in Table XIII. As with the previous sets of *Phytophthora* isolates that were analyzed using flow cytometry, the results are quite consistent within each isolate (low CV). Between isolates of the same species the differences were also limited in this case. For several isolates the analyses are still ongoing. Although analysis with the *Arabidopsis* reference is best in most cases, as the size of the reference should not be too far from the size of the sample, it may be recommendable to also run unknown samples with the *Raphanus* reference first, as in such case there is no confusion possible for samples with a nuclear size that is very close to the reference.

Table XIII - Holoploid nuclear size (in Mbp) of additional *Phytophthora* isolates that were processed using flow cytometry during the third project year. Reference used (Ref) was either *Arabidopsis* (A) or *Raphanus* (R). Number of measurements refers to the number of days that nuclear size was determined. Within each day, three measurements were conducted.

| ***Phytophthora* clade** | ***Phytophthora* species** | **isolate code** | **Ref** | **number of measurements** | **avg size (Mbp)** | **stdev (Mbp)** | **CV** |
|---|---|---|---|---|---|---|---|
| 1 | *tentaculata* | SW22 | A | 2 | 259,8 | 9,4 | 3,6 |
| 3 | *pseudosyringae* | SW17 | R | 2 | 158,0 | 6,1 | 3,8 |
| 4 | *quercetorum* | TJ25 | A | 3 | 124,8 | 1,2 | 1,0 |
| 5 | *heveae* | TJ13 | A | 3 | 137,4 | 2,8 | 2,0 |
| 5 | *heveae* | TJ14 | A | 2 | 129,7 | 3,1 | 2,4 |
| 5 | *heveae* | TJ16 | A | 2 | 140,1 | 3,0 | 2,2 |
| 8 | *lateralis* | JA03 | A | 2 | 151,6 | 6,2 | 4,1 |
| 8 | *lateralis* | JA04 | A | 2 | 144,8 | 4,5 | 3,1 |
| 8 | *syringae* | TJ22 | A | 2 | 171,4 | 2,4 | 1,4 |
| 9 | *hydropathica* | TJ18 | A | 3 | 136,2 | 1,9 | 1,4 |
| 9 | *hydropathica* | TJ19 | A | 3 | 136,0 | 3,9 | 2,9 |
| 9 | *polonica* | TS01 | A | 2 | 120,1 | 0,4 | 0,3 |

### 4.1.4. GBS analysis

#### 4.1.4.1. Optimizations

Mycelial production and tissue maceration techniques: Of the different mineral media, production of mycelial biomass was best on C&N and worst on P4 (about 50% less). Growth on V8 was similar to growth on P4. Only for *P. kernoviae* the amount of mycelium was higher on V8 than on the mineral media, but growth on C&N was still quite acceptable (about 60% of growth on V8).

Comparison of the tissue maceration techniques revealed that bead beating (in its different versions) resulted in the best yield but also in a considerable amount of shearing. Using only

a freeze-thaw cycle gave insufficient yield. Manual grinding in N2 gave an acceptable yield and only limited shearing and was preferred.

DNA extraction: The results of the comparison of DNA extraction techniques from *P. ramorum* and *P. cambivora* on mycelium produced in either C&N or V8 medium are shown in Table XIV. Evaluation was in terms of yield, DNA quality, shearing, price, efficacy of restriction digest and user friendliness. We selected the Nucleospin kit to continue our experiments. Reasons was the yield was sufficient (though lower than the other techniques), DNA quality was high, both based on 260/280 ratio and the lack of shearing and blocking in electrophoresis-based evaluation, good restriction digests, user friendliness, low risk for contamination and not unimportantly, the limited price.

Table XIV - Result of the optimization of DNA extraction from *Phytophthora* species for GBS. Results for the media (V8 and C&N) and the species (*P. ramorum* and *P. cambivora*) were not different and are combined in the table.

| DNA extr. technique | DNA yield | DNA quality (260/280 ratio) | Electrophoresis-based evaluation | Restriction digest | price | User friendliness, time, safety and risk for contamination |
|---|---|---|---|---|---|---|
| CTAB | 67700 ± 34267 | 1,91 ± 0,13 | -- | - | +++ | + |
| Nucleospin | 10967 ± 5684 | 2,47 ± 1,25 | ++ | ++ | ++ | +++ |
| Quickpick | 11165 ± 8611 | 1,88 ± 0,23 | + | + | ++ | ++ |
| Invisorb | 24000 ± 16083 | 2,43 ± 0,15 | + | - | ++ | +++ |
| DNEasy | 27760 ± 23633 | 2,22 ± 0,24 | ++ | + | + | +++ |
| Puregene | 66000 ± 29348 | 1,80 ± 0,09 | + | + | +++ | ++ |

Restriction digest: Of the enzymes tested, the desired electrophoresis pattern was obtained with the enzymes *Hpa*II (4-cutter, same restriction site as *Msp*I and *Pst*I (6-cutter) with all the *Phytophthora* species tested. Some enzymes did not perform well (e.g. *Bam*HI), some cut in repetitive sequences (e.g. *Pae*I). We chose *Hpa*II instead of *Msp*I (isoschizomers) because *Hpa*II does not cut when internal C-nucleotides are methylated, which is more commonly the case in repetitive sequences. We want to avoid such repetitive sequences in GBS. An example of a gel-electrophoresis-based evaluation of a restriction digest is shown in Figure 19. Regarding the duration of the restriction and the amount of enzyme tested, a 15 minute digest at half strength enzyme concentration was still sufficient for a good digest pattern.

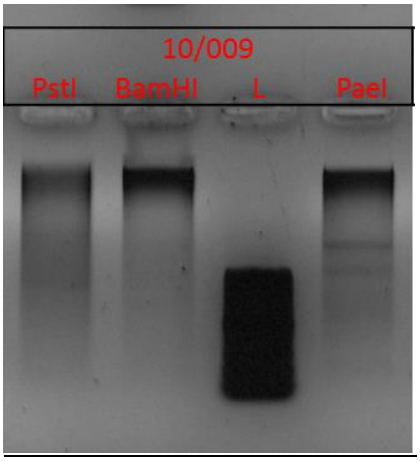



Figure 19 – Electrophoresis-based evaluation of the restriction digest of the enzymes PstI, BamHI and PaeI on DNA of isolate 10/009 (Phytophthora cinnamomi). L = Ladder.

Amount of adaptor: Our fear of increasing the adaptor amount to a level where adaptor dimers would become a problem was unfounded. Even at 0.5 pmol there was no problem after the cleanup step: only in one sample out of 96 where adaptor dimers could be observed in the profile. Therefore 0.5 pmol was chosen as the optimized amount of adaptors.

Number of PCR cycles, amount of PCR primers, annealing temperature and elongation time: Regarding the number of PCR cycles, our initial tests involved 16 to 20 cycles. However, later tests showed that even 12 cycles were sufficient to obtain enough product for all samples (all genome sizes tested). Figure 20 shows the results of such an experiment, as evaluated using the Qiaxcel machine (shows the spectrum but is not fully reliable in terms of quantity). Figure 21 shows the same results, as evaluated using the Quantus machine (does not show the spectrum but reliable in terms of DNA quantity). When using only 12 cycles, the amounts of DNA obtained are lower for the two *Phytophthora* species with the smallest genomes compared to 14 and 16 cycles. However, the amounts obtained after 12 cycles were already sufficient for our purpose and this number was chosen to avoid the introduction of bias due to additional cycles.

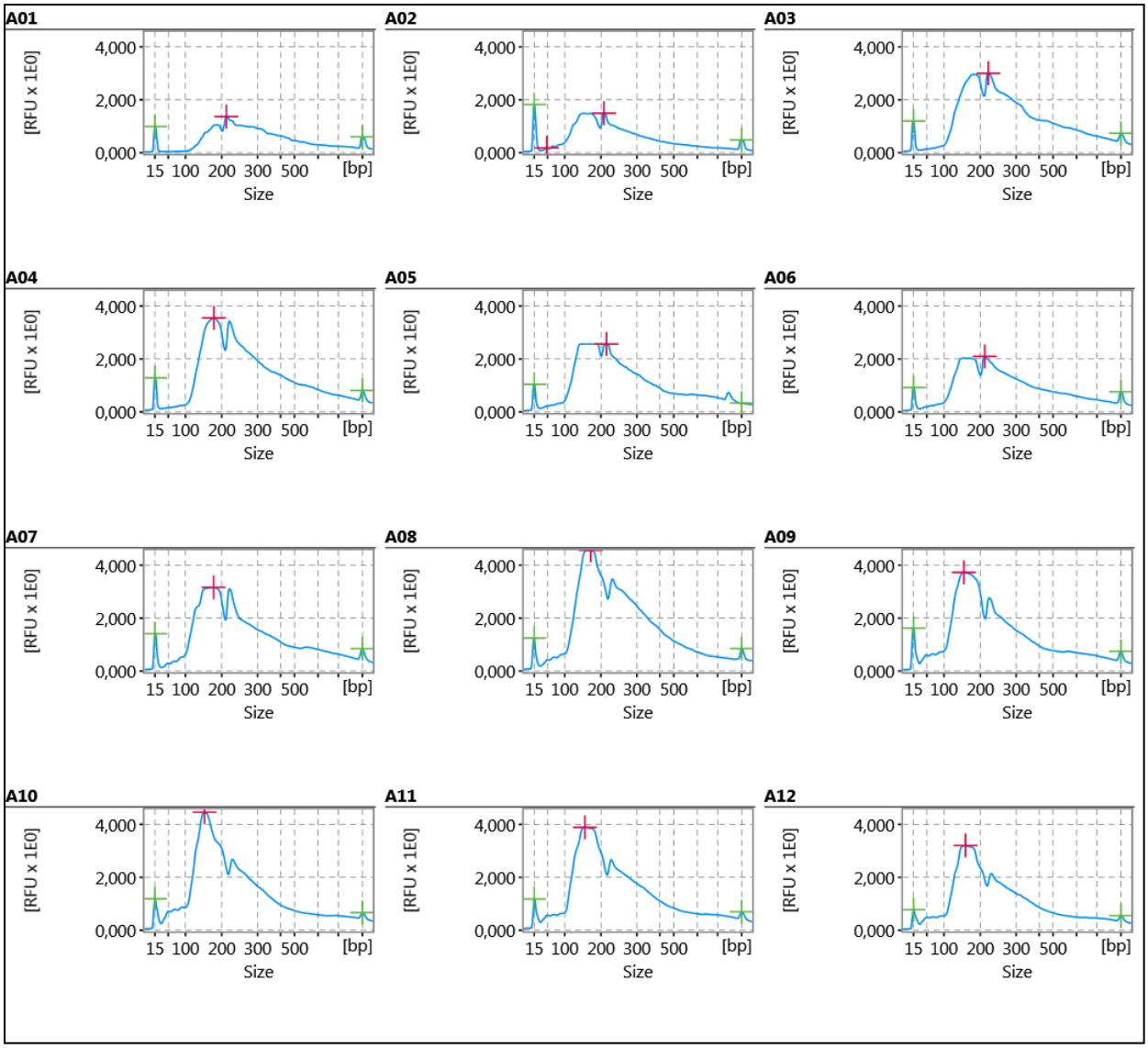



Figure 20 - Analysis of 12 libraries(A01 to A12) using the Qiaxel machine representing either 12, 14 or 16 PCR cycles for samples A01-A04, A05-A08, and A09-A12, respectively. Samples A01, A05, A09 are from *P. citrophthora* (genome of 125 Mbp). Samples A02, A06 and A10 are from *P. cryptogea* (genome of 217 Mbp). Samples A03, A07, and A11 are from *P. inundata* (292 Mbp). Samples A04, A08, A12 are from *P. cambivora* (465 Mbp).

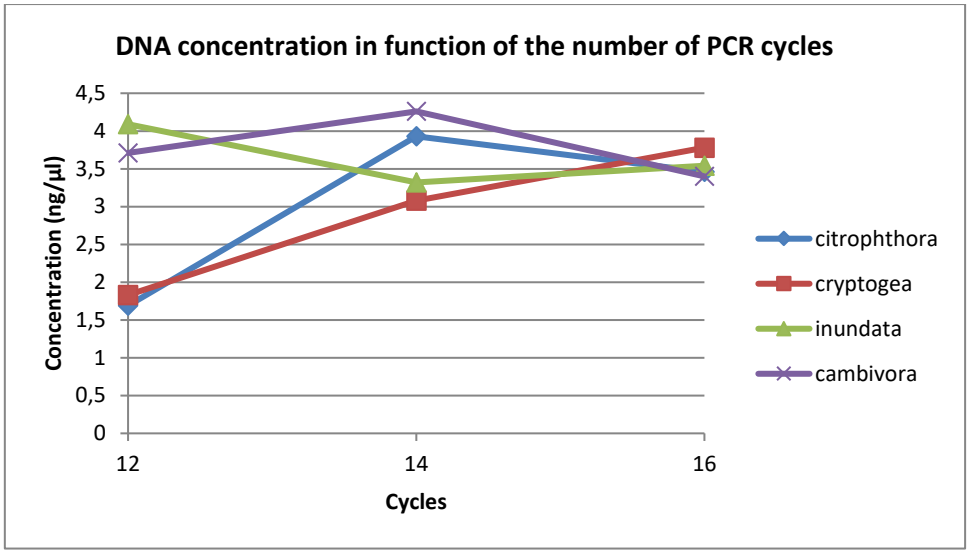



Figure 21 - Analysis of the same samples as shown in Figure 20, but using the Quantus machine (DNA quantification).

The amount of PCR primers was set at 2 µL of 10 µM for each primer but the concentration seemed of minor importance (within the range tested).
The initiative to lower the annealing temperature was because initially, the amount of library product seemed low. However, this involved visualization before cleanup, when the amount of salts in the product interfered with the Qiaxcel visualization system. When the library was evaluated after cleanup, this problem did not produce itself. Therefore, the annealing temperature was reset to 65°C.
Regarding the elongation time, little difference was seen in library profiles produces with the different elongation times. The elongation time was reset to the initial 30 s.

First library preparation and submission for sequencing: The 96 libraries from the first run were evaluated individually after PCR and final cleanup using the Qiaxcel machine. DNA concentrations were measured, amounts were adjusted based on (estimated) genome size and the pooled library was sent in for sequencing (see Figure 22).

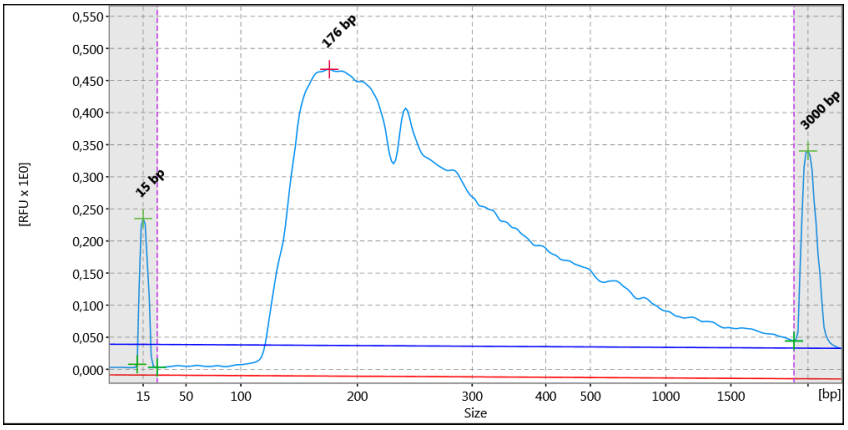



Figure 22 - Electrophoregram of the pool of the first set of libraries sent in for GBS as evaluated using the Qiaxcel. The peaks around 15 and 3000 bp are system markers. The depression around 230 bp is probably a Qiaxcel system artifact (and thus has not biological relevance). Most valuable are the fragments between 100 and 300 bp, which form the bulk of the library.

### 4.1.4.2. Analysis of the GBS data

Once the Illumina sequence data is downloaded from the provider, first the read quality is checked using the program FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). This generates a score of 0-40 for the base quality at the different positions in the sequences. An example of such a result is shown in Figure 23 (forward read only). It is normal that the base quality decreases towards the end of the fragment. In our case the base quality was still excellent for sequences of 125 bp and very reasonable for sequences up to 150 bp. In general, the read quality of all batches processed so far was very good.

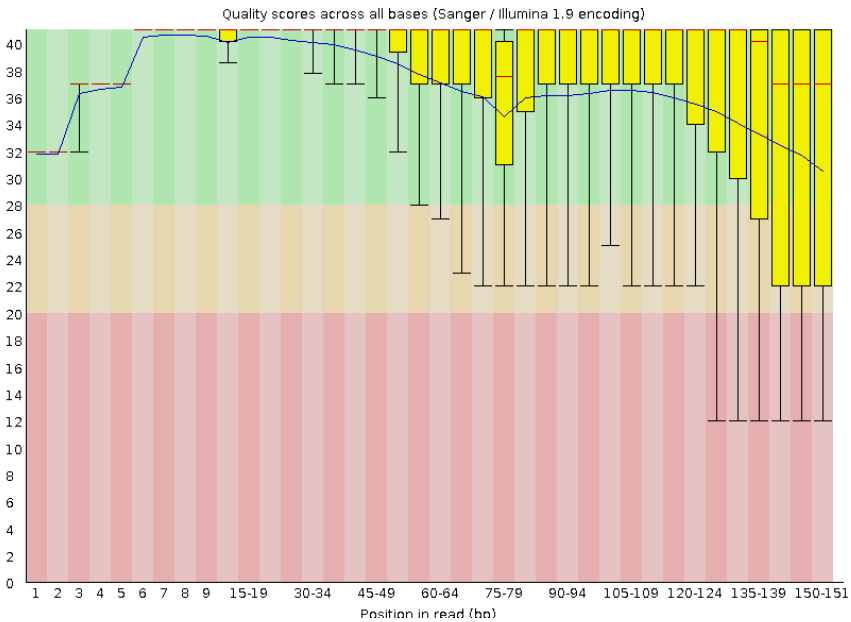



Figure 23 - Read quality plot for the forward reads of GBS run 2. Red dashes (within yellow bars) = median, blue line = average, yellow boxes = interquartile range (25% to 75%), error bars = (10 to) 90% range

The second step in the analysis involves the preprocessing of the data. This consists of demultiplexing (based on the presence of the specific barcode sequences for the separate 96 samples), trimming of the barcode and the restriction site remnants, merging of the overlapping fragments (fragments are sequenced in both directions but only in case of sufficient overlap are they merged) and quality filtering. During this quality filtering only the following sequences are kept for further analysis: only merged reads of at least 30 bp, a minimum read quality of 25, a minimum base quality of 20 and a maximum number of 10 "Ns". An example of the effect of quality filtering on the numbers of reads is presented in Table XV. In this example, 284 443 177 merged reads were obtained that passed all the quality controls. Based on 96 isolates this represents an average of almost 3 million usable merged reads per isolate. The preprocessing is conducted with ILVO code that also makes use of existing programs such as GBSX, cutadapt, FastX toolkit, Pear, prinseq-lite.

Table XV - Effect of quality filtering on the number of reads in batch 3. The percentages represent the remaining portion compared to the initial number.

| | **total** | **% remaining** | **per isolate** |
|---|---|---|---|
| number of paired reads | 362 323 580 | 100 | |
| paired reads after demultiplexing | 332 440 762 | 92 | average 3 499 376 |
| | | | minimum 1 556 937 |
| | | | maximum 8 367 255 |
| merged reads | 296 224 149 | 82 | |
| merged reads after quality filtering | 284 629 121 | 78.6 | |
| reads without restriction sites | 284 443 177 | 78.5 | average:2 962 949 |

The third step in the analysis of the data is the locus identification (grouping of the sequences of the same locus) and analysis of inferred loci. This is conducted using the GibPSs (Genotype Individuals based on Paired Sequences or single reads) program (Hapke and Thiele 2016). The grouping is based on the sequence similarity of the sequences. Loci are only identified when they are based on a minimum number of reads. This grouping allows for a defined number of SNPs, so that each locus can contain its different alleles. The analysis of the inferred loci involves removal of indel variants and removal of loci that are sequenced too deeply. The latter typically consist of repeat sequences, which are of little value.

The average (± stdev) length of the loci was 129.4 ± 67.2 bp. Typical histograms of the number of loci of the different sizes are presented in Figure 24. It shows the expected inverse relationship between length and number of loci with length. In some isolates the number of large fragments plateaus, while for most isolates it continues to decrease. The former may be due to incomplete restriction.

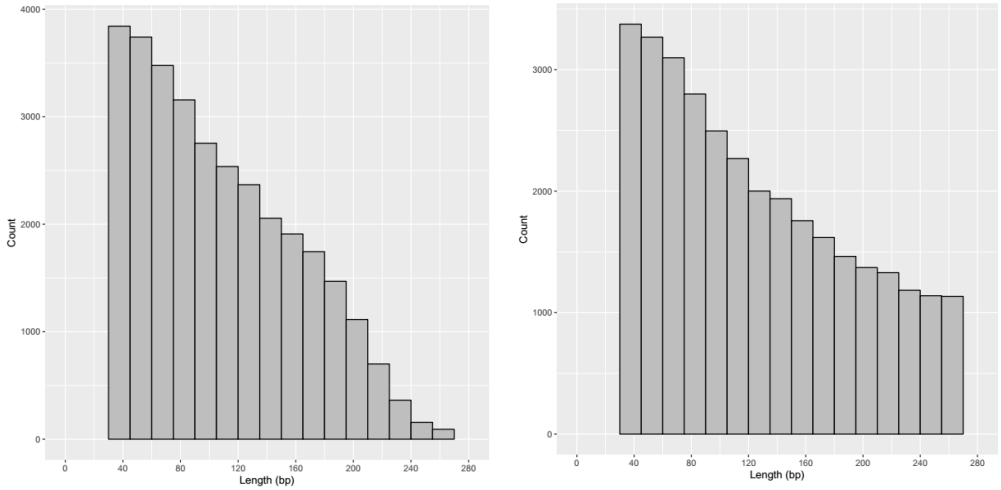



Figure 24 - Histograms showing the number of merged fragments by size of the fragments for isolate *P. niederhauserii* AB2 (left) and isolate *P. cinnamomi* 11/022 (right).

The number of loci identified per isolate varied from approximately 13 000 to 54 000, with an average of 25 000. As the average locus length was 129.4 bp this represents an average of 3 225 000 bp of the genome per isolate. With a haploid genome size of about 100 Mbp per *Phytophthora* species (larger as well as smaller exist of course) this represents about 3.2% of the genome. This is considered large enough to be representative of the sample.

The number of loci per isolate depended on the clade, species, and hybrid nature (or not). Given that in step two we obtained an average of about 3 000 000 reads per isolate and an average of 25 000 loci per isolate, the average read depth per locus is about 120. The sequence data of the loci of each sample are exported and saved for the fourth and final step, which can be conducted using samples from different runs.

This fourth step is the pairwise comparison of the data of the individual samples (selected from one or multiple GBS runs and sequencing runs) and is conducted using ILVO code. It results in a matrix in which the number of loci that each two samples have in common are determined. Common loci are determined based on sequence similarity, in which 10% of SNPs are allowed. Of the common loci, the percentage in which at least one allele is 100% identical is also calculated. As explained in 3.1.2.3, the data on the number of common loci and the level of sequence similarity within the common loci allows detection and characterization of the samples in terms of hybrid status.

To determine the reliability and reproducibility of the technique, we analyzed replicates of the same isolate, starting from separate DNA extractions. This was performed within the same run as well as between runs. Results are presented in Table XVI. The conclusion was that although there is some variance, overall the technique is quite reproducible.

In the meantime, six GBS runs have been conducted and results of four runs have been received and processed. The details of the 312 isolates that have been processed in the four first runs are presented in Table XVII. The number of isolates processed (312) is less than the maximum possible (= 384 = 4 runs x 96 wells/run) because some isolates have been included more than once in a run or in more than one run. Only 8 samples out of 312 produced no results, presumably due to a technical problem with the processing of those isolates. Also, 27 (out of 312) isolates have been excluded because GBS processing revealed they were latently contaminated with bacteria and needed further purification. Such contamination reveals itself via a number of common (bacterial) loci among a few contaminated non-related species. In further runs all isolates are undergoing additional checks for purity to avoid this contamination issue. This contamination was a problem, but it was small and it showed the strength of the GBS technique to reveal this. The 312 isolates have been reduced to 145 isolates for the fourth and final step in the data analysis, in part because of the contaminated or failed status mentioned above but mostly because for some species we had a relatively large number of representatives with closely resembling profiles (based on the analysis of the isolates in runs 1 to 3). Some of those very similar isolates were omitted because they do not contribute information to the matrix in which all isolates are mutually compared, while they inflate the data analysis time exponentially.

Table XVI - Number of loci identified using GBS in replicate analysis of the same isolate, within (n=3) and between (n=2) runs. ND = not determined

| | | Within run | | Between run | |
|---|---|---|---|---|---|
| Species | isolate | average | stdev | average | stdev |
| *ramorum* | 02/001 | 22538 | 677 | 23078 | 764 |
| *cactorum* | 10/045 | 18390 | 159 | ND | ND |
| *hedraiandra* | 10/051 | 18617 | 479 | ND | ND |
| *x serendipita* | HC21 | 21817 | 1218 | 21943 | 179 |

Table XVII - Details of the species and isolates that have been processed using GBS in runs 1 to 4. The table also lists the average number of loci within a species and the average number of loci in common within the given species. Isolates marked in color were included in the comparison matrix (part of the 145 isolates referred to in the text) and were used to calculate the number of loci (in common).

| species | species code | clade | Isolate codes | loci (1) | | loci in common (2) | | (2)/(1) *100 |
|---|---|---|---|---|---|---|---|---|
| | | | | average | CV (%) | average | CV (%) | |
| cactorum | CAC | 1a | 10/024, 11/013, 10/045, 06/002, 07/008, 11/039, 15/027, TJ012, JA02=15/004, 13/014, 13/015, 14/014, 14/068, 15/002, 15/010, 15/016, 15/053, R02, MR03, SW09, 07/009, 10/013, 15/015, MR07, MR08 (was SER), MR10 (was HED, MR16 (was HED), SW10 (was SER), TJ011 (was HED) | 17857 | 2,5 | 17370 | 1,5 | 97,3 |
| hedraiandra | HED | 1a | 10/044, 10/052, 11/029, 10/051, | 18394 | 7,3 | 15392 | 8,3 | 83,7 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | 06/018, 06/019, 16/014n, MR10 (was CAC), MR16 (was CAC), TJ011(was CAC) | | | | | |
| serendipita (like) | SER | 1a | 08/005, 11/027, 11/043, 14/060, 05/012, 05/039, 05/044, 14/067, 11/008, 12/028, 12/024, 13/007, SW10 (was CAC), MR08 (was CAC) | 21063 | 3,0 | 19129 | 5,1 | 90,8 |
| tentaculata | TEN | 1b | SW21, SW23, SW22 | 25102 | 10,3 | 23068 | 0,0 | 91,9 |
| andina | AND | 1c | CBS_115547 | 24685 | | | | |
| infestans | INF | 1c | 16/053, JA07 | 20818 | 0,5 | 18431 | 0,0 | 88,5 |
| mirabilis | MIR | 1c | CBS_678.85 | 20469 | | | | |
| nicotianae | NIC | 1d | 13/006, 13/036, 13/048, 13/003 | 16922 | 0,6 | 15590 | 0,0 | 92,1 |
| pelgrandis | PEL | 1d | 15/009, 15/008, 15/007, 15/006 | 30917 | 7,1 | 28422 | 6,6 | 91,9 |
| botryosa | BOT | 2a | CBS_235.30 | 14517 | | | | |
| citrophthora(like) | CIP | 2a | 10/083, 10/087, 13/016, 14/077, 10/049, 14/084, 12/041 | 16053 | 3,1 | 15203 | 2,8 | 94,7 |
| colocasiae | COL | 2a | CBS_192.91 | 14331 | | | | |
| occultans | OCC | 2a | 05/034, 10/037, 13/037 | 12923 | | | | |
| terminalis | TER | 2a | 10/099 | | | | | |
| capsici | CAP | 2b | SW11 | 17449 | | | | |
| multivesiculata | MVC | 2c | CBS_545.96 | 19878 | | | | |
| citricola E/pini | CITE/ PIN | 2c | 14/013 | | | | | |
| multivora | MUV | 2c | 04/006, 05/048, 05/049, 05/050, 10/014, 10/055, 10/053 | 18450 | 1,1 | 16965 | 1,2 | 91,9 |
| pini | PIN | 2c | 14/002, 10/030, 14/001 | 18209 | | | | |
| plurivora(like) | PLU | 2c | 05/035, 10/033, 14/065, 10/034, 12/023, 13/012, 13/018, 14/010, 14/048, 14/051, CH17, CH45, 05/024, 11/015, 11/017, 11/019, 14/080, 16/003, MR54, MR55, MR57, MR59, 13/033, 14/057, 14/086, 05/052, 12/022, 11/020, 11/010, 11/011 | 17287 | 2,3 | 16785 | 1,4 | 97,1 |
| pseudosyringae | PSR | 3 | SW17, SW18, AC10, AC11, MR60 | 26804 | 2,0 | 25336 | 3,2 | 94,5 |
| quercetorum | QCT | 4 | TJ024, TJ025, TJ026 | 15022 | | | | |
| quercina | QRC | 4 | TJ028, TJ029, TJ027, TJ031, MR61, SW19 | 19563 | 3,4 | 18748 | 2,7 | 95,8 |
| castanea | CAS | 5 | TJ034, TJ035, TJ033 | 18337 | 0,5 | 16771 | 4,0 | 91,5 |
| heveae | HEV | 5 | TJ013, TJ014, TJ016 | 17543 | 0,2 | 16709 | 0,0 | 95,2 |
| humicola | HUM | 6a | CBS_200.81 (contaminated) | approx. 27000 | | | | |
| inundata(like) | INU | 6a | 08/004, 09/001, 09/003 | 30294 | | | | |
| chlamydospora | CHL | 6b | 12/040, 04/004, 07/003, 07/005, 04/001, 10/003 | 30872 | 4,2 | 29564 | 3,8 | 95,8 |
| gonapodyides | GON | 6b | 07/004, 07/007, 14/049, 06/001, MR29, MR30, MR31, MR33, MR35, MR37, 05/017, 05/025, 05/003, 05/004, 05/015, 05/020 (some diversity within GON) | 29862 | 4,8 | 23976 | 8,7 | 80,3 |
| gregata | GRE | 6b | MR38, MR39 | 26398 | 2,9 | 25593 | 0,0 | 97,0 |
| lacustris(like) | LAC | 6b | CH05, CH14, 05/019, CH01, CH09, CH20, CH22, CH25, CH27, CH41, CH43, MR40, MR41, CH07, CH46, MR43, MR44 (hybrid?), MR45, MR46, MR48, 05/013, 06/014, 07/002 | 27566 | 11,6 | 21969 | 4,1 | 79,7 |
| chlamydospora x lacustris | | 6b | 13/005 | | | | | |
| megasperma | MEG | 6b | 14/071, 14/073, 09/002, 13/013, 14/070, 14/072 (hybrid?), 14/074, 14/069, 14/083, 14/085, 16/013, 05/043, 15/069, MR50 | 37248 | 15,2 | 29475 | 8,4 | 79,1 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| riparia | RIP | 6b | MR64 | 23375 | | | | |
| rosacearum | ROS | 6b | MR65 | 23093 | | | | |
| uniformis-like | UNI-like | 6b | MR63 (was RIP) | 21248 | | | | |
| alni | ALN | 7a | SW02, TJ001, AC01, AC03, AC02, MR72, MR73, TJ003 | 49555 | 8,5 | 45788 | 0,0 | 92,4 |
| cambivora | CAM | 7a | 10/010, TJ070, AC12, AC13, MR21, MR23, MR24, TJ080, 05/027, 05/006, 09/004 | 39745 | 6,8 | 29171 | 17,1 | 73,4 |
| fragaria | FRA | 7a | 05/058 | 25225 | | | | |
| heterohybrida | HET | 7a | TJ037, TJ038, TJ036 | 34453 | | | | |
| incrassata | INC | 7a | TJ060, TJ061, TJ063, TJ062 | 53849 | 6,9 | 50599 | 0,0 | 94,0 |
| multiformis | MUF | 7a | SW04, TJ004, TJ005, TJ006, AC04 | 39413 | 15,9 | 34843 | 0,0 | 88,4 |
| rubi | RUB | 7a | CBS_967.95 | 24958 | | | | |
| uniformis | UNI | 7a | TJ008, SW06, AC05, AC07, AC06, MR67, MR68, TJ007, MR69, MR70, MR71 | 25392 | 2,8 | 24541 | 2,1 | 96,6 |
| niederhauserii | NIE | 7b | SW15, AB02, AB03, AB04, SW24, SW25, SW26, SW14 | 33179 | 4,4 | 28345 | 12,8 | 85,4 |
| taxon princessi | PRI | 7b | AB05 | 30352 | | | | |
| vignae | VIG | 7b | SW27 | 27719 | | | | |
| cinnamomi | CIN | 7c | TJ088, TJ089, 10/009, 11/022, 11/024, 14/003, 14/061, 15/018, 10/069, 15/019, 15/001, 15/040, 05/063, 05/115 | 30433 | 6,7 | 27201 | 4,5 | 89,4 |
| cryptogea(like) | CRY | 8a | 13/029, MR26, MR27, 12/018, 14/011A, 12/025, 12/016, 05/022 (was ERY) | 27941 | 3,5 | 25572 | 3,1 | 91,5 |
| drechsleri | DRE | 8a | 05/062 | 25542 | | | | |
| erythroseptica | ERY | 8a | CBS_129.23, 05/059 (was CRY) | 19248 | 0,1 | 17463 | 0,0 | 90,7 |
| brassicae | BRA | 8b | CBS_179.87, 08/006 | 16878 | 10,3 | 15508 | 0,0 | 91,9 |
| porri | POR | 8b | 10/114A, 08/012 | 14072 | | | | |
| lateralis | LAT | 8c | 13/001 | 23438 | | | | |
| ramorum | RAM | 8c | 11/038, 12/012, 13/024, 14/004, 02/001, MR62, 02/002 | 22889 | 5,0 | 21962 | 0,0 | 96,0 |
| syringae | SYR | 8d | 04/005, TJ021, TJ022, MR66 | 22579 | 1,8 | 20929 | 3,6 | 92,7 |
| polonica | POL | 9a | SW16 | 14192 | | | | |
| hydropathica | HYD | 9b | TJ010, TJ018, TJ019 (hybrid?), TJ017 | 31337 | 13,3 | 23318 | 11,2 | 74,4 |
| gallica | GAL | 10 | SW13 | 22490 | | | | |
| kernoviae | KER | 10 | 12/006,10/022 | 14202 | | | | |
| | | | average | **24315** | | | | 90,2 |
| | | | Minimum | 12923 | | | | |
| | | | Maximum | 53849 | | | | |

The average number of loci per species was 24315 and varied from 12923 in *P. occultans* to 53849 in *P. incrassata*. The species with larger numbers of loci (> 27000) have a high likelihood to have undergone (historical) hybridization events. Examples are *P. pelgrandis, P. chlamydospora, P. megasperma, P. alni, P. cambivora, P. heterohybrida, P. incrassata, P. multiformis, P. niederhauserii, P. cinnamomi, P. hydropathica*. In most cases the hybrid status of these species has already been established, but in others (e.g. *P. cinnamomi*), it has not. Some hybrids may have a smaller number of loci (e.g. *P. serendipita* is a hybrid of *P. cactorum* and *P. hedraiandra* but only has 21063 loci because its parental species are closely related and already have many loci in common. So the number of loci in itself is indicative but not a sufficient criterion to spot a hybrid. Within a species the number of loci is quite stable: the average CV is 5.3%. This number is even somewhat inflated because some potential hybrids are still counted within their main parental species, which often has a

significantly lower number of loci than the potential hybrid. Within a clade the number of loci is also quite stable, except in clades that contain hybrid as well as non-hybrid species. For example, *P. uniformis* (a non-hybrid) has an average of 25392 loci while *P. multiformis* (a hybrid) has an average of 39413 loci. Within a species there are an average ± stdev of 90.2±6.5% of loci in common (pairwise comparison). This number is smaller in species such as *P. hedraiandra, P. gonapodyides, P. lacustris, P. megasperma, P. hydropathica*, etc. in which diverse hybrids are contained or in which more non-hybrid heterogeneity is present, for example because they contain sister species. So the number of loci in common within a uniform non-hybrid species is closer to 95-97%. Also, keep in mind that even replicates of the same isolate do not have 100% of loci in common due to batch to batch and sample to sample differences in efficiency of restriction digest, ligation, PCR, sequencing, etc. Between very closely related non-hybrid species (in the same subclade) the % of loci in common is in the range of 75%-80% (of the isolate with the smallest number of loci). Between species of the same clade (but different subclades), the percentage of loci in common goes down quickly, to approximately 5-10%. Species of different clades only have approximately 2% of loci in common.

Given the (increasing) number of samples analyzed, the pairwise comparison of the loci of the different isolates produces a large results matrix. Because of the size, it is discussed below in parts that mainly correspond to the *Phytophthora* clades, and specifically the clades in which hybrids have been detected. The results of a selection of isolates from *Phytophthora* clade 1 are shown in Table XVIII. The number of loci in common clearly shows the relatedness of the species. For example, *P. cactorum* and *P. hedraiandra* are closely related and share a lot of loci, while *P. nicotianae* and *P. tentaculata* are in different subclades, and only a limited number of loci. Clade 1 contains hybrid species such as *P. x serendipita* (hybrid of *P. cactorum* and *P. hedraiandra*) and *P. pelgrandis* (hybrid of *P. cactorum* and *P. nicotianae*). *P. hedraiandra* and *P. cactorum* each have about 18000 loci (see Table XVII). As can be seen in Table XVIII, they have about 14000 loci in common, so each parental species has about 4000 species-specific loci (18000-14000 loci). The hybrid should therefore have about 14000 common loci + 4000 *P. cactorum*-specific loci + 4000 *P. hedraiandra*-specific loci = 22000 loci. We see in the table that indeed, *P. serendipita* has 22000 to 23000 loci. For *P. pelgrandis* we can make a similar calculation. Its parents are less closely related and have about 1500 loci in common. Together with 18000-1500 = 16500 *P. cactorum* specific loci and 17000-1500=15500 *P. nicotianae*-specific loci this amounts to 33500 loci. And indeed, almost 33000 loci were observed in one of the *P. pelgrandis* isolates.

A semi-automated way to detect hybrids is illustrated in Table XIX and is based on the relative amount of shared loci with potential parents. The table shows the average number of loci in common between each two species as a percentage of the average number of loci in one of the species (in this case, the species listed in the left column). Except for the diagonal, which is inversely related to intraspecies diversity, values above 80% indicate that the species on top is a putative hybrid, with the species to the left as one of its parental species. The inverse combination should also be observed and the highest percentage

indicates the correct direction. For example, *P. serendipita* being a hybrid with *P. cactorum* as one of its parents is based on 94.6% while *P. cactorum* being a hybrid with *P. serendipita* as one of its parents has a lower percentage (80.2%) and thus a lower probability. Therefore *P. serendipita* is the hybrid. This system also detects that *P. andina* is a hybrid with *P. infestans* as one of its parents, which is correct. A few other situations will also create a high percentage (60-80%) and indicates closely related species (e.g. species within the same subclade), but in such case the inverse will be a similar percentage. Hybrids with one common and one non-common ancestor (e.g. *P. pelgrandis* and *P*. *serendipita* both have *P. cactorum* as parent) will also create a relatively high percentage, as they both contain the *P. cactorum* genome. These situations are harder to spot based on the rule mentioned above, but if the species on top already has other strong indications of hybridization, then that is a red flag for this type of situation.

As mentioned above, we not only calculated the number of loci in common between each two isolates, we also determined the percentage of those common loci in which at least one allele was identical. This measure is more geared at determining the level of similarity within a species. Here, it can help to determine how closely related a hybrid isolate is to the different isolates of the parental species in the database. An example of such an analysis (for Clade 1) is presented in Table XX, in which a few additional isolates (compared to the green ones shown in Table XVII) were included. Within a species, groups of isolates lined with red are highly similar. For example, within *P. cactorum*, three such groups of isolates were identified. The middle one is also similar to all the *P. serendipita* isolates (96 to 99% versus only 80-82% and 87-89% for the other groups), suggesting that this middle group of *P. cactorum* isolates is closely related to the parental species of the *P. serendipita* isolates. Within *P. serendipita*, two groups could be distinguished: the two outer isolates are highly similar, and so are the remaining isolates. This suggests that two different hybridization events took place. This result is consistent with their difference in COX profile (one group has mitochondria from *P. cactorum*, the other from *P. hedraiandra*.

Within *P. pelgrandis*, two groups could be distinguished. For one of these the *P. cactorum* parental group could be identified, but not for the other. Within the three *P. nicotianae* isolates there were two groups of isolates, but neither one seemed directly related to the parental group of the *P. pelgrandis* isolates. Therefore, the *P. nicotianae* subgroup that lead to the *P. pelgrandis* hybrids is still unknown.

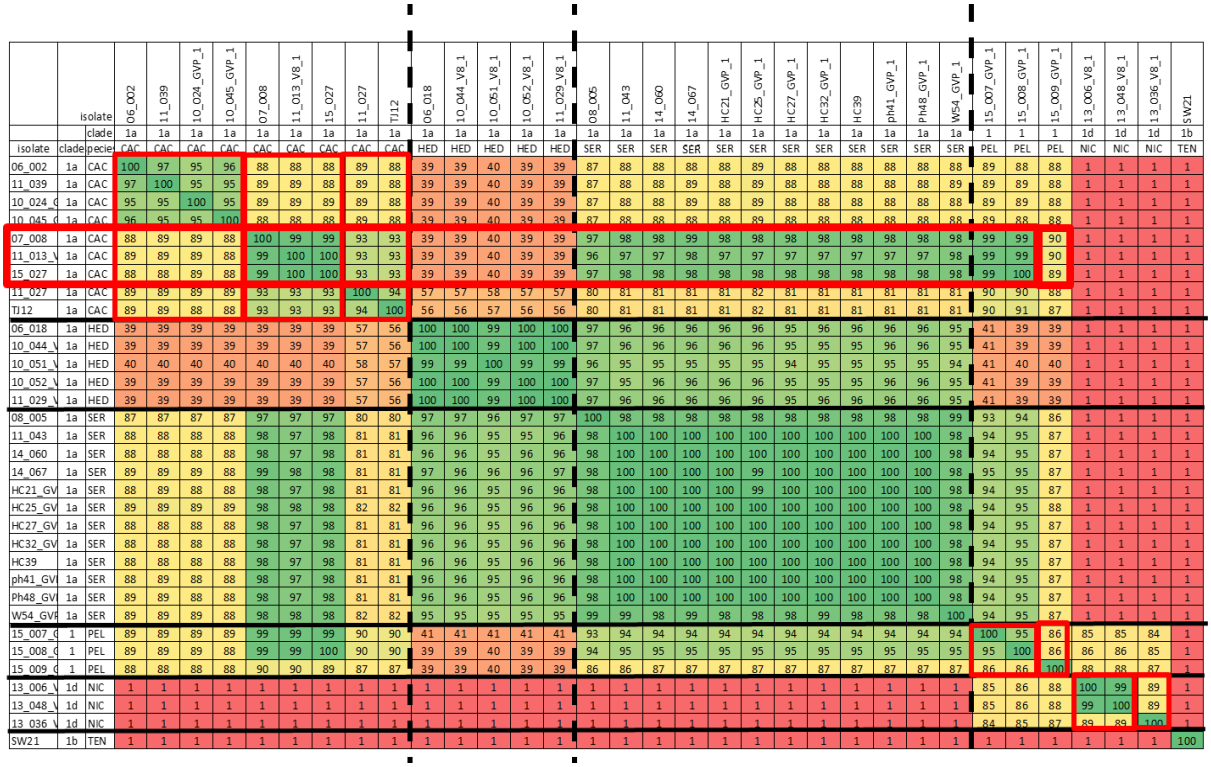
Table XVIII - Matrix table showing the number of common loci of a selection of isolates in **Clade 1** as based on GBS analysis. Abbreviations of the species names are listed in Table XVII. Sections are marked by color depending on the number of loci they have in common. This selection is mostly presented to illustrate the hybrid nature of *P. serendipita* (CACxHED) and *P. pelgrandis* (CACxNIC).

| | clade | 1a | 1a | 1a | 1a | 1a | 1a | 1a | 1a | 1a | 1 | 1 | 1 | 1d | 1d | 1d | 1b |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| clade | pecie | HED | HED | HED | SER | SER | SER | CAC | CAC | CAC | PEL | PEL | PEL | NIC | NIC | NIC | TEN |
| 1a | HED | 19103 | 18040 | 18996 | 18470 | 18468 | 18568 | 13941 | 13880 | 15931 | 12742 | 13947 | 14079 | 1690 | 1686 | 1674 | 1669 |
| 1a | HED | 18040 | 18096 | 18050 | 17744 | 17705 | 17749 | 13469 | 13428 | 15333 | 12510 | 13503 | 13554 | 1659 | 1651 | 1643 | 1630 |
| 1a | HED | 18996 | 18050 | 19075 | 18486 | 18489 | 18594 | 13950 | 13889 | 15913 | 12746 | 13959 | 14088 | 1690 | 1684 | 1674 | 1669 |
| 1a | SER | 18470 | 17744 | 18486 | 22748 | 22507 | 22596 | 17791 | 17942 | 19295 | 16389 | 18007 | 17932 | 1775 | 1771 | 1760 | 1757 |
| 1a | SER | 18468 | 17705 | 18489 | 22507 | 22850 | 22742 | 17842 | 17984 | 19323 | 16408 | 18050 | 17983 | 1779 | 1772 | 1763 | 1761 |
| 1a | SER | 18568 | 17749 | 18594 | 22596 | 22742 | 23046 | 17881 | 18033 | 19369 | 16423 | 18091 | 18039 | 1780 | 1774 | 1763 | 1760 |
| 1a | CAC | 13941 | 13469 | 13950 | 17791 | 17842 | 17881 | 18229 | 17621 | 17235 | 16039 | 17593 | 17686 | 1668 | 1664 | 1655 | 1639 |
| 1a | CAC | 13880 | 13428 | 13889 | 17942 | 17984 | 18033 | 17621 | 18141 | 17281 | 16286 | 17863 | 17687 | 1655 | 1649 | 1643 | 1632 |
| 1a | CAC | 15931 | 15333 | 15913 | 19295 | 19323 | 19369 | 17235 | 17281 | 21463 | 15894 | 17350 | 17373 | 1769 | 1763 | 1753 | 1758 |
| 1 | PEL | 12742 | 12510 | 12746 | 16389 | 16408 | 16423 | 16039 | 16286 | 15894 | 29985 | 28949 | 28472 | 14239 | 14142 | 14068 | 2194 |
| 1 | PEL | 13509 | 13175 | 13512 | 17394 | 17412 | 17441 | 17013 | 17283 | 16816 | 28279 | 30795 | 29803 | 14595 | 14560 | 14406 | 2248 |
| 1 | PEL | 14079 | 13554 | 14088 | 17932 | 17983 | 18039 | 17686 | 17687 | 17373 | 28472 | 30957 | 32745 | 15367 | 15369 | 15093 | 2305 |
| 1d | NIC | 1690 | 1659 | 1690 | 1775 | 1779 | 1780 | 1668 | 1655 | 1769 | 14239 | 15138 | 15367 | 16902 | 15630 | 16286 | 1342 |
| 1d | NIC | 1686 | 1651 | 1684 | 1771 | 1772 | 1774 | 1664 | 1649 | 1763 | 14142 | 15117 | 15369 | 15630 | 17018 | 15379 | 1349 |
| 1d | NIC | 1674 | 1643 | 1674 | 1760 | 1763 | 1763 | 1655 | 1643 | 1753 | 14068 | 14877 | 15093 | 16286 | 15379 | 16518 | 1329 |
| 1b | TEN | 1669 | 1630 | 1669 | 1757 | 1761 | 1760 | 1639 | 1632 | 1758 | 2194 | 2294 | 2305 | 1342 | 1349 | 1329 | 27515 |

Table XIX - Matrix table relating to *Phytophthora* **clade 1** species, showing the average number of loci in common between each two species as a percentage of the average number of loci in the species in the column to the left. Three-letter species codes are listed in Table XVII. Values on the diagonal, marked in grey, simply reflect the % of loci in common within each species. Other values are marked from red to green based on descending percentages. If the percentage is high (>80%) then the species on top is a putative hybrid, with the species to the left as one of its parents. Further explanations in the text. The first column is the subclade letter.

| | | avg #loci | CAC | HED | SER | TEN | AND | INF | MIR | NIC | PEL |
|---|---|---|---|---|---|---|---|---|---|---|---|
| a | CAC | 17857 | 97,3 | 75,4 | 94,6 | 9,2 | 6,9 | 6,3 | 6,5 | 9,2 | 93,4 |
| a | HED | 18394 | 73,2 | 83,7 | 87,4 | 9,1 | 6,8 | 6,2 | 6,4 | 9,0 | 71,1 |
| a | SER | 21063 | 80,2 | 76,3 | 90,8 | 8,3 | 6,2 | 5,7 | 5,8 | 8,2 | 77,6 |
| b | TEN | 25102 | 6,6 | 6,7 | 6,9 | 91,9 | 4,7 | 4,3 | 4,4 | 5,4 | 9,0 |
| c | AND | 24685 | 5,0 | 5,1 | 5,3 | 4,7 | | 68,2 | 50,4 | 4,4 | 7,2 |
| c | INF | 20818 | 5,4 | 5,5 | 5,7 | 5,2 | 80,9 | 88,5 | 53,4 | 4,9 | 7,9 |
| c | MIR | 20469 | 5,7 | 5,8 | 6,0 | 5,3 | 60,8 | 54,3 | | 5,1 | 8,3 |
| d | NIC | 16922 | 9,7 | 9,8 | 10,2 | 7,9 | 6,4 | 6,0 | 6,2 | 92,1 | 86,7 |
| d | PEL | 30917 | 54,0 | 42,3 | 52,9 | 7,3 | 5,7 | 5,3 | 5,5 | 47,5 | 91,9 |

Table XX - Matrix table showing the percentage of common loci with at least one identical allele for a selection of isolates in Phytophthora **clade 1**, as based on GBS analysis. The species codes are listed in Table XVII. Within a species, groups of isolates lined with red are highly similar. Further explanations in the text.



| isolate | clade | pecies | 06_002 | 11_039 | 10_024_GVP_1 | 10_045_GVP_1 | 07_008 | 11_013_V8_1 | 15_027 | 11_027 | TJ12 | 06_018 | 10_044_V8_1 | 10_051_V8_1 | 10_052_V8_1 | 11_029_V8_1 | 08_005 | 11_043 | 14_060 | 14_067 | HC21_GVP_1 | HC25_GVP_1 | HC27_GVP_1 | HC32_GVP_1 | HC39 | ph41_GVP_1 | Ph48_GVP_1 | W54_GVP_1 | 15_007_GVP_1 | 15_008_GVP_1 | 15_009_GVP_1 | 13_006_V8_1 | 13_048_V8_1 | 13_036_V8_1 | SW21 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | clade | | 1a | 1a | 1a | 1a | 1a | 1a | 1a | 1a | 1a | 1a | 1a | 1a | 1a | 1a | 1a | 1a | 1a | 1a | 1a | 1a | 1a | 1a | 1a | 1a | 1a | 1a | 1 | 1 | 1 | 1d | 1d | 1d | 1b |
| isolate | clade | pecies | CAC | CAC | CAC | CAC | CAC | CAC | CAC | CAC | CAC | HED | HED | HED | HED | HED | SER | SER | SER | SER | SER | SER | SER | SER | SER | SER | SER | SER | PEL | PEL | PEL | NIC | NIC | NIC | TEN |
| 06_002 | 1a | CAC | 100 | 97 | 95 | 96 | 88 | 88 | 88 | 89 | 88 | 39 | 39 | 40 | 39 | 39 | 87 | 88 | 88 | 88 | 88 | 89 | 88 | 88 | 88 | 88 | 88 | 88 | 89 | 88 | 88 | 1 | 1 | 1 | 1 |
| 11_039 | 1a | CAC | 97 | 100 | 95 | 95 | 89 | 89 | 88 | 89 | 88 | 39 | 39 | 40 | 39 | 39 | 87 | 88 | 88 | 89 | 88 | 89 | 88 | 88 | 88 | 88 | 88 | 89 | 89 | 89 | 88 | 1 | 1 | 1 | 1 |
| 10_024_G | 1a | CAC | 95 | 95 | 100 | 95 | 89 | 89 | 89 | 89 | 88 | 39 | 39 | 40 | 39 | 39 | 87 | 88 | 88 | 89 | 88 | 89 | 88 | 88 | 88 | 88 | 88 | 88 | 89 | 89 | 88 | 1 | 1 | 1 | 1 |
| 10_045_G | 1a | CAC | 96 | 95 | 95 | 100 | 88 | 88 | 88 | 89 | 88 | 39 | 39 | 40 | 39 | 39 | 87 | 88 | 88 | 88 | 88 | 89 | 88 | 88 | 88 | 88 | 88 | 88 | 89 | 88 | 88 | 1 | 1 | 1 | 1 |
| 07_008 | 1a | CAC | 88 | 89 | 89 | 88 | 100 | 99 | 99 | 93 | 93 | 39 | 39 | 40 | 39 | 39 | 97 | 98 | 98 | 99 | 98 | 98 | 98 | 98 | 98 | 98 | 98 | 98 | 99 | 99 | 90 | 1 | 1 | 1 | 1 |
| 11_013_V | 1a | CAC | 89 | 89 | 89 | 88 | 99 | 100 | 100 | 93 | 93 | 39 | 39 | 40 | 39 | 39 | 96 | 97 | 97 | 98 | 97 | 97 | 97 | 97 | 97 | 97 | 97 | 98 | 99 | 99 | 90 | 1 | 1 | 1 | 1 |
| 15_027 | 1a | CAC | 88 | 88 | 89 | 88 | 99 | 100 | 100 | 93 | 93 | 39 | 39 | 40 | 39 | 39 | 97 | 98 | 98 | 98 | 98 | 98 | 98 | 98 | 98 | 98 | 98 | 98 | 99 | 100 | 89 | 1 | 1 | 1 | 1 |
| 11_027 | 1a | CAC | 89 | 89 | 89 | 89 | 93 | 93 | 93 | 100 | 94 | 57 | 57 | 58 | 57 | 57 | 80 | 81 | 81 | 81 | 81 | 82 | 81 | 81 | 81 | 81 | 81 | 81 | 90 | 90 | 88 | 1 | 1 | 1 | 1 |
| TJ12 | 1a | CAC | 89 | 89 | 88 | 88 | 93 | 93 | 93 | 94 | 100 | 56 | 56 | 57 | 56 | 56 | 80 | 81 | 81 | 81 | 81 | 82 | 81 | 81 | 81 | 81 | 81 | 81 | 90 | 91 | 87 | 1 | 1 | 1 | 1 |
| 06_018 | 1a | HED | 39 | 39 | 39 | 39 | 39 | 39 | 39 | 57 | 56 | 100 | 100 | 99 | 100 | 100 | 97 | 96 | 96 | 96 | 96 | 96 | 95 | 96 | 96 | 96 | 96 | 95 | 41 | 39 | 39 | 1 | 1 | 1 | 1 |
| 10_044_V | 1a | HED | 39 | 39 | 39 | 39 | 39 | 39 | 39 | 57 | 56 | 100 | 100 | 99 | 100 | 100 | 97 | 96 | 96 | 96 | 96 | 96 | 95 | 95 | 95 | 96 | 96 | 95 | 41 | 39 | 39 | 1 | 1 | 1 | 1 |
| 10_051_V | 1a | HED | 40 | 40 | 40 | 40 | 40 | 40 | 40 | 58 | 57 | 99 | 99 | 100 | 99 | 99 | 96 | 95 | 95 | 95 | 95 | 95 | 94 | 95 | 95 | 95 | 95 | 94 | 41 | 40 | 40 | 1 | 1 | 1 | 1 |
| 10_052_V | 1a | HED | 39 | 39 | 39 | 39 | 39 | 39 | 39 | 57 | 56 | 100 | 100 | 99 | 100 | 100 | 97 | 95 | 96 | 96 | 96 | 96 | 95 | 95 | 95 | 96 | 96 | 95 | 41 | 39 | 39 | 1 | 1 | 1 | 1 |
| 11_029_V | 1a | HED | 39 | 39 | 39 | 39 | 39 | 39 | 39 | 57 | 56 | 100 | 100 | 99 | 100 | 100 | 97 | 96 | 96 | 96 | 96 | 96 | 95 | 96 | 96 | 96 | 96 | 95 | 41 | 39 | 39 | 1 | 1 | 1 | 1 |
| 08_005 | 1a | SER | 87 | 87 | 87 | 87 | 97 | 97 | 97 | 80 | 80 | 97 | 97 | 96 | 97 | 97 | 100 | 98 | 98 | 98 | 98 | 98 | 98 | 98 | 98 | 98 | 98 | 99 | 93 | 94 | 86 | 1 | 1 | 1 | 1 |
| 11_043 | 1a | SER | 88 | 88 | 88 | 88 | 98 | 97 | 98 | 81 | 81 | 96 | 96 | 95 | 95 | 96 | 98 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 98 | 94 | 95 | 87 | 1 | 1 | 1 | 1 |
| 14_060 | 1a | SER | 88 | 88 | 88 | 88 | 98 | 97 | 98 | 81 | 81 | 96 | 96 | 95 | 96 | 96 | 98 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 98 | 94 | 95 | 87 | 1 | 1 | 1 | 1 |
| 14_067 | 1a | SER | 89 | 89 | 89 | 88 | 99 | 98 | 98 | 81 | 81 | 97 | 96 | 96 | 96 | 97 | 98 | 100 | 100 | 100 | 100 | 99 | 100 | 100 | 100 | 100 | 100 | 98 | 95 | 95 | 87 | 1 | 1 | 1 | 1 |
| HC21_GV | 1a | SER | 88 | 89 | 88 | 88 | 98 | 97 | 98 | 81 | 81 | 96 | 96 | 95 | 96 | 96 | 98 | 100 | 100 | 100 | 100 | 99 | 100 | 100 | 100 | 100 | 100 | 98 | 94 | 95 | 87 | 1 | 1 | 1 | 1 |
| HC25_GV | 1a | SER | 89 | 89 | 89 | 89 | 98 | 98 | 98 | 82 | 82 | 96 | 96 | 95 | 96 | 96 | 98 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 98 | 94 | 95 | 88 | 1 | 1 | 1 | 1 |
| HC27_GV | 1a | SER | 88 | 88 | 88 | 88 | 98 | 97 | 98 | 81 | 81 | 96 | 96 | 95 | 96 | 96 | 98 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 98 | 94 | 95 | 87 | 1 | 1 | 1 | 1 |
| HC32_GV | 1a | SER | 88 | 88 | 88 | 88 | 98 | 97 | 98 | 81 | 81 | 96 | 96 | 95 | 96 | 96 | 98 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 98 | 94 | 95 | 87 | 1 | 1 | 1 | 1 |
| HC39 | 1a | SER | 88 | 88 | 88 | 88 | 98 | 97 | 98 | 81 | 81 | 96 | 96 | 95 | 96 | 96 | 98 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 98 | 94 | 95 | 87 | 1 | 1 | 1 | 1 |
| ph41_GV | 1a | SER | 88 | 89 | 88 | 88 | 98 | 97 | 98 | 81 | 81 | 96 | 96 | 95 | 96 | 96 | 98 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 98 | 94 | 95 | 87 | 1 | 1 | 1 | 1 |
| Ph48_GV | 1a | SER | 89 | 89 | 88 | 88 | 98 | 97 | 98 | 81 | 81 | 96 | 96 | 95 | 96 | 96 | 98 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 98 | 94 | 95 | 87 | 1 | 1 | 1 | 1 |
| W54_GVP | 1a | SER | 89 | 89 | 89 | 88 | 98 | 98 | 98 | 82 | 82 | 95 | 95 | 95 | 95 | 95 | 99 | 99 | 98 | 99 | 98 | 98 | 98 | 99 | 98 | 98 | 98 | 100 | 94 | 95 | 87 | 1 | 1 | 1 | 1 |
| 15_007_G | 1 | PEL | 89 | 89 | 89 | 89 | 99 | 99 | 99 | 90 | 90 | 41 | 41 | 41 | 41 | 41 | 93 | 94 | 94 | 94 | 94 | 94 | 94 | 94 | 94 | 94 | 94 | 94 | 100 | 95 | 86 | 85 | 85 | 84 | 1 |
| 15_008_G | 1 | PEL | 89 | 89 | 89 | 88 | 99 | 99 | 100 | 90 | 90 | 39 | 39 | 40 | 39 | 39 | 94 | 95 | 95 | 95 | 95 | 95 | 95 | 95 | 95 | 95 | 95 | 95 | 95 | 100 | 86 | 86 | 86 | 85 | 1 |
| 15_009_G | 1 | PEL | 88 | 88 | 88 | 88 | 90 | 90 | 89 | 87 | 87 | 39 | 39 | 40 | 39 | 39 | 86 | 86 | 87 | 87 | 87 | 87 | 87 | 87 | 87 | 87 | 87 | 87 | 86 | 86 | 100 | 88 | 88 | 87 | 1 |
| 13_006_V | 1d | NIC | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 85 | 86 | 88 | 100 | 99 | 89 | 1 |
| 13_048_V | 1d | NIC | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 85 | 86 | 88 | 99 | 100 | 89 | 1 |
| 13_036_V | 1d | NIC | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 84 | 85 | 87 | 89 | 89 | 100 | 1 |
| SW21 | 1b | TEN | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 100 |

The same process of analysis was conducted for the isolates in the other clades. However, the most interesting clades in respect to hybrids were clades 1, 6, 7, 8 and 9 and given the main objective of identification and characterization of hybrids, this report will limit itself to those clades.

Table XXI presents the data on common loci of the isolates from clade 6. At first glance, it is obvious that the number of loci in the species of this clade (observable on the diagonal) are on average considerably higher than those in clade 1. But there are very important exceptions. For example, within *P. chlamydospora* (CHL) there is one isolate (12/040) which only has 22438 loci, while the other isolates in this species have more than 30000 loci. This suggests that the other isolates are hybrids. In the case of 04/004, 07/003 and 10/003 these are hybrids between CHL and an unknown species, as there is no other species in our dataset with which they have a large number of loci in common. In the case of 13/005 it is a hybrid between CHL and *P. lacustris* (LAC), or an ancestor of LAC, based on the large number of loci that this isolate also has in common with LAC. Interestingly, isolate 12/040 (with the small number of loci) has a genome size of 140 Mbp based on flow cytometry, while for example CHL isolate 10/033 (with a large number of loci) only has a genome size of 150 Mbp, suggesting that the hybridization in isolate 10/033 was sexual. In allopolyploid hybrids the genome size tends to be significantly higher.

In *P. gonapodyides* (GON) a similar situation was observed, with isolate 07/007 having only 21513 loci, while the other isolates have around 30000 loci. The other GON and GONVAR (with a variant sequence in rDNA ITS) isolates are thus most likely also hybrids, but again with an unknown other species. Other species within clade 6 also tend to have large numbers of loci. For example, there are more than 31000 in *P. inundata* (INU). It is likely a hybrid based on this size but there are no other species in our database with which it has a large number of loci in common. Two isolates of INU that were measured with flow cytometry had a genome size of 300 Mbp, which is large and may indicate an allopolyploid nature of these hybrids. P. megasperma (MEG) likely also contains allopolyploid hybrids: large and variable numbers of loci and large genome sizes (290 Mbp in two of the ones listed).

Table XXI - Matrix table showing the number of common loci of a selection of isolates in *Phytophthora* **Clade 6** as based on GBS analysis. Abbreviations of the species names are listed in Table XVII. Sections are marked by color depending on the number of loci they have in common

| | | | 08_004_GVP_1 | 09_001 | 09_003_GVP_1 | 04_004 | 07_003 | 10_003_GVP_1 | 12_040_GVP_1 | 13_005_GVP_1 | 06_014 | 05_013 | CH05 | CH14 | 07_004_GVP_1 | 07_007_GVP_1 | 14_049 | 05_003 | 05_004 | 05_017 | 09_002 | 13_013 | 14_070 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 6a | 6a | 6a | 6b | 6b | 6b | 6b | 6b | 6b | 6b | 6b | 6b | 6b | 6b | 6b | 6b | 6b | 6b | 6b | 6b | 6b |
| | | | INU | INU | INU | CHL | CHL | CHL | CHL | HLxGC | LAC | LAC | LAC | LAC | GON | GON | GON | ONVA | ONVA | ONVA | MEG | MEG | MEG |
| 08_004_GVP_1 | 6a | INU | 31031 | 30916 | 30988 | 4133 | 4120 | 4081 | 3719 | 4642 | 3804 | 3745 | 3763 | 3710 | 4162 | 3556 | 4125 | 4187 | 4071 | 4101 | 4305 | 4188 | 4262 |
| 09_001 | 6a | INU | 30916 | 31973 | 31625 | 4166 | 4152 | 4090 | 3728 | 4650 | 3832 | 3773 | 3792 | 3738 | 4176 | 3557 | 4150 | 4214 | 4093 | 4127 | 4339 | 4213 | 4298 |
| 09_003_GVP_1 | 6a | INU | 30988 | 31625 | 32050 | 4153 | 4139 | 4089 | 3727 | 4648 | 3825 | 3767 | 3785 | 3731 | 4174 | 3558 | 4141 | 4204 | 4085 | 4117 | 4328 | 4204 | 4286 |
| 04_004 | 6b | CHL | 4133 | 4166 | 4153 | 31311 | 30478 | 29546 | 21045 | 17989 | 6030 | 5966 | 5979 | 5891 | 10024 | 8342 | 10030 | 10190 | 9908 | 9961 | 10193 | 9870 | 10112 |
| 07_003 | 6b | CHL | 4120 | 4152 | 4139 | 30478 | 31062 | 29346 | 21000 | 17934 | 6010 | 5946 | 5962 | 5875 | 9992 | 8319 | 9993 | 10150 | 9878 | 9919 | 10166 | 9839 | 10083 |
| 10_003_GVP_1 | 6b | CHL | 4081 | 4090 | 4089 | 29546 | 29346 | 30051 | 20735 | 17902 | 5913 | 5851 | 5866 | 5777 | 9880 | 8336 | 9798 | 9961 | 9703 | 9741 | 9963 | 9675 | 9890 |
| 12_040_GVP_1 | 6b | CHL | 3719 | 3728 | 3727 | 21045 | 21000 | 20735 | 22438 | 17982 | 5346 | 5293 | 5319 | 5237 | 9017 | 7616 | 8945 | 9092 | 8856 | 8902 | 9099 | 8853 | 9025 |
| 13_005_GVP_1 | 6b | CHL | 4642 | 4650 | 4648 | 17989 | 17934 | 17902 | 17982 | 33652 | 18726 | 18545 | 18522 | 18262 | 9576 | 8407 | 9418 | 9619 | 9357 | 9401 | 9538 | 9326 | 9453 |
| 06_014 | 6b | LAC | 3804 | 3832 | 3825 | 6030 | 6010 | 5913 | 5346 | 18726 | 29397 | 24850 | 24770 | 24138 | 6114 | 5179 | 6063 | 6182 | 6016 | 6064 | 6046 | 5838 | 6000 |
| 05_013 | 6b | LAC | 3745 | 3773 | 3767 | 5966 | 5946 | 5851 | 5293 | 18545 | 24850 | 28671 | 24796 | 24217 | 6033 | 5111 | 5991 | 6105 | 5935 | 5983 | 5966 | 5765 | 5924 |
| CH05 | 6b | LAC | 3763 | 3792 | 3785 | 5979 | 5962 | 5866 | 5319 | 18522 | 24770 | 24796 | 29063 | 24952 | 6073 | 5124 | 6017 | 6149 | 5962 | 6021 | 6010 | 5812 | 5961 |
| CH14 | 6b | LAC | 3710 | 3738 | 3731 | 5891 | 5875 | 5777 | 5237 | 18262 | 24138 | 24217 | 24952 | 27835 | 5968 | 5052 | 5913 | 6023 | 5861 | 5928 | 5911 | 5717 | 5864 |
| 07_004_GVP_1 | 6b | GON | 4162 | 4176 | 4174 | 10024 | 9992 | 9880 | 9017 | 9576 | 6114 | 6033 | 6073 | 5968 | 31314 | 21469 | 24383 | 25514 | 24295 | 24493 | 10824 | 10472 | 10722 |
| 07_007_GVP_1 | 6b | GON | 3556 | 3557 | 3558 | 8342 | 8319 | 8336 | 7616 | 8407 | 5179 | 5111 | 5124 | 5052 | 21469 | 21513 | 18536 | 19383 | 18679 | 18663 | 8916 | 8729 | 8847 |
| 14_049 | 6b | GON | 4125 | 4150 | 4141 | 10030 | 9993 | 9798 | 8945 | 9418 | 6063 | 5991 | 6017 | 5913 | 24383 | 18536 | 30159 | 24519 | 23790 | 23898 | 10702 | 10336 | 10600 |
| 05_003 | 6b | GONVAR | 4187 | 4214 | 4204 | 10190 | 10150 | 9961 | 9092 | 9619 | 6182 | 6105 | 6149 | 6023 | 25514 | 19383 | 24519 | 31344 | 24413 | 24683 | 10950 | 10581 | 10838 |
| 05_004 | 6b | GONVAR | 4071 | 4093 | 4085 | 9908 | 9878 | 9703 | 8856 | 9357 | 6016 | 5935 | 5962 | 5861 | 24295 | 18679 | 23790 | 24413 | 29685 | 23974 | 10595 | 10250 | 10488 |
| 05_017 | 6b | GONVAR | 4101 | 4127 | 4117 | 9961 | 9919 | 9741 | 8902 | 9401 | 6064 | 5983 | 6021 | 5928 | 24493 | 18663 | 23898 | 24683 | 23974 | 30076 | 10712 | 10343 | 10611 |
| 09_002 | 6b | MEG | 4305 | 4339 | 4328 | 10193 | 10166 | 9963 | 9099 | 9538 | 6046 | 5966 | 6010 | 5911 | 10824 | 8916 | 10702 | 10950 | 10595 | 10712 | 36183 | 29920 | 32692 |
| 13_013 | 6b | MEG | 4188 | 4213 | 4204 | 9870 | 9839 | 9675 | 8853 | 9326 | 5838 | 5765 | 5812 | 5717 | 10472 | 8729 | 10336 | 10581 | 10250 | 10343 | 29920 | 30726 | 28676 |
| 14_070 | 6b | MEG | 4262 | 4298 | 4286 | 10112 | 10083 | 9890 | 9025 | 9453 | 6000 | 5924 | 5961 | 5864 | 10722 | 8847 | 10600 | 10838 | 10488 | 10611 | 32692 | 28676 | 34989 |

When we look at the percentage of common loci that have at least one identical allele (Table XXII), we can also make interesting observations. There are two groups within CHL. Isolate 12/040, with the small number of loci, is closely related to the CHLxLAC hybrid 13/005, suggesting 12/040 is related to the parental CHL isolate of this hybrid. Within LAC, GON(VAR), and MEG, there is a large amount of diversity, with most (GON) to all (LAC, MEG) isolates being ungrouped. This could in part be due to their habitat (flooded systems, in which dispersal is easy). In *P. inundata* (a species from a different subclade), this genetic diversity is not observed.

Table XXII - Matrix table showing the percentage of common loci with at least one identical allele for a selection of isolates in *Phytophthora* **clade 6**, as based on GBS analysis. The species codes are listed in Table XVII. Further explanations in the text

| | | | 08_004_GVP_1 | 09_001 | 09_003_GVP_1 | 04_004 | 07_003 | 10_003_GVP_1 | 12_040_GVP_1 | 13_005_GVP_1 | 06_014 | 05_013 | CH05 | CH14 | 07_004_GVP_1 | 07_007_GVP_1 | 14_049 | 05_003 | 05_004 | 05_017 | 09_002 | 13_013 | 14_070 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 6a | 6a | 6a | 6b | 6b | 6b | 6b | 6b | 6b | 6b | 6b | 6b | 6b | 6b | 6b | 6b | 6b | 6b | 6b | 6b | 6b |
| | | | INU | INU | INU | CHL | CHL | CHL | CHL | HLxGC | LAC | LAC | LAC | LAC | GON | GON | GON | ONVA | ONVA | ONVA | MEG | MEG | MEG |
| 08_004_C | 6a | INU | 100 | 100 | 99 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
| 09_001 | 6a | INU | 100 | 100 | 100 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
| 09_003_C | 6a | INU | 100 | 100 | 100 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
| 04_004 | 6b | GON | 2 | 2 | 2 | 100 | 100 | 100 | 88 | 77 | 4 | 4 | 4 | 4 | 9 | 11 | 9 | 9 | 9 | 9 | 8 | 9 | 8 |
| 07_003 | 6b | CHL | 2 | 2 | 2 | 100 | 100 | 100 | 87 | 77 | 4 | 4 | 4 | 4 | 9 | 11 | 9 | 9 | 9 | 9 | 9 | 9 | 9 |
| 10_003_C | 6b | CHL | 2 | 2 | 2 | 100 | 100 | 100 | 88 | 77 | 4 | 4 | 4 | 4 | 10 | 11 | 9 | 9 | 9 | 9 | 9 | 9 | 9 |
| 12_040_C | 6b | CHL | 2 | 2 | 2 | 88 | 87 | 88 | 100 | 97 | 4 | 3 | 4 | 3 | 9 | 10 | 9 | 9 | 9 | 9 | 9 | 9 | 9 |
| 13_005_C | 6b | CHL | 2 | 2 | 2 | 77 | 77 | 77 | 97 | 100 | 69 | 69 | 68 | 68 | 9 | 10 | 9 | 9 | 9 | 9 | 8 | 8 | 8 |
| 06_014 | 6b | GON | 2 | 2 | 2 | 4 | 4 | 4 | 4 | 69 | 100 | 79 | 78 | 77 | 5 | 5 | 5 | 5 | 5 | 5 | 4 | 4 | 4 |
| 05_013 | 6b | GON | 2 | 2 | 2 | 4 | 4 | 4 | 3 | 69 | 79 | 100 | 79 | 80 | 5 | 5 | 4 | 5 | 4 | 4 | 4 | 4 | 4 |
| CH05 | 6b | LAC | 2 | 2 | 2 | 4 | 4 | 4 | 4 | 69 | 78 | 79 | 100 | 83 | 4 | 5 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| CH14 | 6b | LAC | 2 | 2 | 2 | 4 | 4 | 4 | 3 | 68 | 77 | 80 | 83 | 100 | 4 | 5 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| 07_004_C | 6b | GON | 2 | 2 | 2 | 9 | 9 | 10 | 9 | 9 | 5 | 5 | 4 | 4 | 100 | 99 | 75 | 80 | 77 | 77 | 10 | 10 | 10 |
| 07_007_C | 6b | GON | 2 | 2 | 2 | 11 | 11 | 11 | 10 | 10 | 5 | 5 | 5 | 5 | 99 | 100 | 78 | 83 | 80 | 80 | 11 | 12 | 11 |
| 14_049 | 6b | GON | 2 | 2 | 2 | 9 | 9 | 9 | 9 | 9 | 5 | 4 | 4 | 4 | 75 | 78 | 100 | 75 | 74 | 74 | 10 | 10 | 9 |
| 05_003 | 6b | GONV | 2 | 2 | 2 | 9 | 9 | 9 | 9 | 9 | 5 | 4 | 4 | 4 | 80 | 83 | 76 | 100 | 77 | 77 | 10 | 10 | 10 |
| 05_004 | 6b | GONV | 2 | 2 | 2 | 9 | 9 | 9 | 9 | 9 | 4 | 4 | 4 | 4 | 76 | 79 | 73 | 76 | 100 | 75 | 9 | 10 | 9 |
| 05_017 | 6b | GONV | 2 | 2 | 2 | 9 | 9 | 9 | 9 | 9 | 4 | 4 | 4 | 4 | 76 | 79 | 73 | 76 | 76 | 100 | 9 | 10 | 9 |
| 09_002 | 6b | MEG | 2 | 2 | 2 | 9 | 9 | 9 | 9 | 8 | 4 | 4 | 4 | 4 | 10 | 12 | 10 | 10 | 9 | 10 | 100 | 93 | 87 |
| 13_013 | 6b | MEG | 2 | 2 | 2 | 9 | 9 | 9 | 9 | 8 | 4 | 4 | 4 | 4 | 10 | 12 | 10 | 10 | 10 | 10 | 92 | 100 | 88 |
| 14_070 | 6b | MEG | 2 | 2 | 2 | 8 | 9 | 9 | 9 | 8 | 4 | 4 | 4 | 4 | 10 | 11 | 10 | 10 | 9 | 10 | 86 | 88 | 100 |

In clade 7 we find a number of known hybrid species including *P. cambivora, P. heterohybrida*, *P. incrassata, P. alni* and *P. multiformis*. The matrix with the number of common loci is presented in Table XXIII. It confirms the hybrid nature of the species listed above. They have large to very large numbers of loci and in cases where the parents are known (e.g. *P. alni* is a hybrid of *P. uniformis* and *P. multiformis*) their parentage is also confirmed by the data on the number of loci: *P. uniformis* has around 25000 loci, *P. multiformis* has around 43000 loci (one exception) and *P. uniformis* and *P. multiformis* have about 15000 loci in common. Therefore, their hybrid should have about 15000 common + (25000-15000) *P. uniformis*-specific + (43000-15000) *P. multiformis*-specific = 53000 loci, a number which corresponds to the observed number of loci in some of the P. alni isolates. Table XXIII also shows quite some intra-species diversity in *P. cambivora* and *P. alni*. These hybrids also have very large and quite variable genome sizes, from 425 to 580 Mbp in *P. cambivora* and from 530 to 850 Mbp in *P. alni*. These large sizes indicate that they are allopolyploid hybrids. Genome rearrangements can explain their variable genome sizes. We only have one isolate of *P. incrassata* included so far, but it had the largest number of loci in our collection (61728) and a very large genome size (around 650 Mbp), again suggesting it is an allopolyploid hybrid.

Table XXIII - Matrix table showing the number of common loci of a selection of isolates in *Phytophthora* **Clade 7** as based on GBS analysis. Abbreviations of the species names are listed in Table XVII. Sections are marked by color depending on the number of loci they have in common.

| | | | 05_006_GVP_1 | AC13 | TJ70_GVP_1 | 05_027_GVP_1 | AC12 | 10_010_GVP_1 | TJ37 | TJ38 | TJ61 | AC01 | AC03 | SW02_GVP_1 | TJ01_GVP_1 | TJ06_GVP_1 | AC04 | SW04_GVP_1 | TJ04_GVP_1 | TJ05_V8_1 | AC05 | AC07 | SW06_GVP_1 | TJ08_GVP_1 | SW15 | 10_009 | 11_022 | 11_024 | 14_003 | TJ88_GVP_1 | TJ89_GVP_1 | 14_061 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 7a | 7a | 7a | 7a | 7a | 7a | 7a | 7a | 7a | 7a | 7a | 7a | 7a | 7a | 7a | 7a | 7a | 7a | 7a | 7a | 7a | 7a | 7b | 7c | 7c | 7c | 7c | 7c | 7c | 7c |
| | | | CAM | CAM | CAM | CAM | CAM | CAM | HET | HET | INC | ALN | ALN | ALN | ALN | ALN | MUF | MUF | MUF | MUF | UNI | UNI | UNI | UNI | NIE | CIN | CIN | CIN | CIN | CIN | CIN | CIN |
| 05_006_GVP_1 | 7a | CAM | 40228 | 39309 | 30377 | 27383 | 27877 | 26410 | 15078 | 15091 | 19866 | 22026 | 21962 | 21685 | 21299 | 21605 | 17859 | 16423 | 17812 | 17731 | 14103 | 12685 | 17400 | 17201 | 4480 | 3612 | 3661 | 3612 | 3612 | 3580 | 3616 | 3622 |
| AC13 | 7a | CAM | 39309 | 41250 | 30681 | 27681 | 28384 | 26681 | 15294 | 15309 | 20154 | 22336 | 22296 | 21870 | 21481 | 21804 | 18104 | 16526 | 18002 | 17911 | 14215 | 12808 | 17547 | 17329 | 4531 | 3635 | 3686 | 3636 | 3636 | 3601 | 3640 | 3646 |
| TJ70_GVP_1 | 7a | CAM | 30377 | 30681 | 31050 | 23485 | 23446 | 22680 | 13535 | 13545 | 17461 | 19253 | 19129 | 19128 | 19009 | 19139 | 15893 | 14994 | 15892 | 15834 | 13109 | 11760 | 15450 | 15337 | 4155 | 3360 | 3408 | 3361 | 3359 | 3355 | 3370 | 3366 |
| 05_027_GVP_1 | 7a | CAM | 27383 | 27681 | 23485 | 39458 | 38478 | 26267 | 15123 | 15130 | 19770 | 21971 | 21845 | 21664 | 21354 | 21616 | 17840 | 16481 | 17800 | 17739 | 14066 | 12696 | 17285 | 17113 | 4479 | 3606 | 3657 | 3606 | 3606 | 3578 | 3611 | 3614 |
| AC12 | 7a | CAM | 27877 | 28384 | 23446 | 38478 | 41358 | 26643 | 15381 | 15390 | 20141 | 22396 | 22346 | 21903 | 21501 | 21809 | 18180 | 16574 | 18054 | 17998 | 14152 | 12772 | 17544 | 17356 | 4531 | 3635 | 3685 | 3633 | 3634 | 3595 | 3632 | 3645 |
| 10_010_GVP_1 | 7a | CAM | 26410 | 26681 | 22680 | 26267 | 26643 | 44877 | 15358 | 15368 | 20030 | 22257 | 22156 | 21897 | 21554 | 21834 | 18094 | 16622 | 18056 | 17968 | 14111 | 12729 | 17427 | 17229 | 4538 | 3640 | 3689 | 3640 | 3640 | 3612 | 3646 | 3643 |
| TJ37 | 7a | HET | 15078 | 15294 | 13535 | 15123 | 15381 | 15358 | 34418 | 33795 | 19897 | 16243 | 16197 | 15862 | 15728 | 15896 | 14678 | 13483 | 14575 | 14548 | 10165 | 9234 | 12212 | 12095 | 4029 | 3238 | 3286 | 3241 | 3239 | 3204 | 3237 | 3247 |
| TJ38 | 7a | HET | 15091 | 15309 | 13545 | 15130 | 15390 | 15368 | 33795 | 34334 | 19910 | 16257 | 16212 | 15875 | 15741 | 15902 | 14686 | 13499 | 14591 | 14557 | 10166 | 9240 | 12227 | 12098 | 4032 | 3238 | 3285 | 3241 | 3238 | 3204 | 3238 | 3246 |
| TJ61 | 7a | INC | 19866 | 20154 | 17461 | 19770 | 20141 | 20030 | 19897 | 19910 | 61728 | 21681 | 21594 | 21063 | 20888 | 21126 | 19229 | 17446 | 19086 | 19007 | 12685 | 11491 | 15338 | 15164 | 4850 | 3889 | 3936 | 3888 | 3885 | 3842 | 3885 | 3902 |
| AC01 | 7a | ALN | 22026 | 22336 | 19253 | 21971 | 22396 | 22257 | 16243 | 16257 | 21681 | 52974 | 51482 | 48157 | 47014 | 48524 | 42036 | 34817 | 41567 | 41160 | 19443 | 17546 | 25276 | 24883 | 4790 | 3809 | 3878 | 3813 | 3810 | 3772 | 3810 | 3824 |
| AC03 | 7a | ALN | 21962 | 22296 | 19129 | 21845 | 22346 | 22156 | 16197 | 16212 | 21594 | 51482 | 54412 | 48139 | 46587 | 48414 | 42657 | 34421 | 41844 | 41350 | 19447 | 17559 | 25655 | 25186 | 4763 | 3804 | 3874 | 3808 | 3807 | 3765 | 3804 | 3819 |
| SW02_GVP_1 | 7a | ALN | 21685 | 21870 | 19128 | 21664 | 21903 | 21897 | 15862 | 15875 | 21063 | 48157 | 48139 | 49622 | 45064 | 46335 | 38776 | 33204 | 38620 | 38344 | 19499 | 17553 | 25174 | 24832 | 4705 | 3758 | 3827 | 3762 | 3758 | 3728 | 3762 | 3772 |
| TJ01_GVP_1 | 7a | ALN | 21299 | 21481 | 19009 | 21354 | 21501 | 21554 | 15728 | 15741 | 20888 | 47014 | 46587 | 45064 | 47873 | 45709 | 38137 | 33383 | 38113 | 37863 | 19096 | 17167 | 23998 | 23750 | 4681 | 3735 | 3804 | 3740 | 3735 | 3711 | 3738 | 3751 |
| TJ06_GVP_1 | 7a | ALN | 21605 | 21804 | 19139 | 21616 | 21809 | 21834 | 15896 | 15902 | 21126 | 48524 | 48414 | 46335 | 45709 | 49823 | 39499 | 33897 | 39406 | 39093 | 19331 | 17386 | 24630 | 24338 | 4710 | 3749 | 3818 | 3753 | 3749 | 3721 | 3757 | 3762 |
| AC04 | 7a | MUF | 17859 | 18104 | 15893 | 17840 | 18180 | 18094 | 14678 | 14686 | 19229 | 42036 | 42657 | 38776 | 38137 | 39499 | 44180 | 35228 | 42809 | 42299 | 12049 | 10953 | 14677 | 14508 | 4485 | 3582 | 3650 | 3586 | 3583 | 3546 | 3581 | 3597 |
| SW04_GVP_1 | 7a | MUF | 16423 | 16526 | 14994 | 16481 | 16574 | 16622 | 13483 | 13499 | 17446 | 34817 | 34421 | 33204 | 33383 | 33897 | 35228 | 35353 | 35162 | 35008 | 11420 | 10373 | 13516 | 13433 | 4229 | 3380 | 3448 | 3383 | 3381 | 3362 | 3383 | 3394 |
| TJ04_GVP_1 | 7a | MUF | 17812 | 18002 | 15892 | 17800 | 18054 | 18056 | 14575 | 14591 | 19086 | 41567 | 41844 | 38620 | 38113 | 39406 | 42809 | 35162 | 43067 | 41972 | 12023 | 10929 | 14628 | 14464 | 4466 | 3570 | 3638 | 3573 | 3571 | 3535 | 3569 | 3585 |
| TJ05_V8_1 | 7a | MUF | 17731 | 17911 | 15834 | 17739 | 17998 | 17968 | 14548 | 14557 | 19007 | 41160 | 41350 | 38344 | 37863 | 39093 | 42299 | 35008 | 41972 | 42497 | 12000 | 10908 | 14570 | 14421 | 4456 | 3558 | 3627 | 3562 | 3560 | 3530 | 3561 | 3575 |
| AC05 | 7a | UNI | 14103 | 14215 | 13109 | 14066 | 14152 | 14111 | 10165 | 10166 | 12685 | 19443 | 19447 | 19499 | 19096 | 19331 | 12049 | 11420 | 12023 | 12000 | 26703 | 22326 | 19674 | 19545 | 3320 | 2692 | 2737 | 2693 | 2690 | 2684 | 2693 | 2709 |
| AC07 | 7a | UNI | 12685 | 12808 | 11760 | 12696 | 12772 | 12729 | 9234 | 9240 | 11491 | 17546 | 17559 | 17553 | 17167 | 17386 | 10953 | 10373 | 10929 | 10908 | 22326 | 24782 | 17720 | 17592 | 3002 | 2387 | 2431 | 2388 | 2385 | 2379 | 2389 | 2399 |
| SW06_GVP_1 | 7a | UNI | 17400 | 17547 | 15450 | 17285 | 17544 | 17427 | 12212 | 12227 | 15338 | 25276 | 25655 | 25174 | 23998 | 24630 | 14677 | 13516 | 14628 | 14570 | 19674 | 17720 | 26195 | 25471 | 3826 | 3077 | 3123 | 3077 | 3075 | 3052 | 3080 | 3093 |
| TJ08_GVP_1 | 7a | UNI | 17201 | 17329 | 15337 | 17113 | 17356 | 17229 | 12095 | 12098 | 15164 | 24883 | 25186 | 24832 | 23750 | 24338 | 14508 | 13433 | 14464 | 14421 | 19545 | 17592 | 25471 | 25718 | 3803 | 3060 | 3106 | 3061 | 3058 | 3037 | 3061 | 3075 |
| SW15 | 7b | NIE | 4480 | 4531 | 4155 | 4479 | 4531 | 4538 | 4029 | 4032 | 4850 | 4790 | 4763 | 4705 | 4681 | 4710 | 4485 | 4229 | 4466 | 4456 | 3320 | 3002 | 3826 | 3803 | 44423 | 3382 | 3388 | 3383 | 3381 | 3355 | 3380 | 3383 |
| 10_009 | 7c | CIN | 3612 | 3635 | 3360 | 3606 | 3635 | 3640 | 3238 | 3238 | 3889 | 3809 | 3804 | 3758 | 3735 | 3749 | 3582 | 3380 | 3570 | 3558 | 2692 | 2387 | 3077 | 3060 | 3382 | 32863 | 32736 | 32509 | 32586 | 31477 | 32578 | 29691 |
| 11_022 | 7c | CIN | 3661 | 3686 | 3408 | 3657 | 3685 | 3689 | 3286 | 3285 | 3936 | 3878 | 3874 | 3827 | 3804 | 3818 | 3650 | 3448 | 3638 | 3627 | 2737 | 2431 | 3123 | 3106 | 3388 | 32736 | 33439 | 32864 | 32996 | 31650 | 32914 | 29911 |
| 11_024 | 7c | CIN | 3612 | 3636 | 3361 | 3606 | 3633 | 3640 | 3241 | 3241 | 3888 | 3813 | 3808 | 3762 | 3740 | 3753 | 3586 | 3383 | 3573 | 3562 | 2693 | 2388 | 3077 | 3061 | 3383 | 32509 | 32864 | 33051 | 32783 | 31482 | 32672 | 29793 |
| 14_003 | 7c | CIN | 3612 | 3636 | 3359 | 3606 | 3634 | 3640 | 3239 | 3238 | 3885 | 3810 | 3807 | 3758 | 3735 | 3749 | 3583 | 3381 | 3571 | 3560 | 2690 | 2385 | 3075 | 3058 | 3381 | 32586 | 32996 | 32783 | 33224 | 31484 | 32764 | 29825 |
| TJ88_GVP_1 | 7c | CIN | 3580 | 3601 | 3355 | 3578 | 3595 | 3612 | 3204 | 3204 | 3842 | 3772 | 3765 | 3728 | 3711 | 3721 | 3546 | 3362 | 3535 | 3530 | 2684 | 2379 | 3052 | 3037 | 3355 | 31477 | 31650 | 31482 | 31484 | 31710 | 31677 | 28855 |
| TJ89_GVP_1 | 7c | CIN | 3616 | 3640 | 3370 | 3611 | 3632 | 3646 | 3237 | 3238 | 3885 | 3810 | 3804 | 3762 | 3738 | 3757 | 3581 | 3383 | 3569 | 3561 | 2693 | 2389 | 3080 | 3061 | 3380 | 32578 | 32914 | 32672 | 32764 | 31677 | 33130 | 29730 |
| 14_061 | 7c | CIN | 3622 | 3646 | 3366 | 3614 | 3645 | 3643 | 3247 | 3246 | 3902 | 3824 | 3819 | 3772 | 3751 | 3762 | 3597 | 3394 | 3585 | 3575 | 2709 | 2399 | 3093 | 3075 | 3383 | 29691 | 29911 | 29793 | 29825 | 28855 | 29730 | 33334 |

Species in subclades b and c of clade 7 also have large numbers of loci (44000 for *P. niederhauserii* and 33000 for *P. cinnamomi*), which may also point to a hybridization event. In the case of *P. cinnamomi*, the genome size is around 400 Mbp, confirming this suspicion. However, the hybrid nature of these species, which may be the result of an ancient hybridization event, still needs to be confirmed. Within *P. cinnamomi*, the number of loci in common is large, except for one isolate (14/061).

Table XXIV contains the information on the percentage of common loci that have at least one identical allele in clade 7. It shows three groups within *P. cambivora*, which correspond to groups that could be distinguished based on the number of loci in common. Interestingly, isolate TJ70 had a significantly smaller number of loci than the other isolates, but the loci present seem to correspond very closely to those in the first group of *P. cambivora* isolates (05/006 and AC13). Within *P. alni/P. uniformis/P. multiformis* there is little observable diversity except for *P. cambivora* isolate AC03, which seems less related to the *P. uniformis* isolates in our collection than the other *P. cambivora* isolates. *P. cinnamomi* isolate 14/061, which was somewhat different from the other *P. cinnamomi* isolates based on the number of loci in common, also showed different from the other isolates based on the percentage of common loci that have at least one identical allele (84-85% instead of 99-100%).

Table XXV shows the matrix table with the number of loci in common of the isolates in clades 8 through 10. Overall the number of loci is not high and with one exception, there are no indications of hybridization. The exception is in clade 9 with isolate TJ19, which has a relatively large number of loci and is a hybrid with *P. hydropathica* as one of its parents. So even though it is labeled as *P. hydropathica*, this is not fully accurate. Of course, the number of isolates and species in these clades needs to be expanded in order to get a better idea of the true extent of hybridization events in these clades.

Table XXIV - Matrix table showing the percentage of common loci with at least one identical allele for a selection of isolates in Phytophthora **clade 7**, as based on GBS analysis. The species codes are listed in Table XVII. Further explanations in the text.

| | | | 05_006_GVP_1 | AC13 | TJ70_GVP_1 | 05_027_GVP_1 | AC12 | 10_010_GVP_1 | TJ37 | TJ38 | TJ61 | AC01 | AC03 | SW02_GVP_1 | TJ01_GVP_1 | TJ06_GVP_1 | AC04 | SW04_GVP_1 | TJ04_GVP_1 | TJ05_V8_1 | AC05 | AC07 | SW06_GVP_1 | TJ08_GVP_1 | SW15 | 10_009 | 11_022 | 11_024 | 14_003 | TJ88_GVP_1 | TJ89_GVP_1 | 14_061 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 7a | 7a | 7a | 7a | 7a | 7a | 7a | 7a | 7a | 7a | 7a | 7a | 7a | 7a | 7a | 7a | 7a | 7a | 7a | 7a | 7a | 7a | 7b | 7c | 7c | 7c | 7c | 7c | 7c | 7c |
| | | | CAM | CAM | CAM | CAM | CAM | CAM | HET | HET | INC | ALN | ALN | ALN | ALN | ALN | MUF | MUF | MUF | MUF | UNI | UNI | UNI | UNI | NIE | CIN | CIN | CIN | CIN | CIN | CIN | CIN |
| 05_006_G | 7a | CAM | 100 | 99 | 98 | 61 | 61 | 53 | 17 | 17 | 19 | 28 | 28 | 29 | 29 | 29 | 19 | 20 | 20 | 20 | 37 | 36 | 35 | 35 | 4 | 3 | 3 | 3 | 3 | 3 | 3 | 3 |
| AC13 | 7a | CAM | 99 | 100 | 99 | 62 | 61 | 53 | 17 | 17 | 19 | 28 | 28 | 29 | 29 | 29 | 20 | 21 | 20 | 20 | 37 | 36 | 35 | 35 | 4 | 3 | 3 | 3 | 3 | 3 | 3 | 3 |
| TJ70_GVP | 7a | CAM | 98 | 98 | 100 | 62 | 62 | 55 | 19 | 19 | 21 | 31 | 30 | 31 | 31 | 31 | 21 | 22 | 21 | 21 | 39 | 38 | 38 | 38 | 4 | 3 | 3 | 3 | 3 | 3 | 3 | 3 |
| 05_027_G | 7a | CAM | 62 | 62 | 63 | 100 | 99 | 53 | 18 | 18 | 19 | 29 | 28 | 29 | 30 | 29 | 20 | 21 | 20 | 20 | 38 | 36 | 36 | 36 | 4 | 3 | 3 | 3 | 3 | 3 | 3 | 3 |
| AC12 | 7a | CAM | 61 | 61 | 62 | 99 | 100 | 52 | 18 | 18 | 19 | 28 | 27 | 29 | 29 | 29 | 19 | 21 | 20 | 20 | 37 | 36 | 35 | 35 | 4 | 3 | 3 | 3 | 3 | 3 | 3 | 3 |
| 10_010_G | 7a | CAM | 53 | 53 | 54 | 53 | 52 | 100 | 18 | 18 | 19 | 28 | 27 | 29 | 29 | 28 | 19 | 20 | 19 | 19 | 37 | 36 | 35 | 35 | 4 | 3 | 3 | 3 | 3 | 3 | 3 | 3 |
| TJ37 | 7a | HET | 17 | 17 | 19 | 18 | 18 | 18 | 100 | 100 | 25 | 17 | 16 | 17 | 17 | 17 | 15 | 16 | 15 | 15 | 17 | 16 | 15 | 15 | 4 | 2 | 3 | 2 | 2 | 2 | 2 | 2 |
| TJ38 | 7a | HET | 17 | 17 | 19 | 18 | 18 | 18 | 100 | 100 | 25 | 17 | 16 | 17 | 17 | 17 | 15 | 16 | 15 | 15 | 17 | 16 | 15 | 15 | 4 | 2 | 3 | 2 | 2 | 2 | 2 | 2 |
| TJ61 | 7a | INC | 20 | 19 | 22 | 20 | 19 | 20 | 26 | 26 | 100 | 18 | 18 | 18 | 18 | 18 | 18 | 19 | 18 | 18 | 21 | 20 | 19 | 19 | 4 | 3 | 3 | 3 | 3 | 3 | 3 | 3 |
| AC01 | 7a | ALN | 29 | 29 | 31 | 29 | 29 | 28 | 17 | 17 | 17 | 100 | 99 | 99 | 99 | 99 | 98 | 98 | 98 | 98 | 95 | 95 | 96 | 95 | 4 | 3 | 4 | 3 | 3 | 3 | 3 | 3 |
| AC03 | 7a | ALN | 28 | 27 | 30 | 28 | 28 | 27 | 16 | 16 | 17 | 97 | 100 | 97 | 97 | 97 | 96 | 95 | 96 | 96 | 88 | 88 | 90 | 89 | 4 | 3 | 4 | 3 | 3 | 3 | 3 | 3 |
| SW02_GV | 7a | ALN | 29 | 29 | 31 | 30 | 29 | 29 | 17 | 17 | 18 | 98 | 98 | 100 | 98 | 98 | 96 | 96 | 96 | 96 | 96 | 96 | 97 | 96 | 4 | 3 | 4 | 3 | 3 | 3 | 3 | 3 |
| TJ01_GVP | 7a | ALN | 30 | 30 | 31 | 30 | 30 | 29 | 17 | 17 | 18 | 99 | 99 | 99 | 100 | 99 | 98 | 98 | 98 | 98 | 96 | 95 | 96 | 96 | 4 | 3 | 4 | 3 | 3 | 3 | 3 | 3 |
| TJ06_GVP | 7a | ALN | 29 | 29 | 31 | 29 | 29 | 29 | 17 | 17 | 18 | 98 | 98 | 98 | 98 | 100 | 98 | 97 | 97 | 97 | 95 | 94 | 95 | 95 | 4 | 3 | 4 | 3 | 3 | 3 | 3 | 3 |
| AC04 | 7a | MUF | 19 | 19 | 21 | 20 | 19 | 19 | 15 | 15 | 17 | 94 | 94 | 92 | 94 | 94 | 100 | 99 | 99 | 99 | 21 | 20 | 20 | 20 | 4 | 3 | 4 | 3 | 3 | 3 | 3 | 3 |
| SW04_GV | 7a | MUF | 20 | 20 | 21 | 21 | 20 | 20 | 16 | 16 | 18 | 93 | 93 | 91 | 93 | 92 | 99 | 100 | 99 | 99 | 21 | 21 | 20 | 20 | 4 | 3 | 4 | 3 | 3 | 3 | 3 | 3 |
| TJ04_GVP | 7a | MUF | 19 | 19 | 21 | 20 | 19 | 19 | 15 | 15 | 17 | 94 | 94 | 92 | 94 | 93 | 99 | 99 | 100 | 99 | 21 | 20 | 20 | 20 | 4 | 3 | 4 | 3 | 3 | 3 | 3 | 3 |
| TJ05_V8_ | 7a | MUF | 19 | 19 | 21 | 20 | 19 | 19 | 15 | 15 | 17 | 94 | 94 | 92 | 93 | 93 | 99 | 99 | 99 | 100 | 21 | 20 | 20 | 20 | 4 | 3 | 4 | 3 | 3 | 3 | 3 | 3 |
| AC05 | 7a | UNI | 37 | 37 | 39 | 38 | 37 | 37 | 17 | 17 | 21 | 94 | 88 | 95 | 95 | 94 | 21 | 21 | 21 | 21 | 100 | 100 | 100 | 99 | 4 | 2 | 3 | 2 | 2 | 2 | 2 | 3 |
| AC07 | 7a | UNI | 36 | 36 | 37 | 36 | 36 | 36 | 16 | 16 | 20 | 94 | 87 | 95 | 94 | 93 | 20 | 21 | 20 | 20 | 100 | 100 | 100 | 99 | 4 | 2 | 3 | 2 | 2 | 2 | 2 | 2 |
| SW06_GV | 7a | UNI | 35 | 35 | 38 | 36 | 35 | 35 | 15 | 15 | 19 | 95 | 89 | 96 | 96 | 95 | 19 | 20 | 20 | 20 | 100 | 100 | 100 | 99 | 4 | 2 | 3 | 2 | 2 | 2 | 2 | 2 |
| TJ08_GVP | 7a | UNI | 35 | 35 | 37 | 36 | 35 | 35 | 15 | 15 | 19 | 94 | 88 | 95 | 95 | 94 | 20 | 20 | 20 | 20 | 99 | 99 | 99 | 100 | 4 | 2 | 3 | 2 | 2 | 2 | 2 | 2 |
| SW15 | 7b | NIE | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 100 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| 10_009 | 7c | CIN | 3 | 3 | 3 | 3 | 3 | 3 | 2 | 2 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 2 | 2 | 2 | 2 | 5 | 100 | 99 | 99 | 99 | 99 | 99 | 84 |
| 11_022 | 7c | CIN | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 3 | 3 | 3 | 3 | 5 | 100 | 100 | 99 | 99 | 100 | 100 | 84 |
| 11_024 | 7c | CIN | 3 | 3 | 3 | 3 | 3 | 3 | 2 | 2 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 2 | 2 | 2 | 2 | 5 | 99 | 99 | 100 | 99 | 99 | 99 | 84 |
| 14_003 | 7c | CIN | 3 | 3 | 3 | 3 | 3 | 3 | 2 | 2 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 2 | 2 | 2 | 2 | 5 | 99 | 99 | 99 | 100 | 99 | 99 | 84 |
| TJ88_GVP | 7c | CIN | 3 | 3 | 3 | 3 | 3 | 3 | 2 | 2 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 2 | 2 | 2 | 2 | 5 | 99 | 99 | 99 | 99 | 100 | 99 | 85 |
| TJ89_GVP | 7c | CIN | 3 | 3 | 3 | 3 | 3 | 3 | 2 | 2 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 2 | 2 | 2 | 2 | 5 | 100 | 100 | 99 | 99 | 100 | 100 | 84 |
| 14_061 | 7c | CIN | 3 | 3 | 3 | 3 | 3 | 3 | 2 | 2 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 2 | 2 | 2 | 5 | 84 | 84 | 84 | 84 | 85 | 84 | 100 |

The isolate that was originally identified as *P. erythroseptica* (058/022) is here still listed as such but was probably misidentified given the number of loci it has in common with the isolates of *P. cryptogea*. So it should most likely be renamed to *P. cryptogea*.

It is also striking to see that *P. kernoviae* (clade 10) only has a bit over 14000 loci, the lowest in our collection. Its genome size is around 120 Mbp, which proportionally is not all that low and for example comparable to *P. ramorum*, which has approximately 135 Mbp nuclear content but around 22000 loci. Based on multi-gene phylogenies, clade 10 is ancestral to the other clades, which could help explain this relatively small number loci.

Table XXV - Matrix table showing the number of common loci of a selection of isolates in *Phytophthora* **clades 8 through 10** as based on GBS analysis. Abbreviations of the species names are listed in Table XVII. Sections are marked by color depending on the number of loci they have in common.

| | | | 12_016_GVP | 12_018 | 13_029_GVP | 14_011_GVP | 05_022_GVP | 13_001 | 02_001 | 11_038_GVP | 12_012_GVP | 13_024_GVP | 14_004_GVP | 04_005_V8 | TJ21 | TJ22 | TJ10_GVP_ | TJ18_GVP_ | TJ17_2 | TJ19_GVP_ | 10_022 | 12_006_2 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 8a | 8a | 8a | 8a | 8a | 8c | 8c | 8c | 8c | 8c | 8c | 8d | 8d | 8d | 9 | 9 | 9 | 9 | 10 | 10 |
| | | | CRY | CRY | CRY | CRY | ERY | LAT | RAM | RAM | RAM | RAM | RAM | SYR | SYR | SYR | HYD | HYD | HYD | HYD | KER | KER |
| 12_016_GVP_1 | 8a | CRY | 27935 | 27847 | 25935 | 26012 | 25422 | 1484 | 1604 | 1583 | 1598 | 1602 | 1581 | 1026 | 1024 | 1026 | 644 | 651 | 641 | 721 | 203 | 203 |
| 12_018 | 8a | CRY | 27847 | 30613 | 27435 | 27544 | 26860 | 1522 | 1638 | 1607 | 1624 | 1633 | 1603 | 1044 | 1044 | 1046 | 648 | 654 | 649 | 723 | 207 | 207 |
| 13_029_GVP_1 | 8a | CRY | 25935 | 27435 | 29129 | 28296 | 26359 | 1500 | 1616 | 1592 | 1609 | 1615 | 1589 | 1040 | 1039 | 1041 | 642 | 647 | 640 | 716 | 206 | 206 |
| 14_011_GVP_1 | 8a | CRY | 26012 | 27544 | 28296 | 29208 | 26381 | 1494 | 1617 | 1595 | 1610 | 1616 | 1591 | 1033 | 1032 | 1034 | 640 | 647 | 639 | 717 | 204 | 204 |
| 05_022_GVP_1 | 8a | ERY | 25422 | 26860 | 26359 | 26381 | 28905 | 1490 | 1604 | 1585 | 1599 | 1603 | 1582 | 1032 | 1030 | 1033 | 633 | 640 | 631 | 714 | 202 | 202 |
| 13_001 | 8c | LAT | 1484 | 1522 | 1500 | 1494 | 1490 | 23378 | 7752 | 7510 | 7579 | 7644 | 7453 | 1196 | 1191 | 1195 | 575 | 585 | 582 | 645 | 172 | 171 |
| 02_001 | 8c | RAM | 1604 | 1638 | 1616 | 1617 | 1604 | 7752 | 23618 | 22100 | 22416 | 22789 | 21835 | 1261 | 1262 | 1265 | 624 | 632 | 632 | 702 | 187 | 186 |
| 11_038_GVP_1 | 8c | RAM | 1583 | 1607 | 1592 | 1595 | 1585 | 7510 | 22100 | 22216 | 21717 | 21931 | 21281 | 1236 | 1237 | 1240 | 619 | 627 | 624 | 698 | 185 | 184 |
| 12_012_GVP_1 | 8c | RAM | 1598 | 1624 | 1609 | 1610 | 1599 | 7579 | 22416 | 21717 | 22557 | 22215 | 21481 | 1247 | 1248 | 1251 | 623 | 632 | 630 | 703 | 187 | 186 |
| 13_024_GVP_1 | 8c | RAM | 1602 | 1633 | 1615 | 1616 | 1603 | 7644 | 22789 | 21931 | 22215 | 22934 | 21680 | 1246 | 1246 | 1249 | 622 | 631 | 629 | 703 | 187 | 186 |
| 14_004_GVP_1 | 8c | RAM | 1581 | 1603 | 1589 | 1591 | 1582 | 7453 | 21835 | 21281 | 21481 | 21680 | 21931 | 1231 | 1232 | 1235 | 619 | 630 | 626 | 700 | 185 | 184 |
| 04_005_V8_1 | 8d | SYR | 1026 | 1044 | 1040 | 1033 | 1032 | 1196 | 1261 | 1236 | 1247 | 1246 | 1231 | 22757 | 20820 | 20819 | 534 | 541 | 539 | 585 | 165 | 164 |
| TJ21 | 3 | SYR | 1024 | 1044 | 1039 | 1032 | 1030 | 1191 | 1262 | 1237 | 1248 | 1246 | 1232 | 20820 | 22986 | 22882 | 539 | 545 | 544 | 588 | 169 | 168 |
| TJ22 | 3 | SYR | 1026 | 1046 | 1041 | 1034 | 1033 | 1195 | 1265 | 1240 | 1251 | 1249 | 1235 | 20819 | 22882 | 22960 | 539 | 545 | 544 | 588 | 169 | 168 |
| TJ10_GVP_1 | 9 | HYD | 644 | 648 | 642 | 640 | 633 | 575 | 624 | 619 | 623 | 622 | 619 | 534 | 539 | 539 | 29304 | 28171 | 26252 | 21900 | 240 | 239 |
| TJ18_GVP_1 | 9 | HYD | 651 | 654 | 647 | 647 | 640 | 585 | 632 | 627 | 632 | 631 | 630 | 541 | 545 | 545 | 28171 | 29969 | 27462 | 22355 | 242 | 241 |
| TJ17_2 | 9 | HYD | 641 | 649 | 640 | 639 | 631 | 582 | 632 | 624 | 630 | 629 | 626 | 539 | 544 | 544 | 26252 | 27462 | 28302 | 21638 | 239 | 238 |
| TJ19_GVP_1 | 9 | HYD | 721 | 723 | 716 | 717 | 714 | 645 | 702 | 698 | 703 | 703 | 700 | 585 | 588 | 588 | 21900 | 22355 | 21638 | 36013 | 270 | 269 |
| 10_022 | 10 | KER | 203 | 207 | 206 | 204 | 202 | 172 | 187 | 185 | 187 | 187 | 185 | 165 | 169 | 169 | 240 | 242 | 239 | 270 | 14162 | 14115 |
| 12_006_2 | 10 | KER | 203 | 207 | 206 | 204 | 202 | 171 | 186 | 184 | 186 | 186 | 184 | 164 | 168 | 168 | 239 | 241 | 238 | 269 | 14115 | 14211 |

Table XXVI contains the information on the percentage of common loci that have at least one identical allele for the isolates in clades 8 through 10. It shows that within *P. cryptogea* (including isolate 05/022, which should be renamed from *P. erythroseptica* to *P. cryptogea*), there were three groups. No diversity is observed within *P. ramorum*, which is not surprising given that the isolates were of the same lineage. Likely a single isolates of that lineage was introduced and went through a genetic bottleneck after the introduction into Europe. Within *P. syringae* there were two groups. Among the true *P. hydropathica* isolates, there was a bit of diversity. Interestingly, TJ18 has a large number of identical loci (98-99%) with both TJ10 and TJ17 but the latter two isolates only have 93% of identical loci.

Table XXVI - Matrix table showing the percentage of common loci with at least one identical allele for a selection of isolates in *Phytophthora* **clades 8 through 10**, as based on GBS analysis. The species codes are listed in Table XVII. Further explanations in the text.

| | | | 12_016_GVP_1 | 12_018 | 13_029_GVP_1 | 14_011_GVP_1 | 05_022_GVP_1 | 13_001 | 02_001 | 11_038_GVP_1 | 12_012_GVP_1 | 13_024_GVP_1 | 14_004_GVP_1 | 04_005_V8_1 | TJ21 | TJ22 | TJ10_GVP_1 | TJ18_GVP_1 | TJ17_2 | TJ19_GVP_1 | 10_022 | 12_006_2 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 8a | 8a | 8a | 8a | 8a | 8c | 8c | 8c | 8c | 8c | 8c | 8d | 8d | 8d | 9 | 9 | 9 | 9 | 10 | 10 |
| | | | CRY | CRY | CRY | CRY | ERY | LAT | RAM | RAM | RAM | RAM | RAM | SYR | SYR | SYR | HYD | HYD | HYD | HYD | KER | KER |
| 12_016_C | 8a | CRY | 100 | 100 | 86 | 87 | 82 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 0 |
| 12_018 | 8a | CRY | 100 | 100 | 86 | 86 | 81 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 0 |
| 13_029_C | 8a | CRY | 87 | 86 | 100 | 99 | 83 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 0 |
| 14_011_C | 8a | CRY | 87 | 86 | 99 | 100 | 83 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 0 |
| 05_022_C | 8a | ERY | 82 | 81 | 83 | 83 | 100 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 13_001 | 8c | LAT | 1 | 1 | 1 | 1 | 1 | 100 | 4 | 5 | 4 | 4 | 5 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 |
| 02_001 | 8c | RAM | 1 | 1 | 1 | 1 | 1 | 4 | 100 | 100 | 100 | 100 | 100 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 |
| 11_038_C | 8c | RAM | 1 | 1 | 1 | 1 | 1 | 5 | 100 | 100 | 100 | 100 | 100 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 |
| 12_012_C | 8c | RAM | 1 | 1 | 1 | 1 | 1 | 5 | 100 | 100 | 100 | 100 | 100 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 |
| 13_024_C | 8c | RAM | 1 | 1 | 1 | 1 | 1 | 4 | 100 | 100 | 100 | 100 | 100 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 |
| 14_004_C | 8c | RAM | 1 | 1 | 1 | 1 | 1 | 5 | 100 | 100 | 100 | 100 | 100 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 |
| 04_005_V | 8d | SYR | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 100 | 69 | 69 | 1 | 1 | 1 | 1 | 0 | 0 |
| TJ21 | 3 | SYR | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 69 | 100 | 100 | 1 | 1 | 1 | 1 | 0 | 0 |
| TJ22 | 3 | SYR | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 69 | 100 | 100 | 1 | 1 | 1 | 1 | 0 | 0 |
| TJ10_GVP | 9 | HYD | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 100 | 98 | 93 | 85 | 0 | 0 |
| TJ18_GVP | 9 | HYD | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 99 | 100 | 99 | 86 | 0 | 0 |
| TJ17_2 | 9 | HYD | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 93 | 99 | 100 | 85 | 0 | 0 |
| TJ19_GVP | 9 | HYD | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 85 | 86 | 86 | 100 | 0 | 0 |
| 10_022 | 10 | KER | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 100 | 100 |
| 12_006_2 | 10 | KER | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 100 | 100 |

Figure 25 shows the correlation between the number of loci in common and the genome size. The Pearson correlation coefficient was 0.85 and thus quite high, which together with the large number of samples resulted in a very low p-value of less than 0.00001. Although a very clear correlation was present, different parts can be distinguished in this graph. Lined in red are allopolyploid hybrids, which have large genome sizes and large numbers of loci, given that the genomes of their hybrid parents have simply been merged. Lined in green are some of the sexual hybrids, which have a relatively large number of loci but “regular” genome sizes.

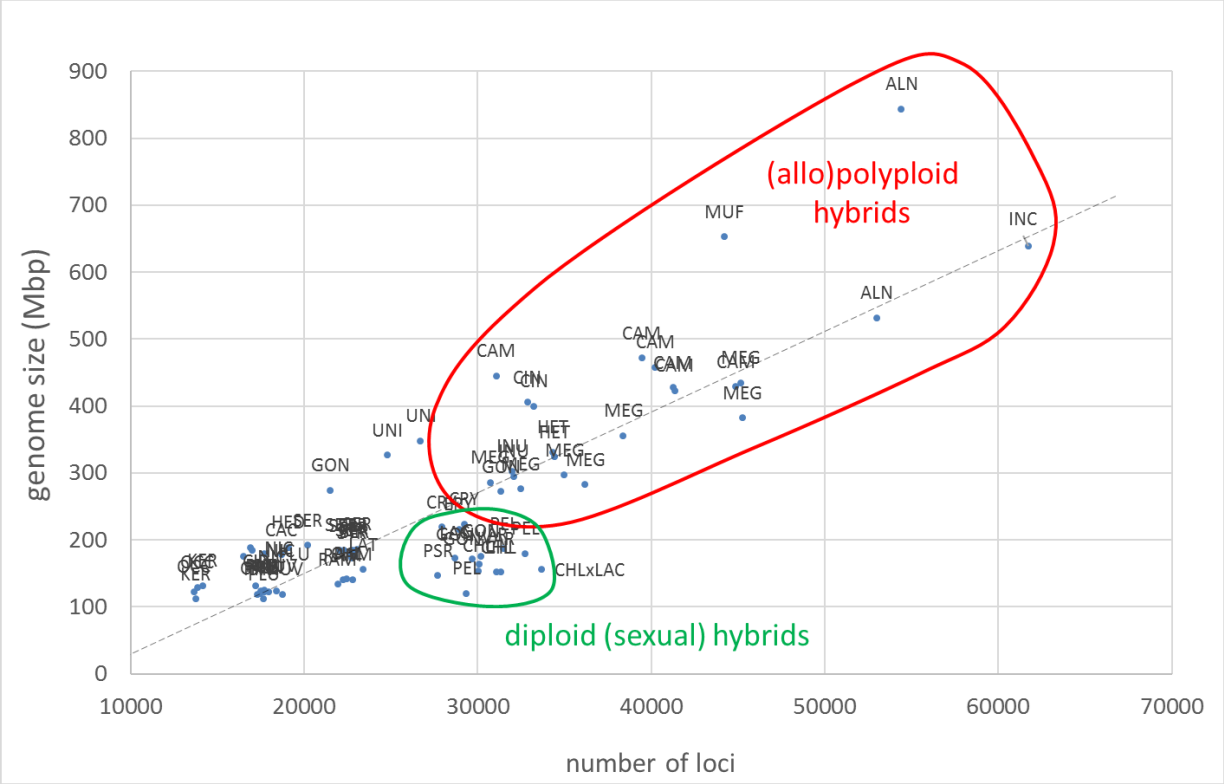



Figure 25 - Correlation between the number of loci in common (based on GBS), and the genome size (based on flow cytometry). The species codes are listed in Table XVII

On top of the number of common loci and the percentage of those that have at least one identical allele, we also calculated the percentage of loci for which there were more than 2 alleles present. The idea is that this measure will help detect allopolyploid hybrids as those hybrids receive the full two loci from each parent and therefore will have a relatively large percentage of loci with more than 2 alleles. Of course, some level of polymorphism is necessary within the parents in order to have more than two alleles, because if both parents are homozygous in a specific allele, the hybrid will not have more than two alleles. Diploid sexual hybrids will also not be detected by this measure. Figure 26 presents the results of this calculation, graphically expressed in combination with the number of loci in those isolates. Most isolates have 0 to 4% of their loci with more than 2 alleles. So even in non-hybrid species this event is not exceptional, and could be due to duplication of the gene within the diploid genome followed by independent mutations. It may also be due to the merging of similar but separate loci into the same locus in case there are insufficient differences between them. But although “regular” isolates also have loci with >2 alleles based on this technique, the allopolyploid hybrids were clearly detected as a separate group, with a percentage exceeding 6% and going to 15%. Interestingly, some species that we suspected to be hybrids, such as *P. cinnamomi, P. gonapodyides* and P. megasperma are confirmed here as likely allopolyploid hybrids.

Taken together, the GBS technique was quite reproducible and useful in the identification of hybrids. Large numbers of loci, large numbers of loci in common with other species, and >6% of loci with more than two alleles per locus all helped in the identification of hybrids. Data on the percentage of common loci with at least one identical allele helped in the further characterization of isolates, including the identification of parental subgroups that were related to the true parents of the hybrids.

The new techniques and especially GBS confirmed the hybrid nature of the following species: *P. serendipita, P. pelgrandis* and *P. andina* in clade 1, *P. chlamydospora* in clade 6, *P. cambivora, P. heterohybrida, P. incrassata, P. alni* and *P. multiformis* in clade 7. It also resulted in the identification of several new hybrid species or isolates. These include *P. inundata, P. lacustris, P. gonapodyides, P. megasperma, P. chlamydospora x lacustris* in clade 6, *P. cinnamomi* and *P. niederhauserii* in clade 7 and *P. hydropathica* in clade 9. Therefore we can conclude that is a powerful technique, fit for purpose.

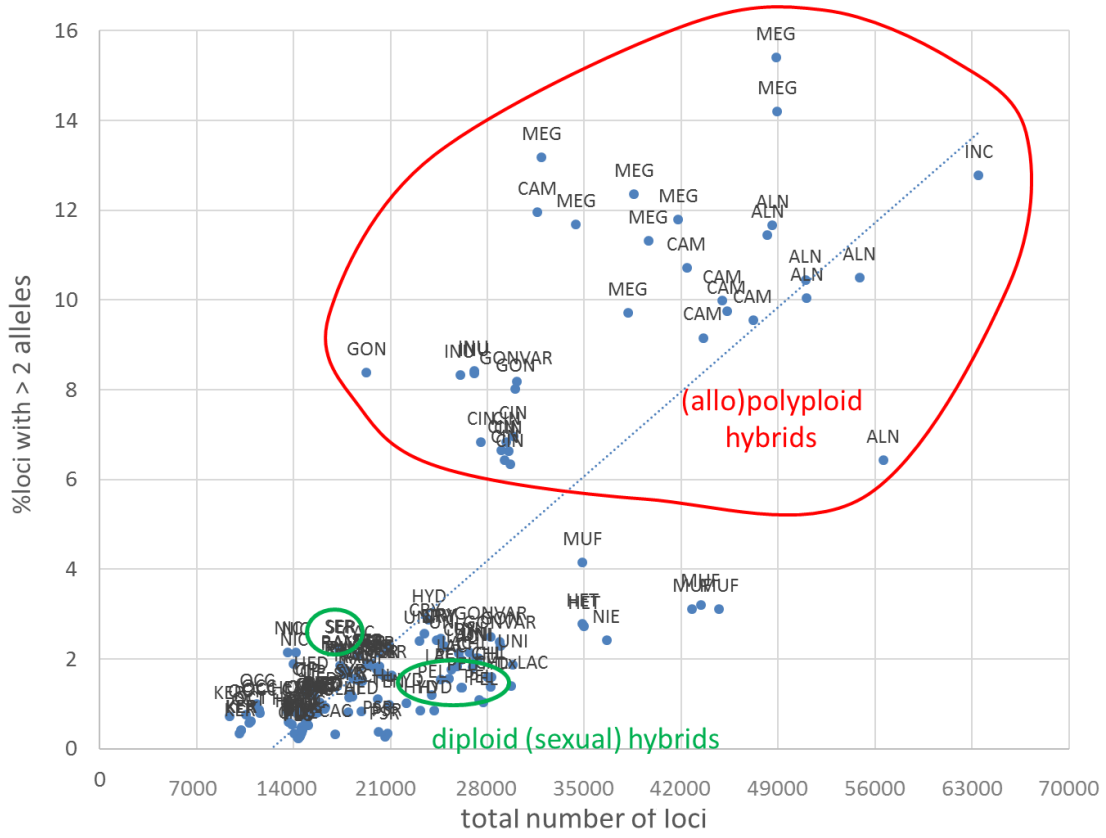



Figure 26 - Correlation between the number of loci (based on GBS), and percentage of loci with more than two alleles. The species codes are listed in Table XVII.

## 4.2. Detection of invasive fungi with an aerial dispersal

### 4.2.1. Efficiency of two types of rods used with Rotorod based on microscopy

In 2014, spores were collected with Rotorods in a douglas fir forest in Maissin (from 25 to 27 April, and from 8 May to 10 May), and in an oak forest in Bièvre (from 9 July to 11 July, and from 27 July to 29 July). The number of spores of less than 10 µm, and of more than 20 µm has been determined by microscopy. As shown in Figure 27, there were significant differences between both types of rods for small spores (P=0.022), with nails being more efficient than matches. In contrast, both types of rods were equivalent for the collection of large spores (P=0.888).

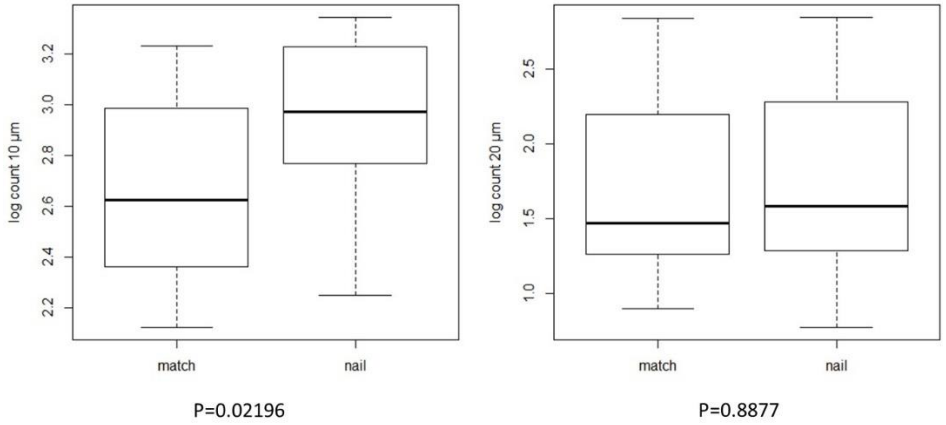



Figure 27 - Boxplot of the log count number of spores against the type of rod used in the Rotorod. On the left, spores <10 µm; on the right, spores > 20 µm.

### 4.2.2. Optimizations of DNA extraction protocols

Comparison of DNA extraction kits: As shown in Figure 28, the mean Ct values were slightly lower with the High Pure Template Preparation Kit for both types of spore traps (filters and nails), especially for the lowest concentration tested. The High Pure Template Preparation kit has therefore been selected for further tests.

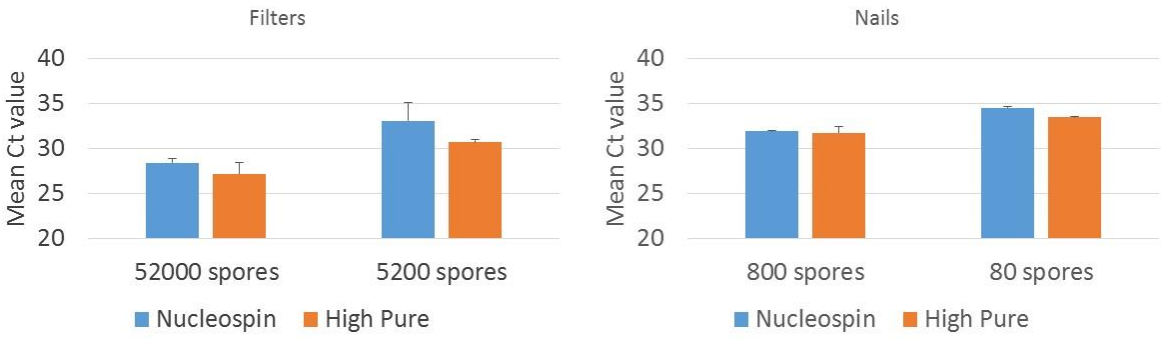



Figure 28 - Mean Ct value after real time PCR for the detection of *Erysiphe alphitoides* on DNA extracted with two commercial kits from filter or double-side tape (on nails) spiked with conidia of the pathogen.

Collection of spores from filter papers: As shown in Figure 29, the mean Ct values were slightly lower with the method “Garbelotto”. This method was therefore selected for further tests.

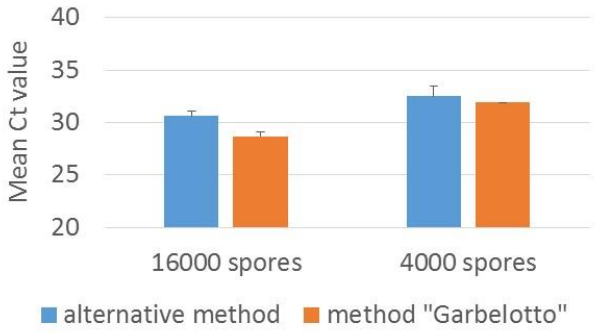



Figure 29 - Mean Ct value after real time PCR for the detection of *Erysiphe alphitoides* on DNA extracted from filter papers spiked with conidia of the pathogen and treated according to two methods for the recovery of spores.

Collection of spores from Burkard and Rotorod samplers (matches): The mean Ct values were slightly different between sampler (P=0.022) but not between the different treatments (P= 0.447). The vortex was preferred to the bead beater in order to make the protocol usable by a vast majority of labs (i.e. some diagnostic labs cannot afford a beat beater). The T4 treatment (vortex + zirconium beads) was selected as it provided the lowest variability between replicates.

DNA recovery: The DNA extraction efficiency has been evaluated by comparing the concentration of spores of *Erysiphe alphitoides* recovered after DNA extraction of known concentrations of spores in suspension or fixed on the spore trapping surface (tape on nail or match for Rotorods, Melinex+vaseline for Burkard sampler or filter Whatman No. 3). The results are presented in Tables XXVII-XXIX. The overall percent recovery was 100% for double sided tape put on nail or match. It was around 85% for the Burkard sampler, and around 70% for the filters.

The low recovery rate observed for the filter is probably due to the loss of some TE 4X added to the filter to recover the spores (~15 ml recovered from the 20 ml added to the Petri Dish due to the absorption of the liquid by the filter).

The results at ILVO were mostly consistent with the results at CRAW. 10000 spores were spiked on either Burkard tape, Rotorod/matches, Rotorod/nails, Whatman nr. 1 filters and Whatman nr. 3 filters. The average Cts (± stdev) were 24.0±0.8, 22.6±0.9, 27.1±1.7, 28.7±0.6, 30.9±0.6, respectively. So rotorod/matches were the best, with Burkard tape somewhat less and filters worst. Rotorod nails consistently did not do well at ILVO. An interaction between the type of nails used ILVO and the DNA extraction technique used at ILVO was suspected for the difference with CRAW. Because no simple solution for this was found, eventually, plastic rods were used, and these performed as well as the wooden rods: Cts (± stdev) were 29.2±1.4 for wooden rods and 29.4±0.5 for plastic rods when spiked with 1000 spores (n=4, p=0.76). Upon spiking, rods of 1.5 versus 2 mm width and ABS plastic versus PLA plastic did not produce significant results either. However, it could be that the capture efficiency of the thinner rods is higher than those of the wider rods, but this was not tested.

Table XXVII – Experiment 1. Percent recovery of spores from Rotorod (nails) and Burkard samplers.

| | Recovery (%) | | |
|---|---|---|---|
| Spore spiked | Spores suspension | Rotorod/ Nail (40 mm) | Burkard Melinex-Vaseline |
| 114 | 100,0 | 99,7 | 82,9 |
| 1140 | 100,0 | 108,0 | 85,6 |
| 2280 | 100,0 | 105,7 | 86,8 |
| Mean recovery | 100,0 | 104,5 | 85,1 |

Table XXVIII – Experiment 2. Percentage recovery of spores from Rotorod (matches)

| | Recovery (%) | |
|---|---|---|
| Spore spiked | Spores suspension | Match |
| 100 | 100,0 | 108,3 |
| 1000 | 100,0 | 98,1 |
| 1500 | 100,0 | 102,3 |
| Mean recovery | 100,0 | 102,9 |

Table XXIX- Experiment 3. Percentage recovery from filter (Whatman No.3)

| | Recovery (%) - test 1 | | | Recovery (%) - test 2 | |
|---|---|---|---|---|---|
| Spores spiked | Spores suspension | Filter (No.3) | Spore spiked | Spores suspension | Filter (No.3) |
| 35,0 | 100,0 | 72,9 | 100 | 100,0 | 67,9 |
| 350,0 | 100,0 | 63,6 | 1000 | 100,0 | 69,1 |
| 3500,0 | 100,0 | 65,7 | 10000 | 100,0 | 90,0 |
| Mean recovery | 100,0 | 67,4 | Mean recovery | 100,0 | 75,7 |

### 4.2.3. Analysis of forest samples by qPCR

In 2014, comparisons were made between 5 types of spore traps (Burkard, two types of RST differing by the width and the length of their rods, and two types of filter paper) for the collection of spores of *Erysiphe alphitoides* (large size spores), *Hymenoscyphus fraxineus* (medium size spores) and *Heterobasidion annosum* (small size spores) in a mixed forest (Floriffoux). Different experiments were conducted from June to September. As shown in Table XXX, clear differences (expressed in Ct values) were observed between devices and between pathogens. The Whatman filters were inefficient for the detection of *H. fraxineus* and *H. annosum* even at periods conducive to the release of spores of these pathogens. For the detection of *E. alphitoïdes*, filter No. 3 seemed slightly more efficient than filter No.1. The Burkard sampler was less efficient than the Rotorods over a two-day period of spore collection. The Rotorod devices detected the three pathogens and provided the lowest Ct values. The Rotorods using nails (thinner rods than Rotorods using matches) was more efficient for the collection of small spores. These results are in agreement with the analysis carried out by microscopy (see subsection 4.2.1).

The results were generally reproducible between two replicates for Rotorod devices and filter papers.

Table XXX - Ct values of real-time PCR for the detection of the 3 fungi from DNA extracted from 5 types of spore traps put in the **non-specific stand** (Floriffoux) in 2014 (collection period = 2 days). Two replicates per trapping system (R1 & R2). – indicates no amplification curve. Cells in grey indicate engine failure (with the Rotorods). Colour codes are linked to the type of trapping system.

| | *Erysiphe alphitoides* | | | | | | | | | *Hymenoscyphus fraxineus* | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | RST-N | | RST-M | | Filter 1 | | Filter 3 | | Bu | RST-N | | RST-M | | Filter 1 | | Filter 3 | | Bu |
| Date | R1 | R2 | R1 | R2 | R1 | R2 | R1 | R2 | R1 | R1 | R2 | R1 | R2 | R1 | R2 | R1 | R2 | R1 |
| 6 June | 29.7 | 30.4 | 30.9 | 29.7 | 34.8 | 34.3 | 36.0 | 34.7 | 36.3 | 31.9 | 33.9 | 31.7 | 31.7 | - | - | - | - | 37.7 |
| 18 June | 28.6 | | 30.5 | 34.2 | 37.2 | 37.7 | 36.7 | 36.3 | 33.7 | 32.7 | | 33.6 | 34.7 | - | - | 34.3 | - | - |
| 25 July | | 32.3 | 34.5 | | 36.5 | 35.9 | 34.3 | 38.1 | 34.5 | | 28.4 | 33.8 | | - | - | - | - | 33.7 |
| 1 August | 32.0 | 32.4 | 32.7 | 32.5 | 37.8 | - | 38.9 | - | 35.7 | 28.8 | 28.2 | 26.9 | 28.1 | - | 38.1 | - | - | 35.7 |
| 13 August | 33.4 | 34.7 | 35.2 | 36.0 | - | 37.2 | 36.6 | 36.7 | 36.6 | 26.1 | 26.9 | 27.3 | 27.2 | - | 36.4 | 36.9 | - | 29.9 |
| 30 August | - | 32.6 | 35.8 | 36.7 | 36.6 | 37.3 | 38.0 | 37.5 | - | 35.9 | 27.7 | - | - | - | - | 37.5 | - | |
| 8-sept | - | - | - | - | - | 37.0 | - | - | - | - | - | - | - | - | - | - | 37.8 | 37.0 |
| 15-sept | 38.2 | - | 38.0 | 36.8 | - | 39.7 | - | - | - | 38.0 | - | - | - | - | - | - | - | - |
| 26-sept | - | 37.7 | 36.6 | 36.6 | + | - | 38.2 | 38.5 | 38.7 | 36.1 | 33.2 | 35.8 | 34.3 | - | - | - | - | 37.0 |

| | *Heterobasidion annosum* | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | RST-N | | RST-M | | Filter 1 | | Filter 3 | | Bu |
| Date | R1 | R2 | R1 | R2 | R1 | R2 | R1 | R2 | R1 |
| 6 June | 35.1 | 35.7 | - | 34.2 | - | - | - | - | - |
| 18 June | 33.2 | | 34.4 | 35.3 | - | - | - | - | - |
| 25 July | | 33.1 | 37.1 | | - | - | - | - | - |
| 1 August | 33.2 | 31.9 | 35.4 | 34.3 | - | - | - | - | 38.4 |
| 13 August | 34.0 | 32.3 | - | 36.2 | - | - | - | - | 35.6 |
| 30 August | 34.8 | 32.8 | 35.4 | 34.5 | - | - | - | - | 35.4 |
| 8-sept | - | - | 35.3 | - | - | - | - | - | - |
| 15-sept | 34.9 | 38.0 | 34.8 | 33.9 | - | - | - | - | 34.2 |
| 26-sept | 34.9 | 33.9 | 36.0 | 35.6 | - | - | - | - | 38.9 |

Experiments were also conducted in specific stands with a high level of inoculum of the three fungi under study. With both types of Rotorods, the three pathogens were detected in their corresponding sites and at the different collection dates. The Ct values were lower than in the non-specific site, therefore confirming that the inoculum level was higher in the specific sites compared to the non-specific one. *Erysiphe alphitoides* and *H. fraxineus* were also detected on filters but the Ct values were often close to the end of the PCR run. In contrast, *H. annosum* was not detected on filters (Table XXXI).

Table XXXI - Ct values after real-time PCR for the detection of 3 fungi with 4 types of spore traps put in **specific forest stands** in 2014 (collection period=2 days). Two replicates per trapping system (R1 & R2). – indicates no fluorescence detection. Colour codes are linked to the type of trapping system.

| plantation/ pathogen | Collection date | RST-Nail 4 cm | | RST-Match | | Filter 1 | | Filter 3 | |
|---|---|---|---|---|---|---|---|---|---|
| | | R1 | R2 | R1 | R2 | R1 | R2 | R1 | R2 |
| Oak/*Erysiphe alphitoides* | 27 June | 26.6 | 28.4 | 28.5 | 29.5 | 37.9 | 35.1 | 35.3 | 33.4 |
| | 9 July | 31.7 | 31.3 | 31.9 | 33.0 | - | 37.8 | 31.8 | 33.0 |
| Ash/ *Hymenoscyphus fraxineus* | 25 July | 25.1 | 20.2 | 22.7 | 20.7 | 31.3 | 32.6 | 31.3 | 31.9 |
| | 7 August | 20.1 | 23.8 | 23.8 | 22.0 | 31.5 | 32.1 | 32.8 | 35.2 |
| Spruce/ *Heterobasidion annosum* | 25 April | 25.2 | 26.2 | 32.9 | 30.5 | - | 34.9 | - | 35.0 |
| | 8 May | 26.0 | 26.1 | 29.5 | 28.4 | - | - | - | - |
| | 4 September | 28.0 | 27.1 | 29.3 | 28.4 | - | - | - | - |
| | 17 September | 26.3 | 26.0 | 31.7 | 28.5 | 37.2 | - | 37.7 | - |
| | 6 October | 24.0 | 23.9 | 29.0 | 28.8 | - | - | - | - |
| | 15 October | 23.8 | 24.0 | 27.2 | 28.7 | - | - | - | - |

Filters were also installed in the forest for a 14-day period. The results, presented in Table XXXII, show that a longer period of spore collection does not improve the test sensitivity, especially for the detection of small spores (*H. annosum*).

Table XXXII - Ct value after real-time PCR for the detection of 3 fungi with 2 types of Whatman filters (No. 1 and 3) put in specific and non-specific forest stands in 2014 **during 14 days**. Two replicates per trapping system (R1 & R2). – indicates no fluorescence detection. Colour codes are linked to the type of trapping system.

| plantation/ pathogen | Stand | Collection date | Filter 1 | | Filter 3 | |
|---|---|---|---|---|---|---|
| | | | R1 | R2 | R1 | R2 |
| Oak/*Erysiphe alphitoides* | Bièvre | 25 June-9 July | - | 32.28 | 32.1 | 31.76 |
| | Floriffoux | 4 June-18 June | 34.56 | 34.81 | 34.9 | 35.47 |
| | | 10 July-24 July | 36.39 | - | 34.8 | 36.76 |
| Ash/ *Hymenoscyphu* | Morialmé | 23 July-7 August | 32.9 | 31.8 | 31.7 | 33.87 |
| | Floriffoux | 10 July - 24 July | - | - | 37.7 | - |
| | | 30 July - 13 August | 37.75 | - | - | - |
| | | 1 Sept. - 15 Sept. | | | | |
| Spruce/ *Heterobasidion annosum* | Maissin | 23 April-8 May | - | - | - | 36.46 |
| | | 2 Sept. - 17 Sept. | 36.71 | - | - | - |
| | | 3 Oct. - 15 Oct. | - | - | - | - |
| | Floriffoux | 15 April - 30 April | - | - | - | - |
| | | 13 May - 28 May | - | - | - | - |
| | | 4 June - 18 June | 31.86 | 34.38 | - | - |
| | | 10 July - 24 July | - | - | - | - |
| | | 30 July - 13 August | - | - | - | - |
| | | 1 Sept. - 15 Sept. | - | - | - | - |
| | | 24 Sept. - 8 Oct. | - | - | - | - |
| | | 22 Oct. - 5 Nov. | - | - | - | - |

In 2015, experiments were repeated in the non-specific forest stand (Floriffoux), from July to September, considering one of the Rotorods tested in 2014 (type 1=nails of 4 cm in length), a “new” Rotorod (type 2=nails of 2.5 cm in length, same width as Rotorod type 1) and filter Whatman No. 3. As shown in Table XXXIII, filters were again inefficient for the collection of small spores (*H. annosum*) while the three fungal pathogens were detected with both types of Rotorods. There were no clear difference between both types of Rotorods.

Table XXXIII – Ct values after real-time PCR for the detection of 3 fungi with 2 types of Rotorods (with nails of 20 mm length and nails of 40 mm length) and Whatman filter No. 3 put in Floriffoux (non-specific forest stand) in 2015 **(spore collection period = 2 days)**. Two replicates per trapping system (R1 & R2). – indicates no fluorescence detection. Colour codes are linked to the type of trapping system.

| | *Erysiphe alphitoides* | | | | | | *Hymenoscyphus fraxineus* | | | | | | *Heterobasidion annosum* | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | RST-N4cm | | RST-N2cm | | Filter No.3 | | RST-N4cm | | RST-N2cm | | Filter 3 | | RST-N4cm | | RST-N2cm | | Filter No.3 | |
| Date | R1 | R2 | R1 | R2 | R1 | R2 | R1 | R2 | R1 | R2 | R1 | R2 | R1 | R2 | R1 | R2 | R1 | R2 |
| 10 July | 34.9 | - | 34.9 | 34.6 | 35.1 | 34.6 | 35.5 | 34.8 | 32.4 | 30.9 | - | - | - | 38.6 | 37.7 | 36.7 | - | - |
| 17 July | 33.7 | 33.6 | 33.7 | 32.9 | 34.5 | 34.1 | 28.6 | 28.4 | 29.7 | 28.8 | - | - | 35.6 | 35.7 | 36.1 | 34.5 | - | - |
| 24 July | 33.0 | - | 33.0 | 32.2 | 36.3 | 36.4 | 26.9 | 28.2 | 26.5 | 27.0 | - | - | 34.2 | 37.1 | 34.8 | 35.5 | - | - |
| 31 July | 35.0 | 34.6 | 35.0 | 35.2 | 38.0 | 37.3 | 24.9 | 25.7 | 28.2 | 25.9 | 35.8 | 35.8 | 33.6 | 33.7 | - | 35.0 | - | - |
| 7 August | | 34.8 | 35.8 | 36.0 | 37.1 | 36.5 | | 26.1 | 29.4 | 29.1 | 35.3 | 34.1 | | 33.9 | 34.9 | 35.01 | - | - |
| 13 August | - | - | - | - | 36.9 | 35.8 | - | - | - | - | 38.5 | 36.8 | - | - | - | - | - | - |
| 28 August | 37.4 | 35.6 | 37.4 | 37.1 | - | 38.2 | 29.8 | 29.1 | 29.6 | 29.3 | - | 36.8 | 33.6 | 33.4 | 33.8 | 33.8 | - | - |
| 4 Sept. | 38.9 | 37.3 | 38.9 | 37.6 | - | - | 28.7 | 29.2 | 29.5 | 29.7 | 37.0 | - | 33.1 | 32.8 | 34.4 | 33.9 | - | - |
| 11 Sept. | 38.4 | 38.2 | 38.4 | 36.0 | 34.7 | 34.1 | 36.5 | 32.2 | 33.1 | 31.4 | - | - | 33.6 | 35.9 | 35.8 | 34.5 | - | - |
| 25 Sept. | 37.7 | - | 37.8 | 38.9 | - | - | - | 35.1 | - | 33.3 | - | - | 33.6 | 35.0 | 36.8 | 33.8 | - | - |

At the nursery site, 6 weeks of sampling during 2014 resulted in 18 filters, 64 RST samples, and 84 one-day-equivalent samples from the Burkard tapes. DNA extraction was performed and the DNA samples were frozen until qPCR analysis. qPCR analysis was started with *Fusarium oxysporum* and the first week of samples. The results are shown in Table XXXIV.

Table XXXIV. Comparison of detection of *Fusarium oxysporum* spores using either Burkard, RST (with wooden rods) and Whatman filters as sampling instruments, in combination with DNA extraction and qPCR. Average±stdev of two samplers for each type. Data are from the first week of sampling at the PCS. Results are expressed as spore equivalents based on a standard curve relating fg of DNA to number of spores. Whatman filters were exposed for one week, while the other samplers were sampling for 1 tot 3 days.

| period | Burkard tape | Rotorod (matches) | Whatman no.1 | Whatman no.3 |
|---|---|---|---|---|
| 28-29/07/2014 | 5.9±8.4 | 67.8±50.8 | | |
| 30-31/07/2014 | 5.5±7.8 | 120.4±29.4 | | |
| 1-3/08/2014 | 48.1±48.0 | 60.2±3.4 | | |
| 28/7-3/8/2014 | | | 0 | 0 |

No detection was made on Whatman filters. Detection was highest with the rotorod samplers. This can not be explained by the DNA extraction efficiency, as the Ct is translated to spore equivalents based on standard curves established from spiked samples. The rotorod samples from the remaining 5 weeks were analysed first. They showed that for most sampling times, the number of spore equivalents per day was less than 10. As the detection of spores with the Burkard was considerably lower during the first week (sometimes only 5 to 10 percent), it made no sense to still process those samples. However, the data confirmed the CRA results that the rotorod samplers have the highest collection efficiency. Based on the comparison carried out in 2014 and 2015 by CRAW, rotorods with nails were the most promising system for the purpose of the study (table XXXV).

Table XXXV. Summary of the experiences with the different type of samplers.

| Type of trapping system | Collection efficiency (2-day period) | | Cost | Ease of installation | DNA recovery |
|---|---|---|---|---|---|
| | small spores | large spores | | | |
| Rotorod with nails 4 cm | +++ | +++ | ++ | ++ | +++ |
| Rotorod with nails 2 cm | +++ | +++ | ++ | ++ | +++ |
| Rotorod with matches | ++ | +++ | ++ | ++ | +++ |
| Burkard sampler | ++ | ++ | - | - | ++ |
| Filter No. 1 | - | + | +++ | +++ | not tested |
| Filter No. 3 | - | + | +++ | +++ | + |

## 4.2.4. High throughput sequencing runs

We carried out 2 NGS runs. In the first run, there were 16 forest samples collected in 2014 from Rotorod with nails, and 14 mock communities. These samples were analyzed with 3 primer sets. In the second NGS run, there were 14 forest samples collected in 2014 (from Rotorod with nails 40 mm) and 22 forest samples collected in 2015 (11 from Rotorods with nails 25 mm, and 11 from Rotorods with nails 40 mm). There were also eight of the 14 mock communities of fungi already analyzed in run 1 (to evaluate the reproducibility of the method). The samples of run 2 were analyzed with the best PCR primers identified in run 1 (primer sets 1 & 2 developed by Toju et al. 2012). In addition to these samples, negative controls were also introduced in the NGS runs 1 and 2 in order to evaluate the procedure set up in the laboratory to control the cross contaminations due to the use of high coverage ITS primers for NGS analysis. These controls were made of sterile water following all the steps of the ITS workflow.

### 4.2.4.1. Quality of the raw data

De-multiplexed Illumina fastq files (96 files R1 and 96 files R2 per run) from which the Illumina barcodes had already been removed from the reads were received from the provider (DNA Vision). The fastq files were merged into two fasta files, one for R1 and one for R2. The quality of the raw sequences in each merged fasta file (R1 and R2) was evaluated with the FASTQC program (see subsection 4.1.4.2 for details on the program). As shown in Figure 30, the read quality of all batches processed was good.

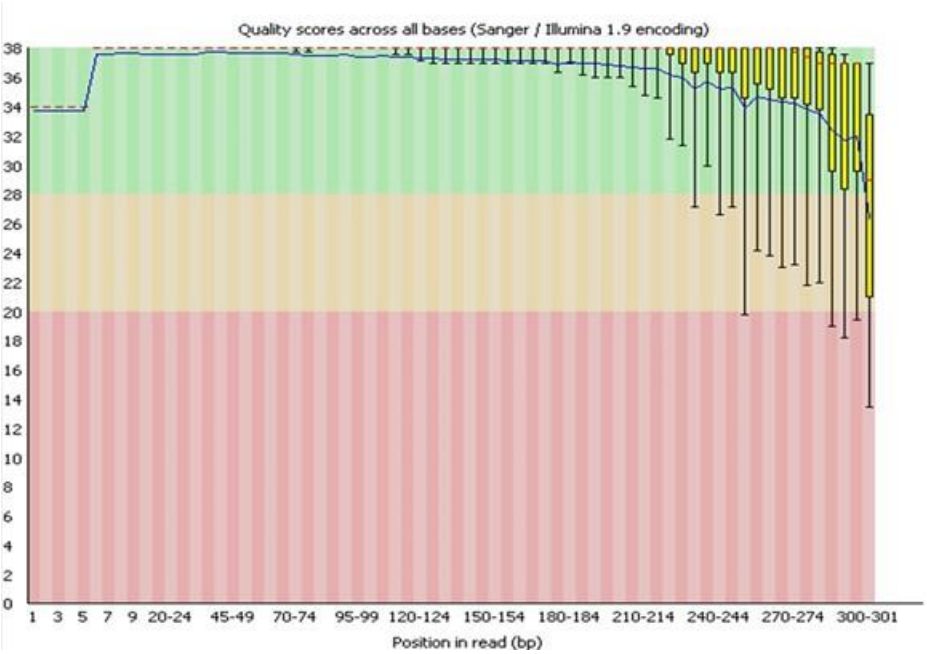



Figure 30 - Result of FASTQC analysis for the forward reads of NGS run 1. Red dashes (within yellow bars) = median, blue line = average, yellow boxes = interquartile range (25% to 75%), error bars = (10 to) 90% range.

### 4.2.4.2. Control of contaminations

As shown in Table XXXVI, the number of reads in the negative controls ranged from 12 to 24 in run 1 (total=118), and from 35 to 75 in run 2 (total=432), these amounts being negligible compared to the total number of reads in each run. The risk of false positive results linked to cross contaminations is therefore very limited.

Table XXXIVI – Number of reads for the two NGS runs, and for the different categories of samples.

| | Forest 2014 | | Forest 2015 | | Mock Communities | | Negative controls | | Total samples | |
|---|---|---|---|---|---|---|---|---|---|---|
| | samples | counts | samples | counts | samples | counts | samples | counts | samples | counts |
| Run 1 | 48 | 1581747 | 0 | 0 | 42 | 1590908 | 6 | 118 | 96 | 3172773 |
| Run 2 | 28 | 1640205 | 44 | 2538251 | 16 | 1083160 | 8 | 432 | 96 | 5262048 |

### 4.2.4.3. Validation of the bioinformatic analysis on mock communities of fungi

- **Specificity - Taxonomic assignment**

With the method based on the Qiime pipeline with the reference ITS database Unite 97, one species of the mock community (*Trametes versicolor*) was correctly assigned at the species level with the three primer pairs. For the other ones, the correct assignment was at the class level with primer sets targeting the ITS1 (Table XXXVII). One species (*Phaeocryptopus gaeumanii*) was assigned as “unidentified fungi” with primer set 2.

Table XXXVII - Percentage of correct assignments at the different taxonomic levels using the bio-informatic method Qiime and the database UNITE 97 on a mock community of 10 fungal species

| Primer set | Division | Class | Order | Family | Genus | Species |
|---|---|---|---|---|---|---|
| ITS1F_KYO2/ITS2_KYO2 (set 1 -ITS1) | 100% | 100% | 70% | 70% | 60% | 10% |
| ITS3_KYO2/ITS4 (set 2- ITS2) | 90% | 90% | 90% | 70% | 60% | 10% |
| ITS1F/ITS2 (set 3- ITS1) | 100% | 100% | 80% | 70% | 40% | 10% |

As the analysis did not assign the sequences of the fungi constituting the mock community at the species level (which is necessary for the objective of the project (i.e. detection of exotic or regulated fungal species with aerial dispersal)), a new method has been developed at CRAW. This method can be used on personal computer (no need of specific equipment) which is an advantage if the protocol is to be proposed as a reference method for diagnostic labs. It is also very rapid (analysis of 96 samples- one run - within one working day). A new reference fungal database adapted to the purpose of the study has also been created.

With the new pipeline/reference ITS database, most of the species constituting the mock community were correctly assigned at the species level (9/10 species with primer pairs 1 and 3, and 8/10 species with primer set 2) (Table XXXVIII). The species *Eutypa lata* (reference strain, CBS) was assigned as *Diatrypella atlantica* with primer pairs 1 and 3, and as *Eutypa*

*caricae* with primer set 2. Sanger sequencing with primers ITS2-ITS5 (targeting the ITS1 region of fungi) confirmed the assignment to the species *Diatrypella atlantica*, suggesting that the problem encountered with the identification of this fungus is not linked to the bio-informatic method used. The other fungus for which the method failed to correctly assign the sequences to the species level is *Hymenoscyphus fraxineus* with primer set 2. In this case, the method did not succeed in distinguish *Hymenosyphus fraxineus* and *H. albidus*, those species being very close to each other at the genetic level.

Table XXXVI - Percentage of correct assignment at the different taxonomic levels using the method developed at CRAW (new pipeline and new reference ITS database) on a mock community of 10 fungal species

| Primer set | Division | Class | Order | Family | Genus | Species |
|---|---|---|---|---|---|---|
| ITS1F_KYO2/ITS2_KYO2 (set 1 -ITS1) | 100% | 100% | 100% | 100% | 90% | 90% |
| ITS3_KYO2/ITS4 (set 2- ITS2) | 100% | 100% | 100% | 100% | 90% | 80% |
| ITS1F/ITS2 (set 3- ITS1) | 100% | 100% | 100% | 100% | 90% | 90% |

- **Performance of the high throughput sequencing method**

In order to evaluate the limit of detection of the NGS method, different concentrations of DNA (from 100 pg to 1 pg/reaction) were tested for each fungal species of the mock community and each primer set. As shown in Table XXXIX, the number of contigs decreased with the DNA concentration for each species considered in this study. However, it was highly variable between the different species for a determined concentration of DNA. At 100 pg/PCR, all the fungi were detected with the three primer sets. At 10 ng/PCR, *Hymenoscyphus fraxineus* was not detected with primers targeting the ITS1. This results could be due to the size of the amplification product generated with primers set 1 (593 bp) and 3 (596 bp) which is not optimal for the Illumina Miseq platform (~250-350 bp). However, the test carried out with primer set 2 did not solve completely the problem suggesting that other parameters should be involved. The number of contigs generated with primer set 3 was lower than with the two other primer sets.

Table XXXVIIX – Mean number of contigs for the 10 fungal species of the mock community, the three sets of primers and the three DNA concentrations used in the first PCR of the NGS protocol (analysis of the data with the bioinformatics method developed at CRAW).

| | Primer Set 1 | | | Primer Set 2 | | | Primer Set 3 | | |
|---|---|---|---|---|---|---|---|---|---|
| Species | 100 pg | 10 pg | 1 pg | 100 pg | 10 pg | 1 pg | 100 pg | 10 pg | 1 pg |
| *Armillaria gallica* | 4414 | 427 | 5 | 1185 | 143 | 5 | 2238 | 114 | 13 |
| *Diatrypella atlantica*/ Eutypa caricae*** | 1341 | 107 | 3 | 1231 | 78 | 2 | 100 | 7 | 3 |
| *Fusarium lateritium* | 6728 | 1094 | 72 | 3582 | 853 | 43 | 1052 | 82 | 3 |
| *Ganoderma adpsersum* | 5601 | 610 | 47 | 2326 | 561 | 43 | 453 | 18 | 5 |
| *Heterobasidion annosum* | 5532 | 649 | 45 | 2167 | 283 | 11 | 430 | 33 | 6 |
| *Hymenoscyphus fraxineus* | 40 | 0 | 0 | 151 | 16 | 0 | 70 | 1 | 0 |
| *Ophiostoma novo-ulmi* | 6091 | 535 | 46 | 1593 | 205 | 26 | 618 | 22 | 5 |
| *Phaeocryptopus gaeumanii* | 2983 | 395 | 41 | 2788 | 178 | 15 | 323 | 39 | 3 |
| *Trametes versicolor* | 5795 | 777 | 25 | 3213 | 777 | 61 | 463 | 28 | 4 |
| *Verticillium dahliae* | 8871 | 121 | 3 | 4574 | 1481 | 132 | 609 | 56 | 4 |

*With primer sets 1 and 3; **: with primer set 2

The number of contigs produced in each replicate of the mock community 1 (100 pg DNA/ PCR) has been compared for the three primer sets. The determination coefficient ranged from 0.986 (for set 1) to 0.997 (for set 2), therefore demonstrating that the method was repeatable.

#### 4.2.4.4. Analysis of forest samples by high throughput sequencing

Forest samples from NGS run 1 were analysed with the new bio-informatic method developed at CRAW (analysis of run 2 is still ongoing). The proportion of contigs representing the fungal species under study in the total number of contigs has been calculated for each sample. The results for the three primer sets are presented in Table XXXX.

With all primer sets, the three fungal species were detected in the specific stands (with high level of inoculum). In contrast, in Floriffoux (mixed forest with the three fungal pathogens in low concentration), only *Erysiphe alphitoides* was detected in both replicates and at the dates of spore releases (May and June). In contrast, the proportion of contigs correspondig to *Hymenoscyphus fraxineus* and *Heterobasidion annosum* was close to 0 while these pathogens were easily detected by real-time PCR from the same DNA samples (see subsection 4.2.3, Table XXX).

These results demonstrate that the NGS method (non-specific method) in the version that we have used is less sensitive than the real time PCR method (specific method) for the detection of airborne inoculum of fungi. Therefore, optimisations are still required to make the NGS method usable for the detection of emergent pathogens. Some parameters of the

bioinformatic pipeline could be modified. However, it seems also needed to optimize the first PCR and/or the second PCR of the Illumina workflow.

Table XXXVIII - NGS protocol with the three primer sets on DNA extracted from Rotorods put in different forest stands in 2014 (run 1). R1/R2= spore trap replicates.

| | | | Primer set 1 | | | Primer set 2 | | | Primer set 3 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Target | | | Target | | | Target | |
| Forest stand | Collection date | Replicate | Total contigs | Species | % total contigs | Total contigs | Species | % total contigs | Total contigs | Species | % total contigs |
| Maissin | 6 october 2014 | R1 | 26819 | *H. annosum* | 49,8 | 14983 | *H. annosum* | 34,0 | 6692 | *H. annosum* | 60,7 |
| | 6 october 2014 | R2 | 32402 | *H. annosum* | 52,0 | 18802 | *H. annosum* | 24,6 | 7544 | *H. annosum* | 60,5 |
| Bièvre | 27 June 2014 | R1 | 2037 | *E. alphitoides* | 22,2 | 14113 | *E. alphitoides* | 15,3 | 793 | *E. alphitoides* | 20,6 |
| | 27 June 2014 | R2 | 2382 | *E. alphitoides* | 21,8 | 8402 | *E. alphitoides* | 10,9 | 1734 | *E. alphitoides* | 23,1 |
| Morialmé | 7 August 2014 | R1 | 20935 | *H. fraxineus* | 1,0 | 14655 | *H. fraxineus* | 2,4 | 4578 | *H. fraxineus* | 1,0 |
| | 7 August 2014 | R2 | 27035 | *H. fraxineus* | 1,2 | 13786 | *H. fraxineus* | 2,3 | 4916 | *H. fraxineus* | 1,0 |
| Carsbourg | 22 May 2014 | R1 | 13808 | *P. gaeumanii* | 1,4 | 7943 | *P. gaeumanii* | 0,9 | 1961 | *P. gaeumanii* | 0,6 |
| | 22 May 2014 | R2 | 6978 | *P. gaeumanii* | 1,6 | 8095 | *P. gaeumanii* | 1,0 | 1381 | *P. gaeumanii* | 0,1 |
| Floriffoux | 28 May 2014 | R1 | 5932 | *E. alphitoides* | 0,6 | 11477 | *E. alphitoides* | 0,2 | 3009 | *E. alphitoides* | 0,2 |
| | 28 May 2014 | R2 | 8692 | *E. alphitoides* | 0,3 | 14953 | *E. alphitoides* | 0,1 | 3952 | *E. alphitoides* | 0,4 |
| Floriffoux | 18 June 2014 | R1 | 6361 | *E. alphitoides* | 5,1 | 20195 | *E. alphitoides* | 2,9 | 7204 | *E. alphitoides* | 4,9 |
| | 18 June 2014 | R2 | 6988 | *E. alphitoides* | 1,2 | 21093 | *E. alphitoides* | 0,3 | 3723 | *E. alphitoides* | 0,4 |
| Floriffoux | 13 August 2014 | R1 | 15201 | *H. fraxineus* | 0,0 | 14213 | *H. fraxineus* | 0,1 | 6002 | *H. fraxineus* | 0,0 |
| | 13 August 2014 | R2 | 14274 | *H. fraxineus* | 0,0 | 17815 | *H. fraxineus* | 0,1 | 5507 | *H. fraxineus* | 0,2 |
| Floriffoux | 8 October 2014 | R1 | 8504 | *H. annosum* | 0,2 | 15188 | *H. annosum* | 0,1 | 4802 | *H. annosum* | 0,0 |
| | 8 October 2014 | R2 | 10237 | *H. annosum* | 0,0 | 16039 | *H. annosum* | 0,1 | 4274 | *H. annosum* | 0,2 |

In contrast to the real-time PCR, the NGS method can detect numerous fungal species present in the same DNA sample. In all forest samples analysed, the number of fungal species detected in one sample ranged from 22 to 154 species. Figures 31-33 illustrate the diversity of fungal species collected on Rotorods in three forest stands in 2014. The proportion of contigs of the genus *Cladosporium* was generally important in all samples. Interestingly, some pathogenic species not detected on trees in the corresponding stand were detected with the NGS method. As an example, in Floriffoux, we detected the species *Gymnopus fusipes* involved in the decline of oak in Europe (see figure 33) while we have never seen fruiting bodies of this fungus in this stand.

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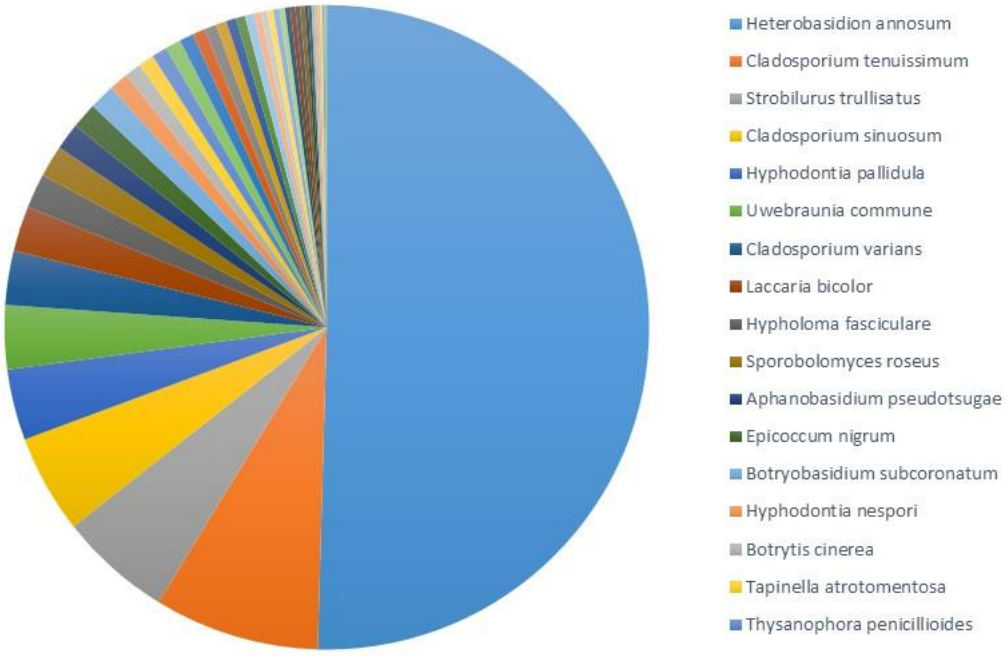


Figure 31 - Distribution of fungal species identified with primer set 1 in Maissin (spore trapping from 6 to 8 October 2014, Douglas fir plantation). Cutoff at 0.1% of the total number of contigs.

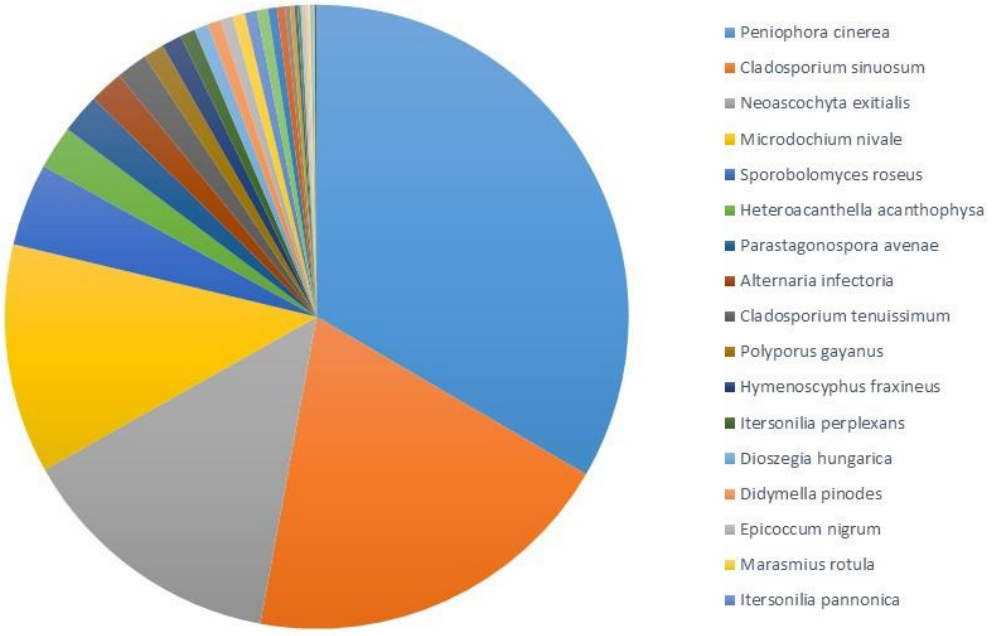


Figure 32 - Distribution of fungal species identified with primer set 1 in Morialme (spore trapping from 5 to 7 August 2014, ash plantation). Cutoff at 0.1% of the total number of contigs.

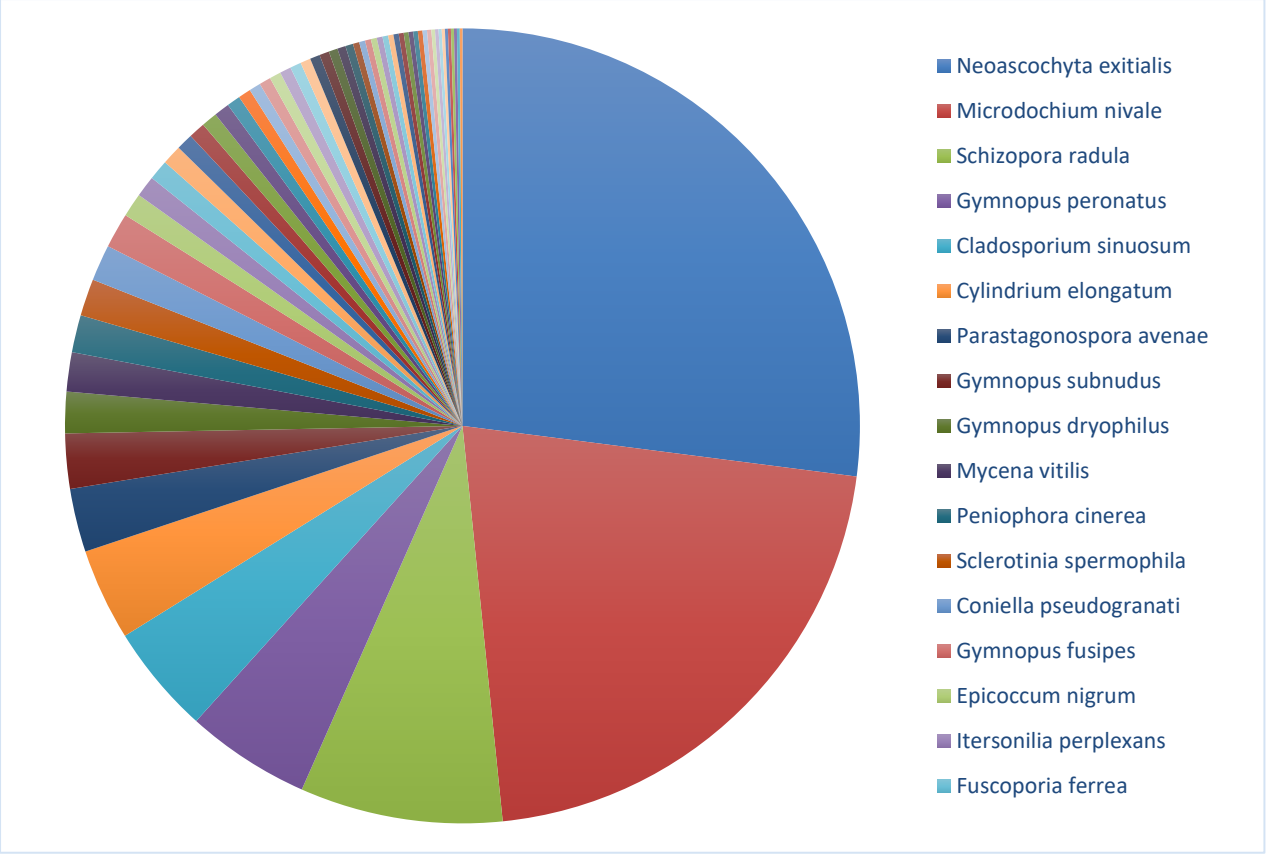


Figure 33 - Distribution of fungal species identified with primer set 1 in Floriffoux (spore trapping from 5 to 7 August 2014, mixed forest). Cutoff at 0.1% of the total number of contigs.

Rarefaction curves were constructed showing the rarefied number of species defined at a 98.7% sequence similarity threshold for the three primer sets (Figure 34). These results indicate that a higher number of species is detected with the primer set 2. The lowest species number was observed for the primer set 3 (ITS1F/ITS2).

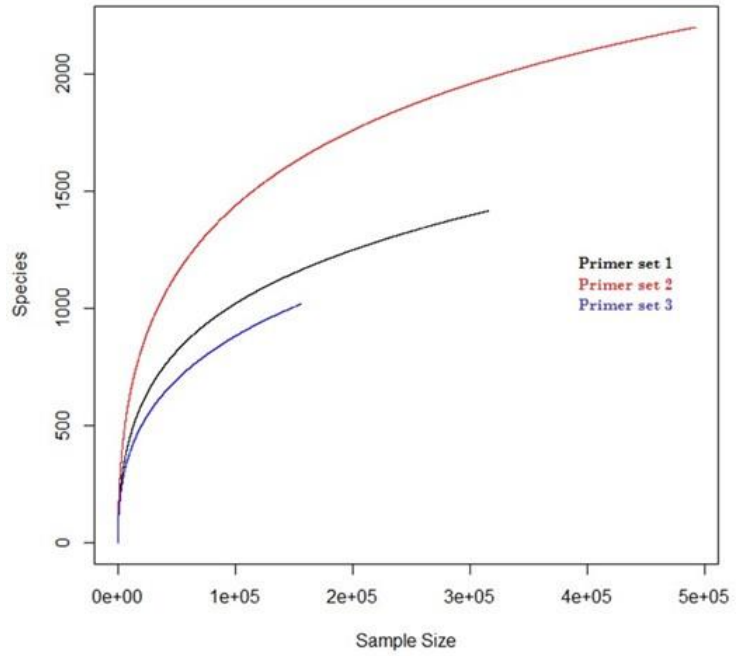


Figure 34 - Rarefaction curves for each of the three primer pairs (set 1 in black, set 2 in red, set 3 in blue) on the forest samples analysed in run 1 (bioinformatic method developed at CRAW).

## 4.3. Conclusions and recommendations

The conclusions and recommendations are listed according to work package.

**WP3. Mechanisms of hybridization in *Phytophthora* species**

A large collection of isolates or DNA samples has been established, purified and maintained. This not only includes many isolates collected by Resipath partners and provided to the Belgian partners but also isolates collected and maintained by the Belgian partners themselves, such as the 116 *Phytophthora* isolates from Belgian beech forests and the 300+ isolates collected via the ILVO diagnostic clinic.

Flow cytometry and GBS have been developed in the first two years as novel methods for detection of hybrids and these methods were now applied to a larger collection of isolates in the third year. Both methods are valuable. The larger our database with GBS results from reference isolates is becoming, the clearer it is that GBS is a very useful and powerful technique. With this technique we could not only confirm the hybrid nature of known hybrids but could also identify the hybrid nature of several other isolates or even species. Although our database is still relatively limited, we could in some cases also clearly identify groups within the parental species that were related (or not) to the parents of the hybrids. Flow cytometry is a general technique, which was more labor intensive than expected, but which was able to help identify allopolyploid hybrids. It also revealed that especially within

allopolyploid hybrids, large intraspecific variation in genome size can be present and thus that chromosomal variation is not unusual in such hybrids.

The results pointed to hybrids in *Phytophthora* clades 1, 6, 7 and 9, but analysis of more isolates is necessary to get a complete picture of the hybrid situation over the different clades. For example, known hybrids in clade 8 were not yet included in this study. Nevertheless, it is already very clear that some clades have a much higher chance of harboring hybrids than others. Clade 6 contains several species that are found in rivers and riparian systems and this clade contains sexual as well as allopolyploid hybrids. Clade 7 contains several species that are found in forests and riparian systems and contains mostly allopolyploid hybrids. Most hybrid species are contained in these two clades but these clades do not contain many species that are commonly found in nurseries. Hence it is possible that it is not necessarily so that the (apparent) increase in *Phytophthora* hybrids is due to the increase in mating in nurseries among formerly separated species. This situation may be different in clade 1, with hybrids that include *P. cactorum* as one of their parents. One further task within Resipath is the analysis of the hybrids in terms of geographic origin, but it is likely that the type of environment (river, forest, nursery) and the clade of *Phytophthora* is a stronger determinant for the chance of finding hybrids than the climate in which the isolate was found.

**WP4. Aerial spore trapping**

A procedure for the analysis of the aerial inoculum of fungi in the forest has been set up in the laboratory. This method includes several steps which have been optimized to meet the objectives of the study: choice of the most efficient trapping system for forest pathogens, DNA extraction method from spore traps with a recovery close to 100%, choice of a NGS workflow providing high quality of reads, new pipeline for the bioinformatic analysis of the NGS data usable on personal computer, and development of a new reference fungal database adapted to the objective of the study (correct assignment of fungi at the species level).

Two NGS runs have been carried out. The first run carried out in 2016 has allowed the comparison of three primer pairs on mock communities (10 fungal species belonging to Ascomycota and Basidiomycota) and forest samples (collected in 2014 in 5 Belgian forests). This analysis has shown the higher capacity of primer set 1 (and to a lesser extend set 2) to correctly assign the fungi at the species level. The test was specific, repeatable and adapted to the detection of a large range of fungal species belonging to different taxa. However, optimisations are still required to improve the sensitivity of the method and to propose its use for the detection of invasive airborne fungal pathogens (present in very low concentration at the beginning of the invasion). A second NGS run carried out on forest samples collected in 2014 and 2015, and on the same mock communities as those used in the first run has been carried out in 2017. This second test will allow to evaluate the reproducibility of the method (same samples in two NGS runs) and to analyse the fungal diversity in two successive years

in the same forest stand. The analysis of run 2 is still ongoing with the bioinformatic method developed at CRAW.

## 4.4. Added value and deliverables of the project

The deliverables of the project are divided into 4 categories

### 4.4.1. New material and databases:

- Reference collection of *Phytophthora* from woody species in Europe (species characterised and hyphal tipped, more than 800 isolates) + corresponding DNA collection (ILVO)
- New reference ITS database adapated to the detection of emergent diseases (as this database can be updated very rapidly) (CRAW)
- New GBS database with DNA loci specific for most relevant species (ILVO)

### 4.4.2. New protocols

- Method based on flow cytometry validated for the characterisation of nuclear content of *Phytophthora* isolates(ILVO)
- Method based on Genotyping By Sequencing valiadated for the characterisation of *Phytophthora* isolates (ILVO)
- New pipeline for the analysis of Illumina MiSeq raw sequences adapted to fungi (with a specificity allowing a detection to the species level) (CRAW)
- Method for the collection of spores in forest habitats (based on the use of rotorods with nails and including the DNA extraction step with 100% recovery) (CRAW)
- Method for the detection of fungal species with an aerial dispersal by using a high throughput sequencing method (optimisations still needed to improve the sensitivity of the method) (CRAW)
- New method for the detection of *Erysiphe alphitoides* by real-time PCR using a Taqman probe (CRAW)

### 4.4.3. Novel data (other than databases)

- Survey data concerning the diversity of *Phytophthora* species in Belgian beech stand (CRAW)
- First report of *Phytophthora pseudosyringae* in beech bark in Belgium (CRAW)
- Identification of new hybrids in *Phytophthora* clades 1, 6, 7 and 9 (ILVO)
- Identification of isolates related to the parental *Phytophthora* species involved in the hybridisation event (ILVO)

### 4.4.4. Added value

- For the laboratories
    - Acquisition of new skills (Flow cytometry, Genotyping by Sequencing, High throughput sequencing and analysis of Illumina miseq data)
    - Cooperation to other projects:

- project Euphresco CERACRY dealing with the characterisation of *Cryphonectria parasitica* isolates by GBS analysis
- project developed at INRA Bordeaux for the survey of species causing powdery mildew in oaks (use of the Taqman method developed in the project for the detection of *Erysiphe alphitoides* – Dr ML Deprez-Loustau)

- For federal / international organisations involved with regulated/emergent plant pathogens

  - It is possible to use the GBS technique to do a detailed identification of (potentially novel) *Phytophthora* species, including the detection of (likely) hybrid species. The technique can similarly be used for other regulated organisms, as long as reference species are included in the analysis.

  - The identification of *Phytophthora* hybrids in several clades seems not to be a rare event. Such results raise a number of questions to be discussed at the international level (EPPO, EFSA, IPPC):
    - if a hybrid *Phytophthora* is produced between a regulated and a non-regulated pathogen, what will be the legal status of the hybrid: is it a regulated pathogen or not?
    - If the hybrid between a regulated and a non regulated species is not considered a regulated pathogen, there is a risk of a false positive result if classical molecular tests (like qPCR) are used for their detection instead of GBS. What will be the position of the Plant Protection Services if such situation occurs?
    - Hybrid species (regardless of the status of the parental species) can be more aggressive than the parental species (the case of *Phytophthora* x*alni* is a good example with dramatic consequences for riparian alders in Europe). If such hybrids are detected, is it necessary to prepare a Pest Risk Analysis? Is there a possibility to rapidly implement the legislation to avoid the spread of this “new” pathogen in areas where it is not yet present?

  - The method developed for the detection of fungi by high throughput sequencing has been validated as an EPPO protocol (diagnostic sensitivity, diagnostic specificity, repeatability, reproducibility). It will be proposed at the EPPO panel on mycological diagnostics (ILVO and CRAW are members) as a new protocol for the detection of pathogenic fungi. Its use could be extended to the detection of other pathogenic organisms (nematodes, bacteria and insects).

  - The survey carried out in beech stands in 2014 and 2015 for the collection of *Phytophthora* can be used by our federal agency to confirm the absence of

regulated *Phytophthora* species in Belgian beech forests (i.e. *Phytophthora ramorum* & *P. kernoviae* were not detected)

- It is possible to start a network of samplers to determine the presence/absence of aerially dispersed pathogens in a given region. The data can be re-analysed with updated versions of the database, so that the historic presence of new pathogens can also be determined.

# 5. DISSEMINATION AND VALORISATION

## 5.1. Workshops with international partners the Rseipath project

- Kick off meeting of the Resipath project in Sweden (21-23 January 2014)
- Meeting with people involved in WP4 in Gembloux (26 March 2014): Partners 3 [France, Anses (R. Ioos, A. Aguayo )], partner 6 [France, INRA (C. Husson)] and partner 7 [Belgium, ILVO (K. Heungens, K. Van Poucke) and CRAW (A. Chandelier)]
- Annual meeting of the project in Lleida (Spain) (9-12 February 2015): all partners.
- Meeting with people involved in WP4 in Merelbeke (26 May 2015): Partners 3 [France, Anses (A. Aguayo)], partner 6 [France, INRA (C. Husson, Marie Grosdidier)] and partner 7 [Belgium, ILVO (K. Heungens, K. Van Poucke) and CRAW (A. Chandelier)]
- The Belgian partners made several presentations at the project meeting in Bordeaux (France), December 5-7, 2016.
    - Kurt Heungens. Introduction to WP3: Mechanisms of hybridization of invading organisms in Europe
    - Kris Van Poucke and Kurt Heungens. Selection of *Phytophthora* reference isolates and potential hybrids from the ILVO plant disease clinic and from collections of colleagues (BE, FR, NL, DE, AT, SE, PT)
    - Sanne Torfs, Kris Van Poucke, Sarah Croes, Wendy Van Hemelrijck, Wannes Keulemans, Kurt Heungens. Optimization and validation of aerial fungal spore sampling and molecular quantification using rotorod samplers.
    - Kris Van Poucke and Kurt Heungens. Detailed analysis of *Phytophthora* isolates using flow cytometry and genotyping-by-sequencing
    - Anne Chandelier and Sébastien Massart. Fungal pathogens of forest trees: detection of airborne inoculum by qPCR and NGS
    - Anne Chandelier and Mélanie Gourgue. *Phytophthora* collection from Belgian beech forests and hybrid status of *Phytophthora cambivora*

## 5.2. Participation in international conferences

- Kris Van Poucke, Thomas Goedefroit, Anne Chandelier, Leen Leus and Kurt Heungens. Flow cytometry reveals large differences in genome size in the genus *Phytophthora*. Oral presentation at the 68th International Symposium in Crop Protection. May 17, 2016, Gent.

- Kris Van Poucke, Thomas Goedefroit, Annelies Haegeman, Tom Ruttink and Kurt Heungens. Identification and characterization of *Phytophthora* hybrids using Genotyping-By-Sequencing. Oral presentation at the 12thEFPP- 10thSFP conference. May 29 –June 2, 2017. Dunkerque, France

- Anne Chandelier, Kurt Heungens. Comparison between different spore traps combined with qPCR for the detection of the airborne inoculum of fungi in the forest. Oral presentation at the annual EMN workshop, 21-23 April 2015, Ljubljana, Slovenia.

- Anne Chandelier. *Phytophthora* baiting in streams of Belgian beech forests. Oral presentation at the annual EMN workshop. April 26-28, Gent.

- V. Talgø, A. Chandelier, S. Schmitz, M. B. Brurberg & I. M. Thomsen. Baiting for *Phytophthora* in waterways associated with Christmas tree production in Norway, Belgium and Denmark. IUFRO Unit 2.02.09. 12th International Christmas Tree Research and Extension Conference (6-11 September 2015). Poster

# 6. PUBLICATIONS

Published

Jung, T., Horta Jung, M., Scanu, B., Seress, D., Kovács, D.M., Maia, C., Pérez-Sierra, A., Chang, T.-T., Chandelier, A., Heungens, A., van Poucke, K., Abad-Campos, P., Léon, M., Cacciola, S.O., Bakonyi, J. (2017). Six new *Phytophthora* species from ITS Clade 7a including two sexually functional heterothallic hybrid species detected in natural ecosystems in Taiwan. Persoonia 38: 100-135. DOI: 10.367/003158517X693615

In preparation

- publications of the GBS and flow cytometry results

- publications on the comparison between different spore trapping systems for the collection of fungal spores in the forest and NGS technology.

# 7. ACKNOWLEDGEMENTS

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# 8. REFERENCES

Abarenkov K, Nilsson RH, Larsson KH et al. (2010). The UNITE database for molecular identification of fungi – recent updates and future perspectives. New Phytologists 186, 2, 281-285

Blair JE, Coffey MD, Park SY, Geiser DM, Kang S (2008). A multi-locus phylogeny for Phytophthora utilizing markers derived from complete genome sequences. Fungal Genetics and Biology 45:266-77.

Bodles WJA, Fossdal CG, Woodward S (2006). Multiplex real-time PCR detection of pathogen colonization in the bark and wood of Picea sitchensis clones differing in resistance to Heterobasidion annosum. Tree Physiology 26, 775-782

Chandelier A, Helson M, Dvorak M, Gischer F (2014). Detection and quantification of airborne inoculum of Hymenoscyphus pseudoalbidus using real-time PCR assays. Plant pathology. Doi: 10.1111/ppa.12218

Chee, KH and Newhook, FJ (1965). Nutritional studies with Phytophthora cinnamomi Rands. New Zealand Journal of Agricultural Research 8: 523-529

Cleary M, Ghasemkhami M, Blomquist M, Witzell J (2016). First report of Phytophthora gonapodyides causing stem canker on European beech (Fagus sylvatica) in Southern Sweden. Plant Disease 100, 2174

Cooke, DEL and Duncan, JM (1997). Phylogenetic analysis of Phytophthora species based on ITS1 and ITS2 sequences of the ribosomal RNA gene repeat. Mycological Research 101(6):667-677

Duvivier M, Dedeurwaerder G, De Proft M, Moreau JM, Legrève A (2013). Real-time PCR quantification and spatio-temporal distribution of airborne inoculum of Mycosphaerella graminicola in Belgium. European Joruanl of Plant Pathology 137, 325-341

Erwin, DC and Katznelson, H (1961). Studies on the nutrition of Phytophthora cryptogea. Canadian Journal of Microbiology 7: 15-21

Feau N, Lauron-Moreau A, Piou D, Marçais B, Dutech C, Desprez-Loustau ML (2011). Niche partitioning of the genetic lineages of the oak powdery mildew complex. Fungal Ecology 5, 154-162

Fleischmann F, Gottlein A, Rodenkirchen H, Lutz C, Oßwald W (2004). Biomass, nutrient and pigment content of beech (*Fagus sylvatica*) saplings infected with *Phytophthora citricola, P. cambivoa, P. pseudosyringae* and *P. undulata*. Forest Pathology 34, 79-92

Garbelotto M, Smith T, Schweigkofler W (2008). Variation in rates of spore deposition of *Fusarium circinatum*, the causal agent of Pine Pitch Canker, over a 12-month-period at two locations in Northern California. Phytopathology 98, 137-143

Hansen EM, Reeser P, Sutton W, Brasier C (2015). Redesignation of Phytophthora Pgchlamydo as Phytophthora chlamydospora sp. nov. North American Fungi 10, 1-14

Hapke A, Thiele D (2016). GIbPSs: a toolkit for fast and accurate analyses of genotyping-by-sequencing data without a reference genome. Molecular Ecology Resources 16(4): 979–990

Heuser T, Zimmer W (2002). Quantitative analysis of Phytopathogenic Ascomycota on leaves of pedunculate oaks (Quercus robur L.) by real-time PCR. FEMS Microbiology Letters 209, 295-299

Hohl, HR (1983). Nutrition of *Phytophthora*. Pages 41-53 in: *Phytophthora*: Its Biology, Taxonomy, Ecology, and Pathology. D.C. Erwin, S. Bartnicki-arcia, and P.H. Tsao, eds. APS, St. Paul, MN. 392 pp.

Jung T, Burgess TI (2009). Re-evaluation of P. citricola isolates from multiple woody hosts in Europe and North America reveals a new species, Phytophthora plurivora sp. Nov. Persoonia 22, 95-110

Jung T, Hudler GW, Jensen-Tracy SL, Griffiths M (2005). Involvement of Phytophthora species in the decline of European beech in Europe and the USA. Mycologist 19, 159-166

Jung T, Nechwatal J, Cooke DE, Hartmann G, Blaschke M, Osswald WF, Duncan JM, Delatour C (2003). Phytophthora pseudosyringae sp. Nov., a new species causing root and collar rot of deciduous tree species in Europe. Mycological Research 107, 772-789

Kanoun-Boulé M, Vasconcelos T, Gasper J, Vera S, Dias-Ferreira C, Husson C (2016). Phytophthora xalni and Phytophthora lacustris associated with common alder decline in central Portugal. Forest Pathology 46, 174-176

McCartney HA, Fitt BDL, Schmechel D (1997). Sampling bioaerosols in plant pathology. Journal of Aerosol Sciences 28, 349-364

Ohta T, Tokishita S, Yamagata H (2001). Ethidium bromide and SYBR Green I enhance the genotoxicity of UV irradiation and chemical mutagens in E. coli. Mutation Research 492, 91-97

Op De Beeck M et al. (2014). Comparison and validation of some ITS primer pairs useful for fungal metabarcoding studies. Plos One http://dx.doi.org/10.1371/journal.pone 10097629

Sansford C. (2012). Revised rapid assessment of the need for a detailed Pest Risk Analysis for Phytophthora pseudosyringae (version 4). Fera, Sand Hutton UK YO41 LZ

Scanu B, Webber J (2015). Dieback and mortality of *Nothofagus* in Britain: ecology, pathogenicity and sporulation potential of the causal agent *Phytophthora pseudosyringae*. Plant Pathology, DOI: 10.1111/ppa.12399.

Schena L, Duncan JM, Cooke DEL (2008). Development and application of a PCR based 'molecular tool box' for the identification of *Phytophthora* species damaging forests and natural ecosystems. Plant Pathology 57, 64-75

Schmitz S, Zini J, Chandelier A (2007). Involvement of *Phytophthora* species in the decline of beech *Fagus sylvatica* in Wallonia (Belgium). Communications in agricultural and applied biological sciences. 72 (4): 879-886

Toju H, Tanabe H, Yamamoto S, Sato H (2012). High-coverage ITS primers for the DNA-based identification of Ascomycetes and Basidiomycetes in Envirnomental samples. PlosOne 7, 2-11

Vercauteren A, De Dobbelaere I, Grünwald NJ, Bonants P, Van Bockstaele E, Maes M, Heungens (2010). Clonal expansion of the Belgian *Phytophthora ramorum* populations based on new microsatellite markers. Molecular Ecology 19:92-1079.

# ANNEXES

1. List of *Phytophthora* isolates and species in the ILVO collection
2. *Phytophthora* collection from beech stands maintained at CRAW (“resi collection”)
3. List of DNA samples from the forest used for high throughput sequencing

**Appendix 1.** List of *Phytophthora* isolates and species in the ILVO collection

| species | number | isolation years (range) |
|---|---|---|
| cactorum/hedraiandra (hybrid) | 47 | 2005-2014 |
| cactorum | 12 | 2005-2011 |
| cambivora | 3 | 2005-2009 |
| cambivora/alni | 2 | 2010-2013 |
| cinnamomi | 18 | 2010-2014 |
| citricola | 11 | 2010-2011 |
| citricola E | 1 | 2014 |
| citricola/multivora/plurivora/pini | 36 | 2004-2014 |
| citrophthora/colocasaie/botryosa | 1 | 2005 |
| citrophthora | 8 | 2010-2013 |
| cryptogea | 2 | 2007-2008 |
| cryptogea/andina/boehmeria | 1 | 2013 |
| cryptogea/drechsleri/erythroseptica | 9 | 2004-2014 |
| erythroseptica | 1 | 2005 |
| gonapodyides | 23 | 2004-2007 |
| gonapodyides? | 19 | 2005-2014 |
| hedraiandra | 8 | 2006-2011 |
| ilicis/nemorosa/pseudosyringae/… | 2 | 2013 |
| inundata | 2 | 2008-2009 |
| inundata/humicola | 1 | 2009 |
| kernoviae | 3 | 2006-2012 |
| lacustris/riparia? | 1 | 2013 |
| lateralis | 1 | 2013 |
| megasperma | 8 | 2005-2014 |
| multivora | 4 | 2010-2012 |
| nicotianae | 8 | 2010-2013 |
| occultans | 4 | 2012-2014 |
| Taxon PgChlamydo | 1 | 2010 |
| pini | 1 | 2010 |
| pini/citricola III | 2 | 2013-2014 |
| plurivora | 37 | 2010-2024 |
| plurivora? | 2 | 2010 |
| ramorum | 44 | 2010-2014 |
| syringae | 1 | 2005 |
| Unknown (to be sequenced) | 32 | 2005-2014 |
| **Total** | **324** | **2004-2014** |

**Appendix 2**. Phytophthora collection maintained at CRAW and constituted during the project from Belgian beech stands.

| code CRAW | Year | Collection date | Locality in Belgium | Origin | Baiting material | Species identity |
|---|---|---|---|---|---|---|
| resi001 | 2014 | spring | Rulles | stream | oak leaflet | *P. gonapodyides* |
| resi002 | 2014 | spring | Marenne | stream | rhododendron leaf | *P. pseudosyringae* |
| resi003 | 2014 | spring | Marenne | stream | rhododendron leaf | *P. pseudosyringae* |
| resi004 | 2014 | spring | Marenne | stream | rhododendron leaf | *P. pseudosyringae* |
| resi005 | 2014 | spring | Marenne | stream | rhododendron leaf | *P. pseudosyringae* |
| resi006 | 2014 | spring | Marenne | stream | rhododendron leaf | *P. pseudosyringae* |
| resi007 | 2014 | spring | Aublain | stream | rhododendron leaf | *P. pseudosyringae* |
| resi008 | 2014 | spring | Mortehan | stream | rhododendron leaf | *P. gonapodyides* |
| resi009 | 2014 | spring | Mortehan | stream | rhododendron leaf | *P. gonapodyides* |
| resi010 | 2014 | spring | Mortehan | stream | oak leaflet | *P. gonapodyides* |
| resi011 | 2014 | spring | Voneche | stream | rhododendron leaf | *P. gonapodyides* |
| resi012 | 2014 | spring | Faulx-Les-Tombes | stream | rhododendron leaf | *P. cambivora* |
| resi013 | 2014 | spring | Faulx-Les-Tombes | stream | rhododendron leaf | *P. pseudosyringae* |
| resi014 | 2014 | spring | Faulx-Les-Tombes | stream | rhododendron leaf | *P. gonapodyides* |
| resi015 | 2014 | spring | Rulles | stream | rhododendron leaf | *P. gonapodyides* |
| resi016 | 2014 | spring | Rulles | stream | rhododendron leaf | *P. pseudosyringae* |
| resi017 | 2014 | spring | Rulles | stream | oak leaflet | *P. gonapodyides* |
| resi018 | 2014 | spring | Rulles | stream | rhododendron leaf | *P. pseudosyringae* |
| resi025 | 2014 | spring | Mortehan | Beech bark | | *P. cambivora* (=4557H) |
| resi026 | 2014 | spring | Mochamps | Beech bark | | *P. gonapodyides* |
| resi029 | 2014 | spring | Goé | stream | rhododendron leaf | *P. gonapodyides* |
| resi030 | 2014 | spring | Goé | stream | rhododendron leaf | *P. gonapodyides* |
| resi031 | 2014 | spring | Goé | stream | rhododendron leaf | *P. gonapodyides* |
| resi032 | 2014 | spring | Goé | stream | rhododendron leaf | *P. gonapodyides* |
| resi033 | 2014 | spring | Mortehan | stream | oak leaflet | *P. gonapodyides* |
| resi034 | 2014 | spring | Mortehan | stream | rhododendron leaf | *P. gonapodyides* |
| resi035 | 2014 | spring | Rulles | stream | rhododendron leaf | *P. pseudsyringae* |
| resi036 | 2014 | spring | Rulles | stream | rhododendron leaf | *P. pseudsyringae* |
| resi037 | 2014 | spring | Spa | Beech bark | | *P. cambivora* |
| resi039 | 2014 | spring | Faulx-Les-Tombes | stream | rhododendron leaf | *P. pseudosyringae* |
| resi040 | 2014 | spring | Aublain | stream | rhododendron leaf | *P. pseudosyringae* |
| resi041 | 2014 | spring | Spa | Beech bark | | *P. pseudosyringae* |
| resi042 | 2014 | spring | Brussels | Beech bark | | *P. gonapodyides* |
| resi043 | 2014 | spring | Brussels | Beech bark | | *P. plurivora* |
| resi044 | 2014 | spring | Awenne | stream | rhododendron leaf | *P. cambivora* |
| resi045 | 2014 | spring | Awenne | stream | rhododendron leaf | *P. gonapodyides* |
| resi046 | 2014 | spring | Awenne | stream | oak leaflet | *P. gonapodyides* |
| resi050 | 2014 | spring | Brussels | Beech bark | | *P. pseudosyringae* |
| resi053 | 2014 | spring | Brussels | stream | rhododendron leaf | *P. lacustris* |
| resi054 | 2014 | spring | Brussels | stream | rhododendron leaf | *P. plurivora* |
| resi055 | 2014 | spring | Brussels | stream | rhododendron leaf | *P. cambivora* |
| resi067 | 2014 | summer | Mortehan | Beech bark | | *P. cambivora* |

| | | | | | | |
|---|---|---|---|---|---|---|
| resi069 | 2014 | summer | Rulles | stream | rhododendron leaf | *P. pseudosyringae* |
| resi073 | 2014 | summer | Goé | stream | rhododendron leaf | *P. pseudosyringae* |
| resi074 | 2014 | summer | Goé | stream | rhododendron leaf | *P. chlamydospora* |
| resi075 | 2014 | summer | Goé | Beech bark | | *P. cambivora* |
| resi076 | 2014 | summer | Ethe | stream | rhododendron leaf | *P. plurivora* |
| resi077 | 2014 | summer | Gesves | Beech bark | | *P. cambivora* |
| resi078 | 2014 | summer | Gesves | stream | rhododendron leaf | *P. gonapodyides* |
| resi079 | 2014 | summer | Gesves | stream | rhododendron leaf | *P. plurivora* |
| resi080 | 2014 | summer | Gesves | stream | rhododendron leaf | *P. plurivora* |
| resi081 | 2014 | summer | Gesves | stream | rhododendron leaf | *P. gonapodyides* |
| resi082 | 2014 | summer | Gesves | stream | rhododendron leaf | *P. plurivora* |
| resi083 | 2014 | summer | Awenne | Beech bark | | *P. cambivora* |
| resi084 | 2014 | summer | Awenne | stream | rhododendron leaf | *P. chlamydospora* |
| resi085 | 2014 | summer | Awenne | stream | rhododendron leaf | *P. gonapodyides* |
| resi086 | 2014 | summer | Awenne | stream | rhododendron leaf | *P. gonapodyides* |
| resi087 | 2014 | summer | Awenne | stream | rhododendron leaf | *P. gonapodyides* |
| resi088 | 2014 | summer | Voneche | stream | rhododendron leaf | *P. plurivora* |
| resi089 | 2014 | summer | Voneche | stream | rhododendron leaf | *P. gonapodyides* |
| resi090 | 2014 | summer | Voneche | stream | rhododendron leaf | *P. gonapodyides* |
| resi092 | 2014 | summer | Mochamps | stream | beech leaflet | *P. cambivora* |
| resi093 | 2014 | summer | Mochamps | stream | beech leaflet | *P. cambivora* |
| resi094 | 2014 | summer | Mochamps | stream | beech leaflet | *P. cambivora* |
| resi095 | 2014 | summer | Brussels | Beech bark | | *P. cambivora* |
| resi096 | 2014 | summer | Brussels | Beech bark | | *P. pseudosyringae* |
| resi097 | 2014 | summer | Brussels | Beech bark | | *P. pseudosyringae* |
| resi098 | 2014 | summer | Brussels | Beech bark | | *P. pseudosyringae* |
| resi099 | 2014 | summer | Brussels | Beech bark | | *P. pseudosyringae* |
| resi100 | 2014 | summer | Brussels | stream | rhododendron leaf | *P. lacustris* |
| resi102 | 2014 | summer | Marenne | stream | oak leaflet | *P. gonapodyides* |
| resi103 | 2014 | summer | Marenne | stream | rhododendron leaf | *P. plurivora* |
| resi104 | 2014 | summer | Marenne | stream | rhododendron leaf | *P. plurivora* |
| resi105 | 2014 | summer | Erbisoeul | stream | beech leaflet | *P. gonapodyides* |
| resi106 | 2014 | summer | Erbisoeul | stream | rhododendron leaf | *P. gonapodyides* |
| resi107 | 2014 | summer | Erbisoeul | stream | oak leaflet | *P. gonapodyides* |
| resi108 | 2014 | summer | Aublain | stream | beech leaflet | *P. cambivora* |
| resi109 | 2014 | summer | Aublain | stream | rhododendron leaf | *P. chlamydospora* |
| resi110 | 2014 | summer | Aublain | stream | oak leaflet | *P. chlamydospora* |
| resi113 | 2014 | summer | Morialmé | Beech bark | | *P. cambivora* |
| resi114 | 2014 | summer | Morialmé | Beech bark | | *P. cambivora* |
| resi115 | 2014 | summer | Morialmé | stream | rhododendron leaf | *P. lacustris* |
| resi117 | 2014 | summer | Morialmé | stream | rhododendron leaf | *P. chlamydospora* |
| resi119 | 2015 | summer | Goé | stream | rhododendron leaf | *P. gonapodyides* |
| resi120 | 2015 | summer | Goé | stream | rhododendron leaf | *P. cambivora* |
| resi121 | 2015 | summer | Goé | stream | rhododendron leaf | *P. gonapodyides* |
| resi122 | 2015 | summer | Goé | stream | rhododendron leaf | *P. cambivora* |
| resi123 | 2015 | summer | Spa | stream | rhododendron leaf | *P. cambivora* |

| | | | | | | |
|---|---|---|---|---|---|---|
| resi124 | 2015 | summer | Spa | stream | rhododendron leaf | *P. cambivora* |
| resi125 | 2015 | summer | Spa | stream | rhododendron leaf | *P. cambivora* |
| resi126 | 2015 | summer | Spa | stream | rhododendron leaf | *P. cambivora* |
| resi127 | 2015 | summer | Spa | stream | rhododendron leaf | *P. cambivora* |
| resi128 | 2015 | summer | Mochamps | stream | rhododendron leaf | *P. cambivora* |
| resi129 | 2015 | summer | Mochamps | stream | rhododendron leaf | *P. cambivora* |
| resi130 | 2015 | summer | Mochamps | stream | rhododendron leaf | *P. cambivora* |
| resi131 | 2015 | summer | Mochamps | stream | rhododendron leaf | *P. cambivora* |
| resi132 | 2015 | summer | Mochamps | stream | rhododendron leaf | *P. cambivora* |
| resi133 | 2015 | summer | Mochamps | stream | rhododendron leaf | *P. cambivora* |
| resi134 | 2015 | summer | Mochamps | stream | rhododendron leaf | *P. cambivora* |
| resi135 | 2015 | summer | Mochamps | stream | rhododendron leaf | *P. cambivora* |
| resi136 | 2015 | summer | Awenne | stream | rhododendron leaf | *P. pseudosyringae* |
| resi137 | 2015 | summer | Awenne | stream | rhododendron leaf | *P. cambivora* |
| resi138 | 2015 | summer | Gesves | stream | rhododendron leaf | *P. cambivora* |
| resi139 | 2015 | summer | Rulles | stream | rhododendron leaf | *P. pseudosyringae* |
| resi140 | 2015 | summer | Rulles | stream | rhododendron leaf | *P. pseudosyringae* |
| resi141 | 2015 | summer | Mortehan | stream | rhododendron leaf | *P. cambivora* |
| resi142 | 2015 | summer | Mortehan | stream | rhododendron leaf | *P. cambivora* |
| resi143 | 2015 | summer | Mortehan | stream | rhododendron leaf | *P. gonapodyides* |
| resi144 | 2015 | summer | Mortehan | stream | rhododendron leaf | *P. cambivora* |
| resi145 | 2015 | summer | Mortehan | stream | rhododendron leaf | *P. gonapodyides* |
| resi146 | 2015 | summer | Vonêche | stream | rhododendron leaf | *P. lacustris* |
| resi147 | 2015 | summer | Vonêche | stream | rhododendron leaf | *P. lacustris* |
| resi148 | 2015 | summer | Vonêche | stream | rhododendron leaf | *P. lacustris* |
| resi149 | 2015 | summer | Vonêche | stream | rhododendron leaf | *P. plurivora* |
| resi150 | 2015 | summer | Aublain | stream | rhododendron leaf | *P. chlamydospora* |
| resi151 | 2015 | summer | Aublain | stream | rhododendron leaf | *P. chlamydospora* |

## Appendix 3. List of DNA samples from the forest used for high throughput sequencing

| Run | # | Location | Forest type | Collection date | Targeted fungal species | Spore trap |
|---|---|---|---|---|---|---|
| 1 | 1 | Maissin | Spruce plantation | 6 October 2014 | *Heterobasidion annosum* | 1 |
| | 2 | Maissin | Spruce plantation | 6 October 2014 | *Heterobasidion annosum* | 2 |
| | 3 | Bièvre | Oak plantation | 27 June 2014 | *Erysiphe alphitoides* | 1 |
| | 4 | Bièvre | Oak plantation | 27 June 2014 | *Erysiphe alphitoides* | 2 |
| | 5 | Morialmé | Ash plantation | 7 August 2014 | *Hymenoscyphus fraxineus* | 1 |
| | 6 | Morialmé | Ash plantation | 7 August 2014 | *Hymenoscyphus fraxineus* | 2 |
| | 7 | Carsbourg | Douglas fir plantation | 22 May 2014 | *Phaeocryptopus gaeumanii* | 1 |
| | 8 | Carsbourg | Douglas fir plantation | 22 May 2014 | *Phaeocryptopus gaeumanii* | 2 |
| | 9 | Floriffoux | Mixed forest | 28 May 2014 | All four pathogens | 1 |
| | 10 | Floriffoux | Mixed forest | 28 May 2014 | All four pathogens | 2 |
| | 11 | Floriffoux | Mixed forest | 18 June 2014 | All four pathogens | 1 |
| | 12 | Floriffoux | Mixed forest | 18 June 2014 | All four pathogens | 2 |
| | 13 | Floriffoux | Mixed forest | 13 August 2014 | All four pathogens | 1 |
| | 14 | Floriffoux | Mixed forest | 13 August 2014 | All four pathogens | 2 |
| | 15 | Floriffoux | Mixed forest | 8 October 2014 | All four pathogens | 1 |
| | 16 | Floriffoux | Mixed forest | 8 October 2014 | All four pathogens | 2 |
| 2 | 1 | Floriffoux | Mixed forest | 6 May 2015 | All four pathogens | 1 |
| | 2 | Floriffoux | Mixed forest | 6 May 2015 | All four pathogens | 2 |
| | 3 | Floriffoux | Mixed forest | 10 July 2015 | All four pathogens | 1 |
| | 4 | Floriffoux | Mixed forest | 10 July 2015 | All four pathogens | 2 |
| | 5 | Floriffoux | Mixed forest | 17 July 2015 | All four pathogens | 1 |
| | 6 | Floriffoux | Mixed forest | 17 July 2015 | All four pathogens | 2 |
| | 7 | Floriffoux | Mixed forest | 24 July 2015 | All four pathogens | 1 |
| | 8 | Floriffoux | Mixed forest | 24 July 2015 | All four pathogens | 2 |
| | 9 | Floriffoux | Mixed forest | 31 July 2015 | All four pathogens | 1 |
| | 10 | Floriffoux | Mixed forest | 31 July 2015 | All four pathogens | 2 |
| | 11 | Floriffoux | Mixed forest | 7 August 2015 | All four pathogens | 1 |
| | 12 | Floriffoux | Mixed forest | 7 August 2015 | All four pathogens | 2 |
| | 13 | Floriffoux | Mixed forest | 13 August 2015 | All four pathogens | 1 |
| | 14 | Floriffoux | Mixed forest | 13 August 2015 | All four pathogens | 2 |
| | 15 | Floriffoux | Mixed forest | 28 August 2015 | All four pathogens | 1 |
| | 16 | Floriffoux | Mixed forest | 28 August 2015 | All four pathogens | 2 |
| | 17 | Floriffoux | Mixed forest | 4 September 2015 | All four pathogens | 1 |
| | 18 | Floriffoux | Mixed forest | 4 September 2015 | All four pathogens | 2 |
| | 19 | Floriffoux | Mixed forest | 11 September 2015 | All four pathogens | 1 |
| | 20 | Floriffoux | Mixed forest | 11 September 2015 | All four pathogens | 2 |
| | 21 | Floriffoux | Mixed forest | 25 September 2015 | All four pathogens | 1 |
| | 22 | Floriffoux | Mixed forest | 25 September 2015 | All four pathogens | 2 |
| | 23 | Floriffoux | Mixed forest | 6 June 2014 | All four pathogens | 1 |
| | 24 | Floriffoux | Mixed forest | 6 June 2014 | All four pathogens | 2 |
| | 25 | Floriffoux | Mixed forest | 18 June 2014 | All four pathogens | 1 |
| | 26 | Floriffoux | Mixed forest | 18 June 2014 | All four pathogens | 2 |
| | 27 | Floriffoux | Mixed forest | 25 July 2014 | All four pathogens | 1 |
| | 28 | Floriffoux | Mixed forest | 25 July 2014 | All four pathogens | 2 |
| | 29 | Floriffoux | Mixed forest | 1 August 2014 | All four pathogens | 1 |
| | 30 | Floriffoux | Mixed forest | 1 August 2014 | All four pathogens | 2 |
| | 31 | Floriffoux | Mixed forest | 13 August 2014 | All four pathogens | 1 |
| | 32 | Floriffoux | Mixed forest | 13 August 2014 | All four pathogens | 2 |
| | 33 | Floriffoux | Mixed forest | 15 September 2014 | All four pathogens | 1 |
| | 34 | Floriffoux | Mixed forest | 15 September 2014 | All four pathogens | 2 |
| | 35 | Floriffoux | Mixed forest | 8 October 2014 | All four pathogens | 1 |
| | 36 | Floriffoux | Mixed forest | 8 October 2014 | All four pathogens | 2 |