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Genotypic and phenotypic variation of *Phytophthora infestans* on potato in the two Swedish regions Bjäre and Östergötland in 2015

Ida Petersson

Department of Forest Mycology and Plant Pathology
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Ida Petersson

Supervisor: Dr. Lina Sjöholm, Department of Forest Mycology and Plant Pathology, Swedish University of Agricultural Sciences

Assistant Supervisor: Dr. Björn Andersson, Department of Forest Mycology and Plant Pathology, Swedish University of Agricultural Sciences

Examiner: Doc. Nils Högberg, Department of Forest Mycology and Plant Pathology, Swedish University of Agricultural Sciences

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Abstract

Potato late blight, caused by the oomycete *Phytophthora infestans*, is a very important disease on potato, and one of the most devastating plant diseases in agriculture worldwide. Despite the small area of potato in comparison with other cultivated crops, 40 % of the total use of fungicides in Sweden is used on potato.

The aim of this master thesis was to compare populations of *P. infestans* in two geographically separate regions in Sweden; Bjäre and Östergötland. Bjäre is known for its intense potato production and early infections of *P. infestans*, whereas in Östergötland, infections of late blight occur much later in the growing season. It is believed that the population of *P. infestans* on Bjäre has an increased sexual reproduction as a result of the cultivation practises in the region. The population structure of *P. infestans* was determined by the phenotypic and genotypic characterisation of samples collected from potato fields in the two different areas. The phenotype was assessed through aggressiveness tests and the genotype was determined by using molecular markers (microsatellites).

The results show that the early infections observed on Bjäre could have been derived from oospores or tuber infections, whereas in Östergötland the primary source probably was caused by asexual sporangia. The population of *P. infestans* in Östergötland showed a higher genotypic diversity than the population on Bjäre, which opposed the hypothesis. Weather conditions favouring an asexual spread of a few oospore driven genotypes on Bjäre this year and the sampling methodology could explain the little genotypic variation found on Bjäre. In Östergötland, the observed higher genotypic variation was probably due to several infection sources, where oospore infections might have been involved during the season as a result of both mating types being present in infested tubers.

The isolates from Bjäre showed a higher aggressiveness compared to the isolates from Östergötland when referring to the latency period, but there were no significant differences in lesion growth rate and number of spores per lesion.

As seen in this study, there are many factors that will affect the success of *P. infestans*; establishment, development and spread of the pathogen can vary between regions and years. This study shows that a sexual reproduction can also be of importance in fields with good crop rotation practices in an area that is normally dominated by an asexual spread of the pathogen.

Keywords: Potato late blight, mating type, simple sequence repeat markers (SSR), microsatellites, multiplex PCR, sexual reproduction, asexual reproduction, aggressiveness tests population study

Sammanfattning

Potatisbladmögel, orsakat av oomyceten *Phytophthora infestans*, är en viktig sjukdom på potatis och tillhör en av de mest förödande växtsjukdomarna inom jordbruket världen över. 40 % av den totala fungicidanvändningen i Sverige används i potatis, trots den relativt lilla arealen potatisodling i jämförelse med andra grödor.

Syftet med detta arbete var att jämföra populationer av *P. infestans* mellan två geografiskt åtskilda regioner i Sverige; Bjäre och Östergötland. Bjäre är känt för sin intensiva potatisodling med tidiga angrepp av bladmögel, medan i Östergötland sker infektionerna av *P. infestans* mycket senare under odlingssäsongen. Troligtvis beror de tidiga angreppen på Bjäre på att populationen av *P. infestans* har en mer sexuell förökning till följd av odlingssättet i regionen. Populationsstrukturen av *P. infestans* bestämdes med en fenotypisk och en genotypisk karaktärisering av isolat insamlade från potatisfält i de två områdena. Fenotypen bedömdes genom aggressivitetstester och genotypen bestämdes med hjälp av molekylära markörer (mikrosatteliter).

Resultaten visar att de tidiga angreppen som observerades på Bjäre kan ha uppkommit från oosporer eller knölinfektioner, medan i Östergötland var den primära källan troligen orsakad av asexuella sporangier. I Östergötland visade populationen av *P. infestans* på en högre genotypisk diversitet än populationen på Bjäre, vilket motsade hypotesen. Väderförhållanden som kan ha gynnat en asexuell spridning av ett fåtal genotyper uppkomna från oosporer på Bjäre i år, samt provtagningsmetoden kan vara förklaringar till den lilla genotypiska variationen som hittades på Bjäre. I Östergötland var den högre observerade genotypiska variationen förmodligen ett resultat av flera infektionsskällor, där oospor-infektioner kan ha varit involverade under säsongen som ett resultat av att båda parningstyper fanns i angripna knölar.

Isolaten från Bjäre hade en högre aggressivitet jämfört med isolaten från Östergötland hänvisat till latenstiden. Det fanns ingen signifikant skillnad i lesionstillväxt eller antalet sporer per lesion.

Denna studie visar att många faktorer har en inverkan på *P. infestans* framgång; etablering, tillväxt och spridning av patogenen kan variera mellan både regioner och år. Studien visar också att en sexuell reproduktion kan vara betydelsefull även i fält med bra växtföljd och som normalt domineras av en asexuell spridning av patogenen.

Nyckelord: Potatisbladmögel, parningstyper, simple sequence repeat markers (SSR), mikrosatteliter, multiplex PCR, sexuell reproduktion, asexuell reproduktion, aggressivitetstester, populationsstudie

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1 Introduction

Potato late blight, caused by the oomycete *Phytophthora infestans*, is a very important disease on potato, and one of the most devastating plant diseases in agriculture worldwide (Agrios, 2005). Unlike many other pathogens, which usually are a bigger problem in moist and warm areas, *P. infestans* is more destructive in cool areas with high humidity. *Phytophthora infestans* is infamous for causing the great famine in the 19th century in Europe, especially in Ireland, where hundreds of thousands of people starved, leading to an immigration wave mainly to America (Fry, 2008). Even today, the disease has devastating consequences in agriculture. Its rapid disease development can cause a 100 % infestation of the crop in just a few days after infection. Potato production has high production costs and high yield losses can therefore have serious consequences on a farmer's economy.

Many studies have been made on *P. infestans* and potato late blight, yet knowledge is still lacking about the full biology of the pathogen in order to be able to control the disease in a sustainable way. One of the most intriguing things about *P. infestans* is its population biology and the constantly ongoing changes of the pathogen, making it difficult for breeders to breed for durable crop resistance (Agrios, 2005).

In Sweden, potato is grown on an area of approximately 26 000 hectares, where about 74 % of this area is cultivated for direct human consumption and 26 % for starch production (Jordbruksverket & Statistiska Centralbyrån, 2014). Despite the small area of potato in comparison with other cultivated crops, 40 % of the total use of fungicides in Sweden is used on potato (Greppa Näringen, 2011). One of the reasons for this is that the cultivation is dominated by cultivars highly susceptible to *P. infestans*, e.g. the traditional cultivars Bintje and King Edward. These highly susceptible cultivars were grown on approximately half of the area of consumption potatoes in 2001 (Jordbruksverket, 2001). Cultivars grown for starch production in Sweden are generally more tolerant than those for direct human consumption.

2 Aim and hypothesis

The aim of this master thesis was to compare populations of *P. infestans* in two geographically separate regions in Sweden; Bjäre in Skåne and the area around Mjölby-Skänninge in Östergötland. Bjäre is known for its intense potato production and early infections of *P. infestans*, whereas in Östergötland, infections of late blight occur much later in the growing season. It is believed that the population of *P. infestans* on Bjäre has an increased sexual reproduction as a result of the cultivation practises in the region.

The hypotheses, which this study is based on, are;

- The isolates from Östergötland have a lower genotypic variation compared to the isolates from Bjäre.
- The population of *P. infestans* on Bjäre is more aggressive than the population of Östergötland.

To be able to test these hypotheses, the population structure of *P. infestans* was determined by the phenotypic and genotypic characterisation of samples collected from potato fields in the two different areas. The aggressiveness phenotype was assessed by determining the latency period (time between infection and initial sporulation), lesion growth rate and number of spores per lesion. The genotypes were determined by using molecular markers (microsatellites or SSR, Simple Sequence Repeat markers).

3 Background

3.1 The biology and life cycle of *Phytophthora infestans*

Late blight on potato is caused by the oomycete *Phytophthora infestans*. Oomycetes or "fungal-like protists" are members of the Kingdom Chromista and until the late 20th century, *P. infestans* was classified as a true fungus (Agrios, 2005). A main difference between oomycetes and fungi is the cell wall composition, where oomycetes have a cell wall of cellulose and glucans, while fungi have a composition of chitin and glucans. Another difference is that oomycetes produce biflagellated zoospores as asexual spores and oospores as resting spores, which indicates a taxonomic difference from the Kingdom True Fungi.

Phytophthora infestans is a hemibiotrophic pathogen, which means that the pathogen demonstrates biotrophic as well as necrotrophic features (Lucas, 1998). For example, just like biotrophs, *P. infestans* shows a high degree of host specificity and also produces a parasitic structure for delivery of nutrients from the plant, called haustoria. Biotrophs require healthy plants for a constant nutrient source whereas nectrotrophs kill their host by causing rapid necrosis of the plant tissue. *Phytophthora infestans* needs living tissue for its survival, but has the ability to saprophytically grow in its host and thereby cause necrosis.

Phytophthora infestans can infect tubers, stems and leaves of potatoes (*Solanum tuberosum*), tomatoes (*S. lycopersicum*) and other members of the family Solanaceae (Agrios, 2005), e.g. weeds present in potato fields; black nightshade (*S. nigrum*), bittersweet (*S. dulcamara*) and hairy nightshade (*S. physalifolium*) (Andersson *et al.*, 2003; Flier *et al.*, 2003). The source of inoculum can be both soilborne and windborne (Agrios, 2005).

Phytophthora infestans can survive and spread clonally (asexually), which is the more common life style for this pathogen worldwide. However, where the two mating types of the pathogen coexist, it can also reproduce and survive sexually (see figure 1).

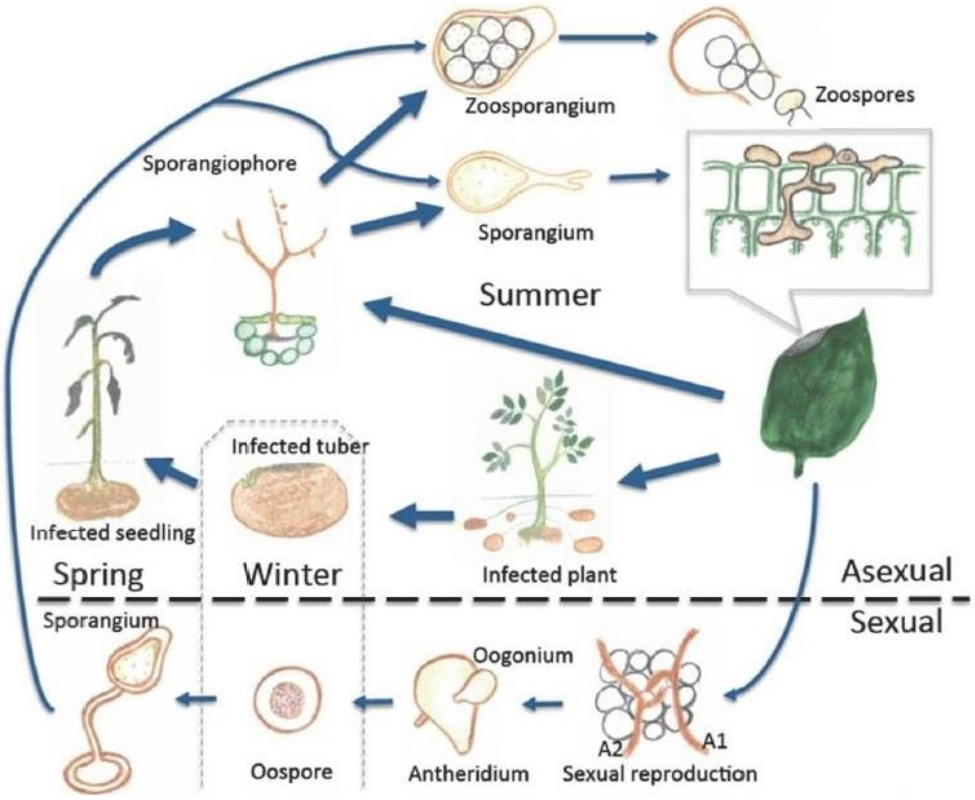

Figure 1: The life cycle of *Phytophthora infestans*. (Illustration made by Alvar Grönberg in Sjöholm (2012). Published with permission from the copyright holder)

3.1.1 The asexual life cycle of the pathogen

The asexual cycle starts with infected tubers, from where infected seedlings emerge (Agrios, 2005). Mycelium inside the plant will, in the presence of water (free water or very high humidity), produce and release sporangiophores through the stomata openings (Fry, 2008). Lemon-shaped, conidia-like sporangia will be produced at the tip of the sporangiophores and can be dispersed for wind-driven airborne propagation. These sporangia each contain three to eight zoospores, which in cooler climates (up to 12-15°C) can be released from the sporangia for host plant infection. In warmer climates (optimum 20-25°C), zoospores are normally not released, instead, sporangia can directly infect new plant tissue by producing a germ tube for penetration of the host (Fry, 2008). Sweden has a cool climate and therefore zoospores are the main source of infection in the asexual cycle.

Zoospores are biflagellate with the tinsel-type and whiplash-type flagella (Fry, 2008) and are spread with water, e.g. with rain splashes to the neighbouring area (Andersson & Sandström, 2000). Zoospores are very short-lived and according to

Fry (2008), they are encysted within 60 minutes. The encysted zoospores can then germinate via a germ tube on the host plant.

Aerial distribution of the pathogen by releasing large amounts of sporangia into the air is one way for the pathogen to spread (Aylor, 2003). However, this kind of spread is limited by the sporangia's sensitivity to UV-light and a short infection period. An aerial distribution of 35-50 km is estimated as maximum for *P. infestans*, which is quite a short distance when comparing it to other pathogens that can be distributed over much larger distances, even between continents (Aylor, 2003).

Phytophthora infestans can survive asexually between seasons as resting mycelia inside potato tubers (Andersson & Sandström, 2000). In this way it is also possible for the pathogen to spread with seed potatoes.

3.1.2 The sexual life cycle of the pathogen

Phytophthora infestans is a heterotallic pathogen, which means that for the sexual reproduction two compatible mating types are required; A1 and A2 (Gallegly & Galindo, 1958). Oospores are formed through oogamy where both mating types are bisexual and can both form a male structure (antheridium) and a female structure (oogonium). When the two mating types meet, a hormone is released, stimulating the production of the sexual structures (Fry, 2008). Fertilization occurs when a female hypha grows through the antheridium, forming an oogonium above the antheridium, followed by fertilization. The fertilized oogonium then develops into a thick-walled oospore. Oospores germinate by producing a germ tube, which in turn produces a sporangium with zoospores that will infect the host plant in the same way as in the asexual cycle.

Oospores are the resting structures of the pathogen and can survive for a long time in the soil, at least for 3-4 years according to Agrios (2005) and at very low temperatures (Drenth *et al.*, 1995).

3.2 Symptoms of late blight on potato

Early symptoms of late blight can be observed as water soaked spots on the leaves, usually at the lower levels of the potato stand (Agrios, 2005). When conditions are favourable, these spots will rapidly become larger and develop into more chocolate-brown necrotic lesions with diffuse margins (see figure 2). Sporangiophores can be seen as a whitish "mould" on the underside of the leaves, usually around the border of the lesion. Stems can also be infected, where brownish lesions will develop (see figure 3). Symptoms can be visible as early as 2-4 days after infection and under heavy infection pressure an entire crop can be destroyed in a very short time (see figure 4).

According to Lehtinen & Hannukkala (2004), infections derived from oospores can be seen at the bottom of the plant stand as "mosaic-like discolorations or lesions in direct contact with the soil", whereas airborne inoculum causes the first symptoms on the upper part of the crop.

On tubers, *P. infestans* can be seen as reddish to brownish marks that extend down under the skin of the potato tuber (Agrios, 2005). When the tubers are heavily infested, the pathogen can cause rotting through secondary infections by bacteria.

Figure 2: Symptoms of *Phytophthora infestans* on potato leaves. (Photo: Ida Petersson)

Figure 3: Symptoms of *Phytophthora infestans* on potato stems. (Photo: Ida Petersson)

Figure 4: An example of what a heavily infested field can look like. Here a field trial in Bayern, Germany, where the unsprayed parcel has a total crop loss caused by *Phytophthora infestans*. (Photo: Ida Petersson)

3.3 History of population dynamics and migration patterns of *P. infestans*

There are some uncertainties about the origin of *P. infestans*, as described in Fry (2008). The pathogen might have its origin in the Toluca Valley in central Mexico (Niederhauser, 1991; Goodwin, 1994), the first place where both mating types were documented and from where several migrations are believed to have taken place. The other theory is that *P. infestans* has its origin in the South American Andes (the origin of potatoes) as claimed by Gómez-Alpizar *et al. (*2007). A recent study has shown evidence for a sexual reproduction in Mexico and a clonal reproduction in the Andes, pointing to a Mexican origin of the pathogen (Goss *et al.*, 2014).

Two big migration events of the pathogen are described in the literature; the "first migration" in the middle of the 19th century when *P. infestans* came to Europe and the US for the first time (Fry, 2008), and the "second migration" in the late 20th century when the A2 mating type was introduced to Europe (Drenth *et al.*, 1994). The year of the first migration has been debated for a long time, but scientists agree on that it occurred around the time of the big Irish famine in the 1840's. Only the A1 mating type was present in Europe at that time, which made scientists believe that *P. infestans* was an asexual pathogen (Drenth *et al.*, 1994). Potato trade from Mexico were also limited, resulting in similar clonal lineages with one single genotype dominating worldwide. Goodwin (1994) and Fry (2008) explain that the spread of the pathogen often occurs with the distribution of infested seed potatoes, but can also happen with infected tomato fruits and other plant materials of these two crops. These are ways of asexual distribution of the pathogen, which can give an explanation for the genetically similarity in population findings in various places in the world (Fry, 2008). One example is the dominating clonal type US-1, which has been found on several continents and is believed to be the clone which caused the Irish potato famine in the 1840's (Goodwin, 1994).

With the "second migration", the US-1 clone was replaced by other populations. The A2 mating type, which is believed to have been introduced to Europe in a second migration from Mexico (Drenth *et al.*, 1994), was found for the first time in Europe, in Switzerland, at the beginning of the 1980's (Hohl & Iselin, 1984). Before that, the presence of both mating types was only documented in Mexico (Gallegly & Galindo, 1958). The A2 mating type was likely introduced with a large export of potato tubers from Mexico to Europe in 1976-1977 (Niederhauser, 1991). Acording to Montarry *et al.* (2010), the reason for this was a drought in 1976, causing a shortage of potatoes in Europe. The coexistence of both mating types resulted in a high genetic diversity of the pathogen, in contrast to the previously dominating clonal lineages (Goodwin, 1994). After the second migration from Mexico, additional migrations via seed potatoes occurred from Europe to the

rest of the world. However, at the same time, the A2 mating type had not yet been reported in the US and Canada, and epidemics of *P. infestans* were still rare. Instead, another clonal lineage was found in North America, which was different from the one discovered earlier (Goodwin, 1994). This indicates a different migration event from Mexico to North America, since the population in Europe, Asia and South America was totally different from the one in North America (Fry, 2008). According to Danies *et al.* (2014), the population in North America is still mainly clonal, even though there have been studies showing implications of sexual reproduction in some counties of the United States (Gavino *et al.*, 2000).

3.4 The current population structure in Europe

Continuous monitoring of late blight in Europe is coordinated by the network EuroBlight (www.euroblight.net) as a part of the EU Directive 2009/128/EC for sustainable use of fungicides and IPM (Integrated Pest Management). Workshop proceedings, information about the population structure in Europe as well as late blight outbreaks are continuously updated on their webpage. Recently, a monitoring across several countries in Europe has been conducted, where genotypes were determined using SSR-markers (Meier-Runge *et al.*, 2014). A few clonal lineages were found to be dominating with the clonal lineage 13_A2 having the highest frequency. In some countries (e.g. in Poland, the Netherlands, Germany and Denmark), most of the isolates did not belong to the clonal lineages found in the rest of Europe. They were classified as unknown with a probable oospore origin. Overall in Europe, populations are considered to be mainly clonal with just a few genotypes present (Flier *et al.*, 2007; Gisi *et al.*, 2011). Both mating types exist but sexual reproduction is still only happening occasionally and seems to be of minor importance (Gisi et al., 2011), except for Poland and one region in the Netherlands where there are strong indications of sexual reproduction (Li *et al.*, 2012; Chmielarz *et al.*, 2014).

A continuous change of populations, where dominating populations can be replaced by others, makes the studies of the population structure challenging. Several studies have reported that the A1 mating type has been dominating in the UK, France and Switzerland for many years. However, in recent years, there has been a shift from A1 to A2 dominated populations, which indicates a new migration of *P. infestans* (Flier *et al.*, 2007; Montarry *et al.*, 2010; Gisi *et al.*, 2011; Cooke *et al.*, 2012a). The most frequent clonal lineage in this new population is 13_A2 (also called Blue-13). Blue-13 is seen as highly aggressive, with increased virulence in terms of overcoming late blight resistance and is resistant to phenylamide fungicides (Cooke *et al.*, 2012a). Theories are suggesting an increased aggressiveness

of Blue-13 as a result of better fitness for survival in combination with a more efficient spread of the population (Montarry *et al.*, 2010; Cooke *et al.*, 2012a).

Another dominating population (6_A1) is existing side by side with 13_A2 in the UK and France, but no evidence of a sexual reproduction has been found so far and both populations are defined as clonal (Montarry *et al.*, 2010; Cooke *et al.*, 2012a). In contrast to the shift from A1 to A2 in many European countries, the opposite has happened in Northern Ireland. Between 2010 and 2011, there was a shift from the 13_A2 to genotype 8_A1, which is also the one constituting the population in Ireland (Cooke *et al.*, 2014).

In the Nordic countries, the population structure of *P. infestans* is totally different compared to the population in Europe (Sjöholm *et al.*, 2013). Both mating types are present, although frequencies vary between regions (Hermansen *et al.*, 2000). The populations show a high genotypic diversity in all four Nordic countries with no dominating clonal lineages, which indicates that oospores are an important source of inoculum (Hermansen *et al.*, 2000; Widmark *et al.*, 2007; Brurberg *et al.*, 2011; Sjöholm *et al.*, 2013). Both mating types have been reported in the Nordic countries since the 1980's, with the first observation of the A2 mating type in Sweden (Kadir & Umaerus, 1987). In Finland, the first reports of the A2 mating type came in the early 1990's (Brurberg *et al.*, 2011). Since then, early outbreaks of late blight and more frequent epidemics of the disease have been observed in the country. This is explained by sexual reproduction of the pathogen, and oospores as an important source of inoculum. The oospores have been accumulating in the soil due to very narrow crop rotations (mostly monoculture in Finland) (Hannukkala, 2014). This also reflects the situation in the North-Eastern Netherlands, where the region with sexual reproduction has a very narrow crop rotation (Li *et al.*, 2012). In Sweden, the very first outbreaks usually occur in the early potato production area with monoculture of potato. However, a more extensive study all over the Nordic countries showed a high genotypic variation also in fields with good crop rotations (Sjöholm *et al.*, 2013).

It is uncertain whether the population in Denmark is sexual or asexual. A study done by Brurberg *et al.* (2011) clearly indicated sexual reproduction and the same conclusion was drawn by Nielsen *et al.* (2014) in a study performed in 2011 and 2012. Two clonal lineages (one of them Blue-13) were detected, although the majority of the samples showed a high genotypic diversity, suggesting that oospores are the main source of inoculum. However, a recent study performed in 2013 showed the opposite result (Montes *et al.*, 2015). There was almost an equal number of A1 and A2 mating types (45 % vs. 55 %), suggesting that sexual reproduction may be possible. Still, there was only a small variation among the collected isolates, drawing the conclusion that Denmark is dominated by clonal lineages. It is however notable that in this study two loci, where high genetic variation is often

detected, were excluded when determining the genotypic composition of the pathogen population.

3.5 The importance of a mixed reproduction system of the pathogen

After the introduction of the A2 mating type to Europe, many studies have been done in order to investigate if sexual reproduction in *P. infestans* is taking place and what the consequences are. In general terms, sexual reproduction of a pathogen will give rise to higher genotypic diversity by sexual recombination, which makes the pathogen more adaptable to a changing environment (McDonald & Linde, 2002). In an evolutionary aspect, a higher genetic diversity may result in a change towards more aggressive populations with better fitness through selection, which has a big impact on the population structure (McDonald & Linde, 2002; Lehtinen & Hannukkala, 2004; Fry, 2008). According to Andrivon (1994), the population structure of *P. infestans* is a constantly changing process, on a global scale as well as a regional and local scale.

Whether an oospore infection is actually possible under field conditions has been discussed over the years, but there is evidence that late blight epidemics derived from oospores are happening (Lehtinen & Hannukkala, 2004; Widmark *et al.*, 2007). The production of oospores is of importance since it gives the pathogen a better ability for long-time survival, as well as by causing infections early in the growing season (Niederhauser, 1991). Early infections derived by oospores has been confirmed by reports from the Nordic countries (Andersson *et al.*, 1998; Lehtinen & Hannukkala, 2004).

Another issue is that the increased adaptability and fitness can have serious consequences in terms of unsuccessful disease control. This was discovered in 1980, when metalaxyl-resistant populations of *P. infestans* were detected in the Netherlands just a few years after the introduction of this fungicide (Davidse *et al.*, 1981). However, Gisi *et al.* (2011) claim that these fungicides have only a limited effect on the occurrence of fungicide resistance in populations, which is rather the result of random genetic changes within pathogen populations.

Although sexual reproduction increases the genotypic diversity, this diversity is likely reduced again by genetic drift or through natural selection by the environment (Andrivon, 1994; Sakai *et al.*, 2001). However, genetic changes are not only possible through sexual recombination. Genetic changes were already seen in populations that were present before the introduction of the A2 mating type and can be the result of mutations (Andrivon, 1994).

According to Zwankhuizen *et al.* (1998), infected tubers in refuse piles were first considered to be the most important source of inoculum, whereas infected

seed tubers, volunteer plants and allotment gardens were of minor importance. In colder climates, like in the Nordic countries, volunteer plants are less important. According to Widmark *et al.* (2007), the quantitatively most important source of infection is infested seed tubers giving a massive production of sporangia resulting in a devastating disease development in a very short time (Fry, 2008). This rapid epidemic allows a few successful genotypes to quickly dominate the population (Zwankhuizen *et al.*, 2000). Li *et al.* (2012) further explains that sexual reproduction of already well established lineages would be "evolutionarily costly" when the environment remains the same. Newly formed genotypes would probably not have the same fitness as the already successful parent. A mixed reproduction system, as that of *P. infestans*, is very effective in terms of generating new genotypes, from where a few, highly successful genotypes can reproduce asexually and cause rapid disease development (Zwankhuizen *et al.*, 2000).

Phytophthora infestans also goes through narrow demographic "bottle necks" at the end of the growing season, especially when conditions are unfavourable for tuber infections (Zwankhuizen *et al.*, 2000; Flier *et al.*, 2007). Only up to 10 % of the genotypes in one season will survive to the next season, according to a report from Drenth *et al.* (1994). Therefore, according to Zwankhuizen *et al.* (2000) and Cooke *et al.* (2012a), a rapid change of the population structure can occur from one year to another, followed by a high production rate of spores during an invasion of the host. A population change can thereby happen by selection of the better fit (Andrivon, 1994), or just by chance. A successful disease development does not necessarily involve the most aggressive genotype.

3.6 Virulence and aggressiveness

Sexual recombination can result in the occurrence of more virulent strains. This has been seen in the Netherlands after the appearance of the A2 mating type (Drenth *et al.*, 1994). However, it is important to distinguish between virulence and aggressiveness. These terms are often mixed up when talking about changes in the pathogen. Virulence means the pathogen's ability to infect the host (a compatible reaction), whereas aggressiveness "describes differences between pathogen strains in the amount of disease they cause on the host", as cited by Lucas (1998, pp 26–28). An increased virulence thereby rises the pathogen's ability to infect cultivars that previously were resistant to *P. infestans*. The pathogen's ability to overcome major resistance genes is a serious problem in resistance breeding against potato late blight. *Phytophthora infestans* has a mixed reproduction system (both sexual and asexual), and according to McDonald & Linde (2002), pathogens with this system, in combination with a high degree of genotype migration, have the highest risk of overcoming major resistance genes. Resistance breeding strate-

gies should therefore focus on obtaining a durable resistance through quantitative resistance.

Aggressiveness is referred to as "epidemic parameters" (Flier *et al.*, 2007) and it can be measured in many different ways, e.g. on detached leaflets, on whole plants, on tubers, in climate chambers or in field trials (Cooke *et al.*, 2006). When performing aggressiveness tests, it is desirable to use a range of cultivars, since the aggressiveness of a population can be adapted to specific cultivars common in the production area studied (Montarry *et al.*, 2008; Lehtinen *et al.*, 2009). It is important to take into consideration that the phenotype (aggressiveness) vary between isolates belonging to the same clonal lineage. A big sample size is therefore required in these kind of tests.

It is not possible to compare different aggressiveness tests with each other, since there might be experimental variations (Lehtinen *et al.*, 2009). Different aggressiveness factors and different testing conditions will give varying results. This was seen in two studies performed by Cooke *et al.* (2012a) and Mariette *et al.* (2015), where one study claimed that Blue-13 is a highly aggressive lineage, whereas the other study reported the dominating Blue-13 to be the weakest among the genotypes tested. Firstly, different aggressiveness factors were assessed and secondly, different incubation temperatures were used. In the study done by Cooke *et al.* (2012a), Blue-13 seemed to be more aggressive in cooler temperatures.

In the literature it is often mentioned that the reason for "new" populations replacing old populations is due to higher aggressiveness, as reviewed by Cooke *et al.* (2006). An increased aggressiveness has been observed in the new US lineages of *P. infestans*, as well as in some of the European lineages, but it is still unclear whether an increased aggressiveness has led to the dominance of these populations. Other factors may have influenced the shift in populations (Gisi *et al.*, 2011).

According to Cooke *et al.* (2006), aggressiveness and fitness of a population are strongly related, but indeed complex. A fitness model includes the ability to reproduce and the ability to survive. Highly aggressive genotypes dominating in one season might be lost before the next season, since the asexual survival is completely dependent on the transmission through seed tubers. There might be a selection for highly aggressive isolates during the season, where successful genotypes will have a rapid disease development, but these genotypes are not guaranteed to survive until the next season. Mariette *et al.* (2015) explains this with the theory that some lineages of *P. infestans* seem to invest in "within-epidemic transmission" whereas other lineages invest in "between-epidemic transmission". The aggressiveness of a clonal lineage may also change over time since there are variations within populations. Consequently, less aggressive genotypes could be favoured as the result of a better fit. The theory of the most aggressive population having the

better fit usually applies to clonal lineages. The situation might be different in regions where sexual recombination is dominating, allowing new, more aggressive strains (Cooke *et al.*, 2006).

3.7 Definitions in population biology

Population biology can be described as the study of populations of an organism, including the biological processes affecting the population structure (McDonald, 2004). In plant pathology, this is referred to diseases caused by populations of pathogens, resulting in economic consequences in terms of yield losses. A fully understanding of the population biology and population dynamics is required in order to apply durable control strategies. In the case of *P. infestans*, it is important to frequently monitor phenotypic and genotypic changes among populations (Lehtinen & Hannukkala, 2004; Li *et al.*, 2012).

Population biology includes the terms *epidemiology* and *population genetics* (McDonald, 2004), where;

- *Epidemiology* describes the disease development, i.e. factors for reproduction and distribution of a population, usually for only one or two growing seasons. Epidemiology can be studied from the field level up to continent level.
- *Population genetics* has an evolutionary focus, describing the genetic changes in a population.

A population can generally be defined as a group of individuals/organisms from the same species, living within the same geographical area (McDonald, 2004; Freeland *et al.*, 2011a). However, population biology is more complex than this definition. The general definition assumes sexual reproduction within a species, whereas in the species of *P. infestans*, a clonal reproduction is also occurring. A mixed reproduction system, in combination with different stages in the life cycle at different times, makes it difficult to define populations of *P. infestans*. In such cases, it is suggested to have a look on the genetic pattern instead, where individuals can be clustered based on genetic similarities. Consequently, individuals within the same geographical region can belong to genetically different populations while other populations can be globally distributed. As already mentioned, since the potato is a cultivated crop, trade and other human interference have a huge influence on the spread and distributions of populations of *P. infestans*.

4 Materials & methods

4.1 Field background

Samples were collected from two regions in Sweden; two conventional grown fields (but with no fungicide treatment) from Bjäre in the county Skåne and two organic fields from the area around Skänninge in the county Östergötland (see figure 5). The two regions will, in this thesis, be referred to as Bjäre and Östergötland.

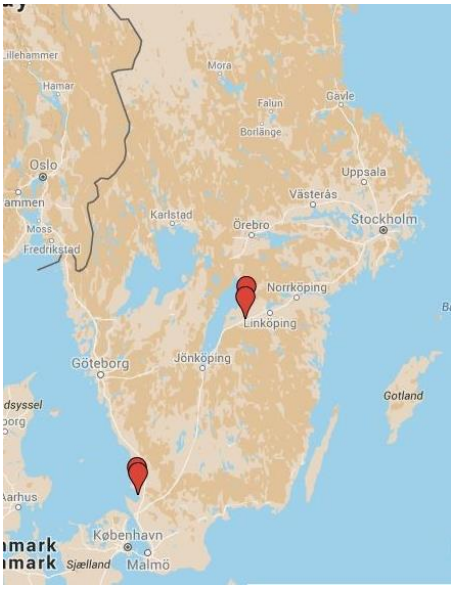

Figure 5: Map of the southern part of Sweden showing the collection sites; Bjäre in the very south and Östergötland further north.

Bjäre is a region in the very southwest of Sweden where early potato is cultivated. Planting normally occurs in March and the fields are often covered with a garden fabric that will protect the crop from frost and allows an early harvest; the

earliest fields are already harvested by the end of May (Widmark *et al.*, 2007). This early harvest makes it possible to plant another crop afterwards, but potato is still planted again in the following year, resulting in a very narrow crop rotation. Early potato has been cultivated in the two fields of Bjäre used in this study for the last 30 years and 50 years respectively. Early outbreaks of late blight is common in this region. In 2015, the earliest infection was reported at the beginning of May. Collection of samples on Bjäre was made on the 15th of May.

In the region Östergötland, potato is planted later than on Bjäre, e.g. by the end of April, as was the case for the two fields used in this study. Outbreaks of late blight usually occur quite late in the season and in 2015, the first symptoms of *P. infestans* were reported on the 23rd of July. Collection of samples in Östergötland was made on the 23rd of July. Potato had not been cultivated for the last 7-10 years in these two fields.

Untreated fields with early infections of *P. infestans* were of interest in this study.

4.2 Sampling

In the fields on Bjäre (field 1, cv. Magda; field 2, cv. Solist), two distinct foci of late blight symptoms were present in the field (see figure 6), but in the fields in Östergötland (field 3 and 4, cv. Solist), the infection of *P. infestans* was evenly spread all over the field, with the exception of one distinct focus in field 3 located in a low area of the field (see figure 7).

Figure 6: A distinct late blight focus in field 2 on Bjäre. (Photo: Ida Petersson)

Figure 7: A distinct late blight focus in field 3 in Östergötland. (Photo: Ida Petersson)

Fifty samples per field were collected by choosing, if possible, two distinct foci in each field. Twenty-five samples from each focus were taken from 5 plants à 5 leaflets per plant (giving a sample size of 50 samples per field). Freshly sporulating single lesion leaflets were selected in order to avoid a mix of genotypes in the same sample (see figure 8).

Figure 8: One sporulating lesion of *Phytophthora infestans* on the potato leaflet. (Photo: Ida Petersson)

The leaf samples were put in zip-lock bags in the field (one leaflet per bag) and marked with the isolate number. Later, a piece of the lesion with sporulating tissue was cut out and divided into two parts; one to be used for isolation of the pathogen by putting it in a cut open potato, and one to be used for DNA sampling using Whatman FTA® cards. The potato with the sample inside was wrapped with a piece of paper towel and marked with the isolate number. To avoid bacterial

growth inside the potato, the cultivar Asterix was used since it has a medium to high level of resistance against bacterial diseases (The European Cultivated Potato Database). The FTA®-protocol (EuroBlight, 2013) was followed for the DNA sampling, where the piece of lesion was pressed (with the sporulating side facing down) onto the sampling area of the FTA card. Any plant residues were removed and the cards were allowed to air dry before they were separately put in zip-lock bags or envelopes. All samples were sent to SLU, Uppsala for further handling.

4.3 Isolation of *Phytophthora infestans*

The 200 isolates of *Phytophthora infestans* from the fields, stored in potatoes, were cultured on Petri dishes containing a 1:1 mixture of rye B (Caten & Jinks, 1968) and pea medium (protocol from Corbière & Andrivon, 2003) amended with antibiotics (ampicillin at 0.2 g/l and 2.5 % pimaricin at 0.4 ml/l (10 mg/ml)) and were kept at room temperature. The samples were re-isolated on new agar plates without antibiotics when the isolates were confirmed to be free from contamination by other microorganisms.

4.4 Determination of mating type

To determine the mating type, all isolates were paired with reference isolates with known mating type (see figure 9). The same methodology as described in Mariette *et al.* (2015) was used, with the exception of the incubation conditions; incubation was made at room temperature with natural light/darkness. Since *P. infestans* in rare cases can be self-fertile (homothallic) and thereby produce oospores against both A1 and A2 (Hermansen *et al.*, 2000), pairing had to be made with both mating types for all isolates.

Determination of mating types was made by André Johansson as a part of his bachelor thesis.

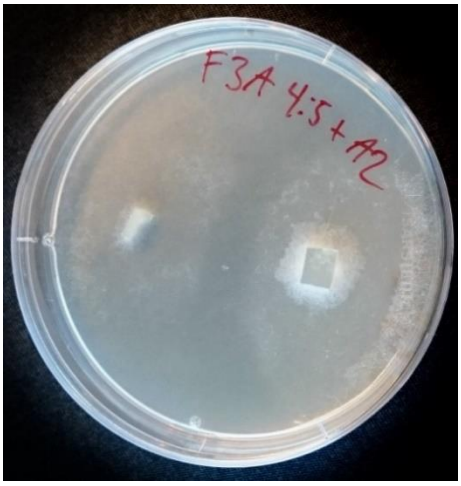

Figure 9: Example of an isolate paired with a reference isolate of mating type A2 of *Phytophthora infestans*. Two halos can be seen in the photo; the two isolates growing towards each other. If oospores are produced, they can be detected at the edges of the hyphae between the two isolates. (Photo: Ida Petersson)

4.5 Phenotyping: Aggressiveness tests

4.5.1 Cultivation of potato plants

Potato leaves of the cultivar Bintje were used for the aggressiveness tests. The potatoes were planted in the greenhouse approximately 7 weeks before the aggressiveness tests.

4.5.2 Preparation of sporangia suspensions

Fifteen isolates per field were randomly selected, giving a total number of 60 isolates being used for the aggressiveness tests. Agar mycelia plugs were transferred from the previously isolated culture plates to freshly cut potato slices and incubated in a climate chamber at a temperature of 10°C and a humidity of 80 %, with 8 hours of darkness and 16 hours of light. Ten potato slices (cv. Mandel) were used per isolate to ensure that sufficient amounts of sporangia were produced for the experiment. The potato slices were put on a plastic net in sealed plastic boxes lined with wet filter paper (see figure 10). The boxes with the potato slices were randomly placed in the climate chamber.

In addition to the isolation on potato slices, a re-isolation of the pathogen was made on a new rye pea agar plate for each isolate, to be saved as a backup culture.

Figure 10: Isolation of *Phytophthora infestans* on potato slices. (Photo: Ida Petersson)

Figure 11: Mycelial growth of *Phytophthora infestans* on potato slices. (Photo: Ida Petersson)

Sporangia were harvested when sufficient amounts were produced on the majority of the potato slices, 9 days after incubation (see figure 11). Sporangia were rinsed off from the slices with deionized water and collected in petri dishes (Lehtinen *et al.*, 2009). The sporangia were counted using a haemocytometer and the concentration was adjusted to 10^4 sporangia per ml. The sporangia suspensions were then placed in 4°C for 3 hours, to induce zoospore formation.

4.5.3 Inoculation of potato leaflets

Leaflets of the same approximate size and age were selected for the inoculation (5 replicates per isolate) and placed in petri dishes with 1.5 % water agar. The inoculation was made by depositing a droplet of 20 µl of sporangial suspension (~200 sporangia) on the abaxial side of the leaflet close to the middle vein (see figure

12). All petri dishes were placed randomly in a climate chamber at a temperature of 15°C and a humidity of 85 %, with 8 hours of darkness and 16 hours of light. After 30 hours, the droplets were removed using a filter paper and the first assessment was made.

Figure 12: Droplet of sporangial suspension on the leaflet. (Photo: Ida Petersson)

Figure 13: Lesion growth of *Phytophthora infestans* on a leaflet. (Photo: Ida Petersson)

4.5.4 Assessment of aggressiveness

Three factors were evaluated; latency period (days after inoculum), lesion growth rate and number of spores produced per lesion. The latency period was evaluated twice daily by checking the isolates for sporulation using a dissection microscope. The lesion growth rate was evaluated by measuring the diameter in millimetre in two directions once per day (see figure 13). The size of the lesion was calculated using the formula for the area of an ellipse.

The whole experiment was conducted for 10 days, after which each potato leaflet was put in a 15 ml Falcon tube with 5 ml of 70 % ethanol and placed in 4°C until the determination of sporulation per lesion.

The samples were vortexed for 10 seconds in order to release the sporangia from the leaflets. The determination of the sporangial concentration was made using a haemocytometer. From the mean value of sporulation per ml, the number of spores per lesion could be calculated by multiplying the mean value with 5 (for 5 ml ethanol in the Falcon tube). Isolates unable to infect the potato leaflets were treated as missing values and were not included in the further statistical analyses.

4.6 Genotyping

4.6.1 DNA-extraction

A six mm cork borer was used to punch out one disc from each of the 200 sampled areas of the FTA®-cards. The disc was then transferred to a 1.5 ml Eppendorf

tube, where 400 µl of the FTA® purification reagent (Whatman Inc.) was added. The tube was shortly vortexed before incubated for approximately four minutes at room temperature. The FTA® purification reagent in the tube was discarded, and the procedure with the purification reagent wash was repeated once. After discarding the reagent again, 400 µl of TE-buffer (10 mM Tris-HCl, 0.1 mM EDTA) was added and the tube was vortexed and incubated at room temperature for another ~four minutes. The step with TE-buffer wash was also repeated once. After discarding the buffer, a pipette tip was used to transfer the disc to a 0.5 ml microcentrifuge tube. Eighty µl of the TE-buffer was added and the sample was placed in a mini-centrifuge in order to spin down the disc to the bottom of the tube. In the last step of the DNA-extraction, the tube was placed on a heat block at 93°C for 5 minutes, before it was cooled down on ice and stored in the freezer.

4.6.2 PCR (Polymerase Chain Reaction)

The extracted DNA from the FTA-cards was amplified by a one-step multiplex PCR reaction. Twelve *P. infestans*- specific simple sequence repeat (SSR) primers (forward and reverse), marked with fluorescence labels, were used in order to amplify 12 specific loci of interest for the genotype analysis (Li *et al.*, 2013). However, primer D13 was excluded from the multiplex PCR assay, since this SSR marker did not work in the multiplex PCR kit. It was used in a separate PCR reaction.

A QIAGEN Type-it® Microsatellite PCR kit was used for the PCR reaction in the multiplex assay, where the manufacturer's protocol was followed. The PCR reaction for D13 was carried out with the same methodology as described in Grönberg *et al.* (2011), but with a primer concentration of 3µM (forward and reverse). The PCR programme for the multiplex assay and for primer D13 can be seen in table 1.

Five reference isolates with known genotypes and mating types were used in the experiment; 95.17.3.2 (C2), 97.38.2.2 (C3), 96.9.5.1 (C4) and 96.13.1.3 (C5) with their origin in Scotland, and US467 (C6) with its origin in the United States (Li *et al.*, 2013).

Table 1: *PCR programme for the multiplex assay and for primer D13.*

	Multiplex assay		D13	
Step	Temperature	Time	Temperature	Time
Initial Denaturation	95°C	5 minutes	94°C	3 minutes
33 cycles:				
Denaturation	95°C	30 seconds	94°C	30 seconds
Primer annealing	58°C	90 seconds	50°C	30 seconds
Primer elongation	72°C	20 seconds	72°C	1 minute
Final Extension	60°C	30 minutes	72°C	25 minutes
Cooling down	4°C	∞	4°C	∞

To check if the PCR had succeeded, the PCR products were analysed by performing an electrophoresis on 1.2 % Agarose gel (containing SB buffer and Nancy-520 fluorescent stain) followed by visualization under UV-light.

The next step was to send the samples off for a fragment analysis to SciLifeLab, Uppsala, but before doing that, all samples had to be diluted with milliQ-water and set to a final volume of 12 µl per sample. The final concentrations of the samples were estimated depending on the results of the electrophoresis, e.g. how strong the bands were. The weaker the bands, the more DNA was required for the analysis in order to assure a sufficient result. The results of the fragment analysis were interpreted by using the software programme GeneMarker®, version 2.6.4 (Soft Genetics), where the length of the fragments of the alleles, in each of the loci analysed, could be identified by looking for peaks in the product size range. *Phytophthora infestans* can be either homozygous (showing one peak) or heterozygous (showing two peaks) in an SSR locus. More than two peaks can also be visualized, indicating differences in ploidy, as described by Cooke *et al.* (2012). In cases where the size of the alleles could not be determined (very weak or missing peaks), they were set to zero in the dataset.

4.7 Statistical analyses

4.7.1 Phenotypic data: Aggressiveness tests

All statistical analyses were performed by using the statistical software JMP, version Pro 12. The data derived from the aggressiveness tests (latency period, lesion growth rate and number of spores produced per lesion) was analysed by nested ANOVA (Analysis of Variance) with replicates nested in treatments. Comparison between fields as well as between locations were made. When significant differences between the fields were found with the nested ANOVA, least square means were compared by performing a Student's t-test for each pair to detect where the differences were located.

4.7.2 Genotypic data

Isolates identical in all loci were considered as one clone and if differences in allele sizes were detected, those isolates were distinguished as unique genotypes (Sjöholm *et al.*, 2013). Analyses were both performed on a field level, where one field was considered as one population, and on a location level where the fields from Bjäre and Östergötland respectively, were grouped together.

All analyses were based on a clone corrected dataset, where copies of the same genotypes were removed, leaving only one for the analyses. The analyses were

performed by using GeneAlEx, version 6.502, a Microsoft Excel add-in (Peakall & Smouse, 2006).

The following analyses were performed:

I. Calculation of differences within populations by using the inbreeding coefficient (F_{IS}) with the formula:

$$F_{IS} = \frac{H_e\text{-}H_o}{H_e}$$

where H_e is the expected heterozygosity and H_o is the observed heterozygosity for the subpopulation (field).

A H_o higher than H_e is an indication of sexual reproduction (Freeland *et al.*, 2011a). A negative F-value also indicates heterozygosity and a positive value indicates homozygosity. The closer to 1 the F-value, the more homozygosity whereas a value close to zero indicates no inbreeding.

II. Calculation of the pairwise genetic distance between populations by using the fixation index (F_{ST}) with the formula:

$$F_{ST} = \frac{H_T\text{-}H_e}{H_T}$$

where H_e is the expected heterozygosity and H_T is the observed heterozygosity of the total population (the entire clone corrected dataset).

F_{ST} values of 0-0.05 indicate a small genetic differentiation between populations, whereas values of 0.05-0.25 indicate a moderate genetic differentiation (Freeland *et al.*, 2011b). Values over 0.25 are considered as having a high genetic differentiation.

III. AMOVA (Analysis of Molecular Variance), where the molecular variation within and between populations was calculated.

IV. PCoA (Principal Coordinate Analysis), for visualizing the genetic pattern from a distance matrix, showing dissimilarities and any clustering based on the genetically distance between populations.

In addition to these tests, the G/N ratio was calculated by dividing the number of unique multilocus genotypes with the total number of genotypes from each field (Sjöholm *et al.*, 2013). A high G/N ratio (close to 1) means a high genotypic diversity, whereas a low G/N ratio indicates a clonal reproduction.

5 Results

5.1 Mating types

All of the analysed isolates from Bjäre (field 1 and 2) were determined as mating type A2. Regarding the fields from Östergötland (field 3 and 4), both mating types were present in both fields. See table 2 for the frequencies of each mating type.

Table 2: Assessment of mating types, showing the percentage of each of the mating types of *Phytophthora infestans* found in the fields of Bjäre (field 1 and 2) and Östergötland (field 3 and 4).

	Field			
Mating type	1	2	3	4
A1			52 %	22 %
A2	100 %	100 %	20 %	33 %
Oospores produced against both mating types			8 %	0 %
No oospore formation or unknown			20 %	45 %
No. of isolates tested	19	25	25	18

5.2 Phenotypic variation: Aggressiveness tests

Due to contamination with bacterial growth, samples for the aggressiveness tests could only be taken from 96 of the 200 isolates from the field samples, limiting the size of the test. Further, ten of the isolates did not sporulate at all in the preparation of sporangia suspensions and were therefore excluded from the test.

The statistical analysis of the aggressiveness tests showed a significant difference in latency period ($P < 0.0001$) and in lesion growth rate (mm/day) between fields ($P < 0.0023$). The test also revealed a big variation between the isolates within the fields ($P < 0.0001$ for latency period and $P < 0.0003$ for lesion growth rate). No significant difference in number of sporangia per lesion could be found

between the fields (P < 0.0811), but a significant difference within the fields was present (P < 0.0001).

When grouping the fields by location (field 1 and 2 for Bjäre and field 3 and 4 for Östergötland), a highly significant difference in latency period was found (P < 0.0001). However, there was no significant difference in lesion growth rate (P < 0.8508) or in number of sporangia per lesion (P < 0.9591) between the two locations.

All tests were performed with a 95 % confidence level. The least square means for each test are displayed as bar charts (see figure 14-17).

Figure 14 shows the statistical differences between fields (least square means) for the latency period.

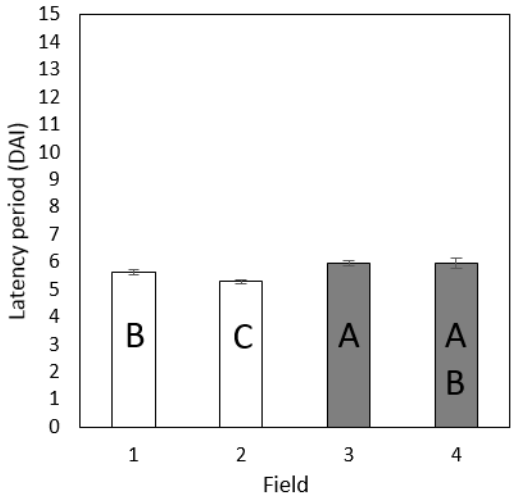

Figure 14: The bars represent least square means in latency period (days after inoculum) between fields for *Phytophthora infestans* isolates collected from potato leaves. Error bars represent the standard deviations. Field 1 (N = 69) and field 2 (N = 63) are from Bjäre and field 3 (N = 60) and field 4 (N = 14) are from Östergötland. Least square means with the same letter are not significantly different.

In figure 15, it can be seen that field 3 is significantly different from the other fields, whereas field 1, 2 and 4 are not significantly different. This explains the results of no significant difference in lesion growth rate when the fields are grouped by location (see figure 17).

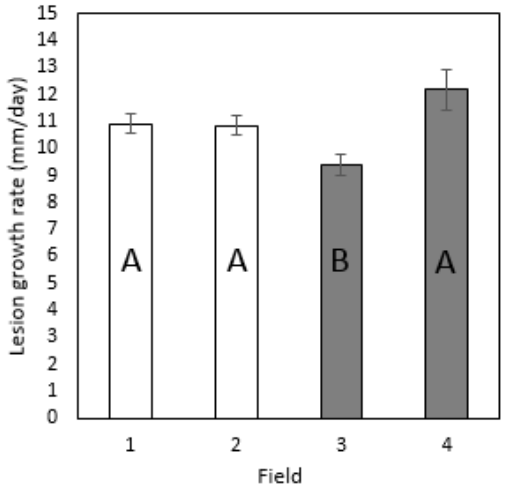

Figure 15: Lesion growth rate (mm/day) between fields for *Phytophthora infestans* isolates collected from potato leaves. Error bars represent the standard deviations. Field 1 (N = 69) and field 2 (N = 63) are from Bjäre and field 3 (N = 60) and field 4 (N = 14) are from Östergötland. Least square means with the same letter are not significantly different.

No significant difference between the fields was detected regarding the number of sporangia per lesion (see figure 16).

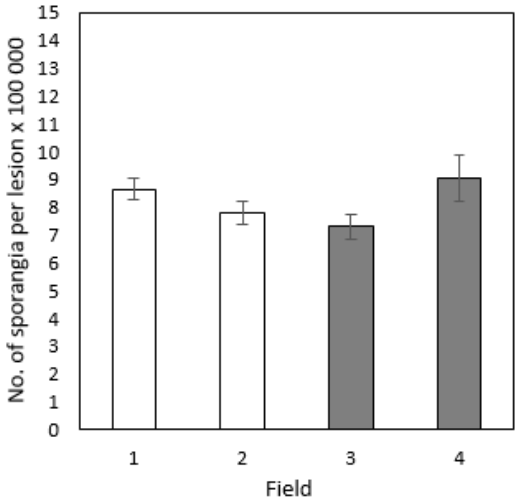

Figure 16: Number of sporangia per lesion (x 100 000) between fields for *Phytophthora infestans* isolates collected from potato leaves. Error bars represent the standard deviations. Field 1 (N = 69) and field 2 (N = 63) are from Bjäre and field 3 (N = 60) and field 4 (N = 14) are from Östergötland.

When the fields are grouped by location, the only significant difference is the latency period between the two locations (see figure 17).

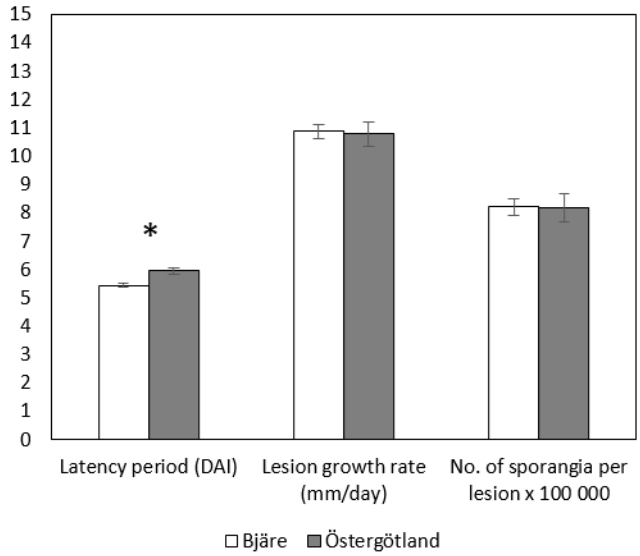

Figure 17: The bars represent least square means in latency period (days after inoculum), lesion growth rate (mm/day) and number of sporangia per lesion (x 100 000) between locations for *Phytophthora infestans* isolates collected from potato leaves. Error bars represent the standard deviations and the asterisk indicates a significant difference between the locations ($P < 0.0001$).

5.3 Genotypic variation

The PCR reaction, analysed with electrophoresis, gave various results. Some of the reactions resulted in very strong bands, indicating sufficient amounts of PCR products, whereas other reactions resulted in very weak bands on the gel (see figure 18 and 19).

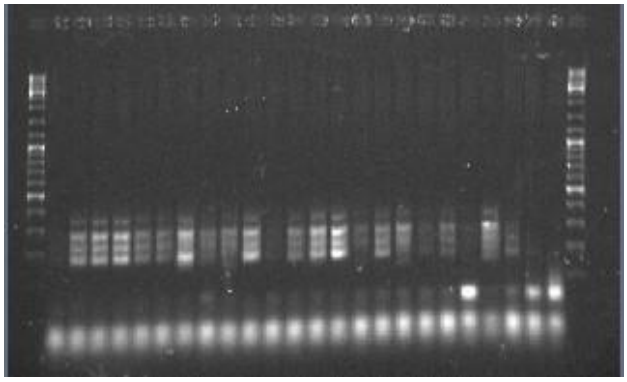

Figure 18: Part of the results from the electrophoresis, showing isolate 182-200 and three references in the multiplex assay.

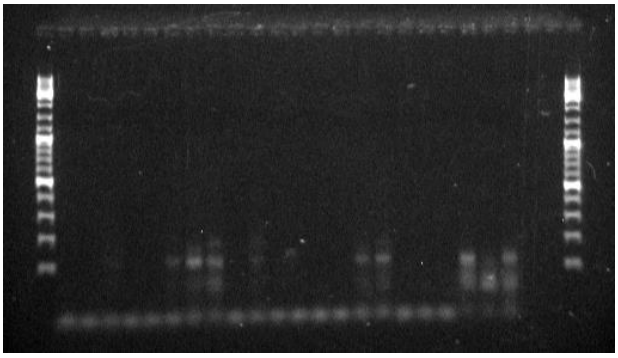

Figure 19: Part of the results from the electrophoresis showing isolate 182-200 and three references with primer D13 in the PCR reaction.

SSR locus PinfSSR4 failed in all samples, therefore it was excluded from the genotype analyses.

The total number of genotypes found among the 200 isolates was 105, giving a G/N ratio of 0.53. The G/N ratio of each field can be seen in table 3. The observed heterozygosity (H_o) was in general higher than the expected (H_e), except for field 4 where H_o and H_e were very similar (see table 3).

Table 3: Genetic data for *Phytophthora infestans* populations. Field 1 and 2 represent Bjäre and field 3 and 4 represent Östergötland.

Field/pop.	N	G	G/N	H_o	S.E.	H_e	S.E.	F	S.E
1	50	26	0.52	0.494	0.114	0.375	0.078	-0.326	0.126
2	50	15	0.30	0.501	0.100	0.422	0.074	-0.208	0.112
3	50	25	0.50	0.361	0.126	0.271	0.072	-0.266	0.221
4	50	39	0.78	0.368	0.079	0.383	0.051	0.025	0.137

N = number of isolates, G = number of genotypes, G/N = number of genotypes divided by number of isolates, H_o = observed heterozygosity, H_e = expected heterozygosity, F = fixation index, S.E. = Standard error

Significant differences can be seen between populations, as illustrated in table 4 and figure 20. All pairwise F_{ST} values in this study showed a moderate variation between populations, with the lowest differentiation between field 1 and 2 (Bjäre) and the highest differentiations between field 1 and 3 as well as 2 and 3 (both populations of Bjäre vs. one of Östergötland).

Table 4: Pairwise F_{ST}-values between populations of *Phytophthora infestans*, where field 1 and 2 are from Bjäre and field 3 and 4 are from Östergötland.

Field/pop.	1	2	3	4
1	0.000			
2	0.073	0.000		
3	0.218	0.226	0.000	
4	0.131	0.160	0.178	0.000

The F_{IS} (inbreeding coefficient) values for each locus can be seen in table 5. The overall mean value is negative, which indicates a high genotypic diversity within all populations.

A positive F_{IS} value indicates a decrease in number of heterozygotes and is a sign of inbreeding/clonal reproduction with non-random mating (Holzman *et al.*, 2009). A negative F_{IS} value indicates an increase of heterozygosity and thereby an increase in genetic variation, which is a result of random mating within a population and outbreeding.

Table 5: F_{IS} values within all four populations of Bjäre and Östergötland for each locus.

Locus	F_{IS}
G11	0.006
Pi02/SSR3	-0.135
SSR11	-0.140
D13	0.219
SSR8	-0.194
Pi04	-0.422
Pi70	0.674
SSR6	-0.417
Pi63	-0.706
SSR2	-0.440
Pi4B	-0.047
Mean	-0.146
SE	0.112

The AMOVA analysis showed that 69 % of the genotypic variation is within the populations, while 31 % of the variation is between populations (see figure 20).

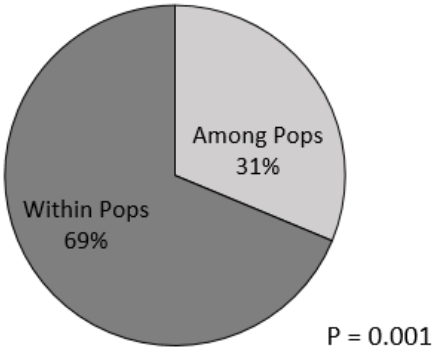

Figure 20: Analysis of molecular variance (AMOVA) within and between populations of *Phytophthora infestans*. Samples from one field are defined as one population. Comparisons between all fields were made, where two fields were from Bjäre and two were from Östergötland.

The variation between populations can be illustrated in the PCoA graph (see figure 21), where 19.30 % of the variation can be explained by axis 1 and 15.32 % by axis 2. The genotypes from field 1 and 2 are overlapping, indicating just a small difference, but a stronger differentiation can be seen among the other populations.

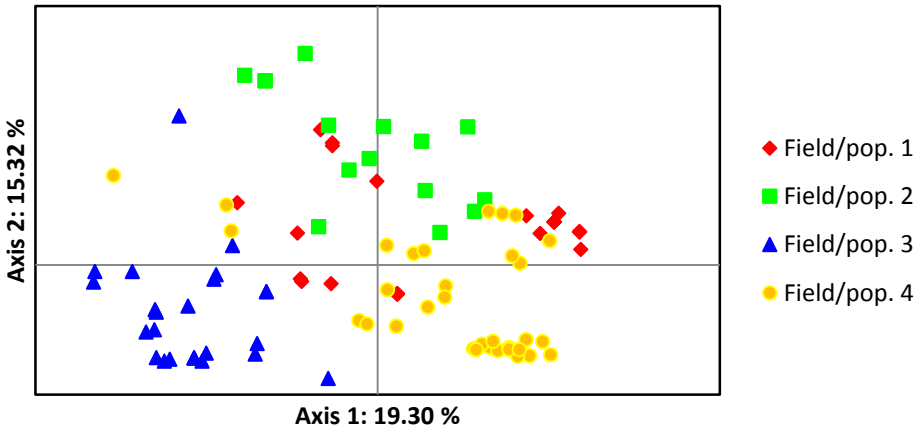

Figure 21: Principal Coordinate Analysis (PCoA) of *Phytophthora infestans* isolates, illustrating the genetic pattern between populations. Field 1 and 2 are from Bjäre and field 3 and 4 are from Östergötland.

When the fields were grouped together into locations, the results were slightly different than when each field was considered as one population.

The G/N value was lower on Bjäre than in Östergötland, indicating a higher genotypic variation in Östergötland (see table 6).

The observed heterozygosity (H_o) was higher than the expected heterozygosity (H_e) on Bjäre, but was lower in Östergötland. This indicates a higher degree of heterozygosity on Bjäre than in Östergötland.

Table 6: Genetic data for *Phytophthora infestans* populations where Bjäre and Östergötland are defined as the two populations.

Location	N	G	G/N	H_o	S.E.	H_e	S.E.	F	S.E
Bjäre	100	41	0.41	0.497	0.100	0.426	0.08	-0.157	0.089
Östergötland	100	64	0.64	0.357	0.083	0.418	0.054	0.125	0.150

N = number of isolates, G = number of genotypes, G/N = number of genotypes divided by number of isolates, H_o = observed heterozygosity, H_e = expected heterozygosity, F = fixation index, S.E. = Standard error

The pairwise F_{ST} value was 0.122, which indicates a moderate differentiation between the two locations Bjäre and Östergötland. Regarding the F_{IS} values (table 7), there was an overall positive mean value, indicating a low genetic variation within locations.

Table 7: F_{IS} values within the locations of Bjäre and Östergötland for each locus.

Locus	F_{IS}
G11	0.243
Pi02/SSR3	-0.068
SSR11	-0.041
D13	0.147
SSR8	0.438
Pi04	-0.304
Pi70	0.840
SSR6	-0.321
Pi63	-0.581
SSR2	-0.261
Pi4B	0.110
Mean	0.018
SE	0.120

The AMOVA analysis showed that 82 % of the genotypic variation is within the regions, while 18 % of the variation is between the regions (see figure 22). When

figure 20 and 22 are compared with each other, a higher variation between all four fields compared to when the fields are grouped together into locations can be seen.

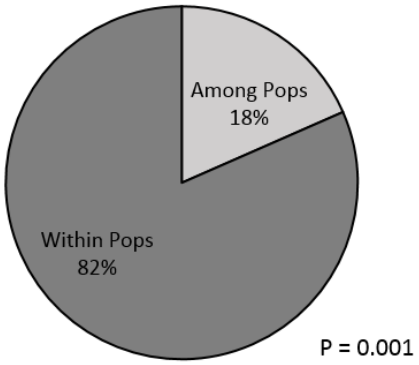

Figure 22: Analysis of molecular variance (AMOVA) within and between populations of *Phytophthora infestans*. Samples from each region are defined as one population. A comparison between the region Bjäre and Östergötland was made.

The variation between the locations can be illustrated in the PCoA graph (see figure 23), where 19.30 % of the variation can be explained by axis 1 and 15.32 % by axis 2. There is a clear population differentiation between the locations Bjäre and Östergötland.

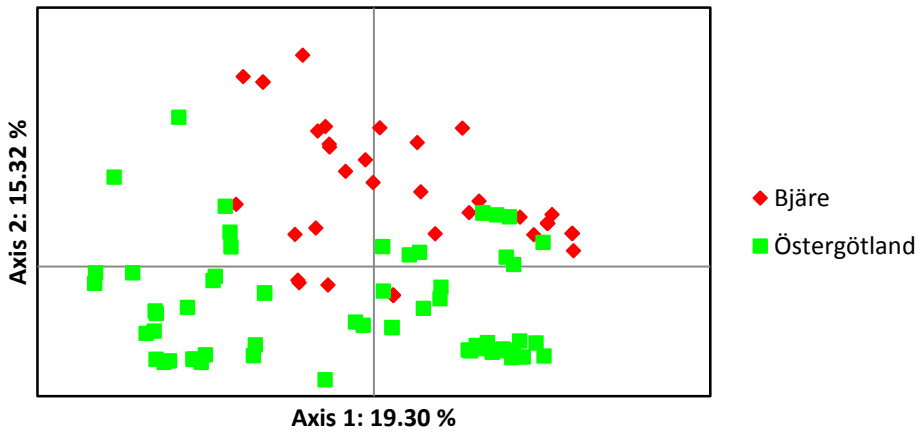

Figure 23: Principal Coordinate Analysis (PCoA) of *Phytophthora infestans* isolates, illustrating the genetic pattern between populations from Bjäre and Östergötland.

6 Discussion

The results show clear differences between the fields and locations in both the aggressiveness tests and the genotyping, which has never been found in earlier studies in Sweden (Lehtinen *et al.*, 2009; Brurberg *et al.*, 2011; Sjöholm *et al.*, 2013).

The overall G/N ratio was on a moderate level, making it difficult to decide whether a clonal reproduction or a sexual reproduction is dominating in the two regions. It could be a mix of those reproduction systems, even though the overall F_{IS} values indicate an increase in heterozygosity. The highest genotypic diversity quantified as the G/N ratio was observed in Östergötland. In this study, differences in single alleles of the multilocus genotypes were enough to distinguish unique genotypes, whereas in other studies, genotypes are often grouped into clonal lineages (e.g. Cooke *et al.*, 2012a). Grouping genotypes into lineages will affect the outcome, especially in regions where clonal lineages can be suspected to dominate. Yuen & Andersson (2013) states that, depending on the factors measured and how the results are interpreted, conclusions for either clonal reproduction or sexual reproduction can be drawn.

The number of genotypes could have been overestimated when calculating the G/N ratio, because of difficulties in clone correcting the dataset due to missing values. Genotypes with missing values were sometimes interpreted as copies when the surrounding genotypes were the same in all other loci, but for some loci, e.g. D13, with a high allelic diversity, the genotypes were kept as unique genotypes. The most important conclusion is still that the lower G/N ratio for Bjäre indicates more clonal populations, whereas in Östergötland there are indications for a higher impact of sexual reproduction. However, the relation between the observed heterozygosity (H_o) and the expected heterozygosity (H_e) shows a different result, where the smallest difference between H_o and H_e was seen in one of the fields in Östergötland (field 4).

Regarding the F_{IS} values when fields were grouped by locations, there was an overall positive mean value, indicating a low genotypic variation within locations.

This low variation can be explained by larger geographic distances between the populations when fields were grouped by location. The genotypes would remain within the regions, rather than travel between them and little outbreeding is therefore occurring.

What was the reason for the lower genetic diversity found on Bjäre? The genotypic diversity was much lower than previous studies from Sweden have shown. One explanation could be the sampling methodology. Studies and surveys around Europe are often based on a few samples per field (e.g. 1-2 isolates) and a large number of fields (e.g. Knapova & Gisi, 2002; Brurberg *et al.*, 2011). According to Meier-Runge *et al.* (2014), the method with just a few samples from many places is a good method to detect dominating clonal lineages. In this study, only four fields were sampled, but with a high number of isolates (50) per field, over two foci. *Phytophthora infestans* has a rapid disease development with a very short time between infection and the start of asexual spread via sporangia and/or zoospores (Widmark *et al.*, 2007). Five samples per plant were collected, increasing the risk of sampling clones from nearby plants. Also, distinct disease foci in a field is an indication of clonal spread of *P. infestans*. Such foci were observed in the fields on Bjäre.

A Dutch study conducted by Zwankhuizen *et al.* (2000) showed that the primary source of inoculum can vary between years. Weather conditions are suggested to be the reason for these variations. In dry years, oospore derived infections will be most evident, while in wet years, populations will be dominated by a few aggressive genotypes derived from infected tubers, which will spread rapidly and mask a sexual reproduction. Infections from oospores require moisture, but too much water will decrease the germination of oospores (Van Bekkum *et al.*, 2007). This means that even though oospores are seen as an important source of inoculum in Sweden, there could be years when an asexual reproduction is favoured over sexual reproduction. In fact, differences in oospore formation have been observed between locations and seasons, according to Andersson *et al.* (2009). A few genotypes that happened to be of the same mating type on Bjäre could have been spread asexually as the result of the wet spring this year. The mild winter in 2014/2015 might also have favoured volunteer tubers in the fields of Bjäre, which could have resulted in early infections of *P. infestans*.

The start of the late blight epidemics in Östergötland is usually later compared to Bjäre. Most potato fields in Östergötland are believed to be infected by wind borne source of inoculum. The start of the epidemic this year and the evenly distributed infections of *P. infestans* also indicates an asexual spread. However, the mating type distribution and the high genotypic variation in Östergötland (see chapter 5.3) indicates involvement of a sexual recombination. Widmark *et al.* (2007) claims that several independent infections from different sources (tubers,

oospores and windborne inoculum) are possible in the same field. Any or all of these events could have been the reason for the high genotypic variation, especially in field 4 (Östergötland). Field 4 was the most heavily infested of all fields and the symptoms were much older with dry necrotic spots. It is noteworthy that potato has not been cultivated for the last 7-10 years in the fields from Östergötland, making it unlikely for oospores in the soil being the primary source of inoculum. The observed high genotypic variation generated through sexual recombination could have survived between seasons in tubers. The same theory was explained by Zwankhuizen *et al.* (2000), where a sexual reproduction was reported in fields with no history of potato production or a very large crop rotation. The late blight susceptible potato cultivar Solist was planted in both fields of Östergötland. Whether the seed potato came from the same batch is unknown. There have also been discussions about whether or not a single tuber can carry several genotypes, and Widmark *et al.* (2007) claims that it is unlikely to happen. However, a recent study has proven that a single tuber can actually carry many genotypes (Andersson, personal communication).

Oospores in the soil can continuously germinate during the growing season, introducing new genotypes by releasing sporangia, especially during wet periods (Van Bekkum *et al.*, 2007). On Bjäre, it would have been desirable to take several samplings at different times to see if the variation would have changed throughout the season. However, several samplings were not possible in the fields of Bjäre in this study, since the fields were harvested just shortly after the first findings.

The genotyping indicated not only a sexual reproduction in Östergötland, but also on Bjäre. With the lack of good crop rotation practises on Bjäre in combination with no fungicide treatments, expectations for oospore production were high. Both a Finnish and a Dutch study indicate a short crop rotation being the reason for epidemics derived from oospores (Lehtinen & Hannukkala, 2004; Li *et al.*, 2012), where a high number of sexually produced genotypes reduce the dominance of clonal lineages (Li *et al.*, 2012).

The fact that exclusively A2 isolates were sampled in both fields on Bjäre is very hard to explain, since the results in this study supports earlier studies which indicate a presence of sexually reproducing populations of *P. infestans* in this region. Both mating types were present in both fields of Östergötland. There are discussions in the literature whether or not the frequencies of both mating types have to be equal for a sexual recombination. Both mating types can be present in approximately the same percentage (e.g. in France) and still not generate a sexual reproduction (Montarry *et al.*, 2010). According to Sjöholm *et al.* (2013), the frequency is of less importance since one single mating can result in huge amounts of oospores.

Before the 1990's, late blight was not considered a problem on Bjäre since the fields were usually harvested before any symptoms appeared (Andersson *et al.*, 2009). This has changed lately, where earlier and earlier infections are observed. Oospore derived infections and the narrow crop rotation could be the reason (Bødker *et al.*, 2006; Hannukkala *et al.*, 2007; Andersson *et al.*, 2009). Another reason for earlier outbreaks could be climatic changes, as described in Wiik (2014). Different conditions in temperature and humidity have been reported to favour or disfavour different genotypes and can explain why there are different populations in different countries (Mariette *et al.*, 2015). Even though it might be difficult to predict the population structure of *P. infestans* in a changing climate, identification of clonal lineages and their behaviour helps to choose the optimal control methods. However, in Sweden and the other Nordic countries, it is of very little help since the genotypic diversity is much higher than in most parts of Europe, which makes it difficult to achieve optimal disease control.

What is the reason for the big impact of sexual reproduction of *P. infestans* in the Nordic countries, when in Europe it only happens occasionally? Climatic reasons have been suggested, where the cold climate in the Nordic countries is believed to limit an early asexual spread from infected tubers, giving an advantage of *P. infestans* to spread via oospores. The cold winters may also prevent germination and biological degradation of oospores between seasons and this has also been observed in Mexico (the Toluca Valley) where winters are cold and dry (Widmark *et al.*, 2007). This will lead to a higher number of germinating oospores when the crop is planted in the next spring, making an early infection possible.

Sexual recombination can generate aggressive genotypes. In this study, a higher aggressiveness of the isolates from Bjäre compared to the isolates from Östergötland was observed when referring to the latency period, but no significant differences were found between the regions in the lesion growth rate and the number of spores per lesion. The latency period seems to be a key in the aggressiveness of *P. infestans*, where the first genotypes to be colonizing the host plant will have an advantage over later colonizers (Yuen, 2012). Sporulation capacity (number of spores per lesion) has been observed to be sensitive to test conditions, which could have an influence on the results (Lehtinen *et al.*, 2009). Larger variations in aggressiveness were observed between fields than between locations, indicating the importance of local phenotypes. Especially field 3 (Östergötland) was significantly different from the other fields regarding the lesion growth rate. There was also a significant difference in the latency period between fields.

The isolates from Bjäre were collected a couple of months earlier than the isolates from Östergötland, which means that they had to be continuously cultured in the laboratory for a longer period. This can lead to losses in aggressiveness, as claimed by Cooke *et al.* (2006). However, the populations of Bjäre still tended to

show a higher aggressiveness than the populations of Östergötland. Instead, the result indicates the importance of the condition of the leaf samples. There were some problems with the isolates from field 4 in Östergötland. The lesions in the field were much drier with less sporulation than in the other fields in the study. This reduced quality resulted in a small sample size of field 4 for the aggressiveness tests. Because of this, the distribution curve also showed a positive skewness, indicating a higher proportion of high values. This may have resulted in an overestimation of the aggressiveness in field 4. There was also a general bacterial contamination of the isolates from all fields, which further reduced the amount of samples that could be used in the aggressiveness tests. In the future, it would be better to apply the sampling method described by Grönberg *et al.* (2011), where collected samples are directly treated in the field, by e.g. immediately transferring single lesion leaflets to water agar.

It is important to keep in mind that laboratory tests cannot be directly related to the field situation, since laboratory and field conditions are different (Cooke *et al.*, 2006). As an example, the sporangia infection of leaflets in the laboratory is made with hundreds of sporangia per leaflet, whereas under field conditions one or a few sporangia may land on the leaflet and not all of them will cause an infection. However, the aggressiveness tests done under laboratory conditions show differentiations between populations that can be reflected in the field (Andersson, 2007).

Since the population structure of *P. infestans* is constantly changing, it is difficult to say whether the pathogen population on Bjäre in general is more aggressive than in Östergötland. The population on Bjäre might have been more aggressive this year when referred to the latency period, but that does not exclude the fact that this aggressiveness will be lost (or increased) the next year when new genotypes appear. Also, even though sexual reproduction allows earlier infections, it does not necessarily lead to higher aggressiveness. This was observed in a Finnish study, where the disease development in terms of epidemics remained the same even though infections appeared earlier in the season (Hannukkala *et al.*, 2007).

To determine whether *P. infestans* is more aggressive on Bjäre, a study over several years has to be made. Another thing that needs to be further investigated is the appearance of sexual reproduction in Östergötland, to see if oospores will be established between years and if there is a risk in terms of more aggressive genotypes as well as earlier infections. Earlier infections will require earlier spraying programmes, which means an increase in the use of fungicides (Hannukkala *et al.*, 2007).

An asexual spread of the pathogen is easier to predict than infections derived by oospores, e.g. with decision support systems based on weather data (Hannukkala *et al.*, 2007). With more frequent infections from oospores, today's systems will not work and the challenge for the future is to come up with solutions for predict-

ing oospore driven infections. Most important in order to decrease the risk of early attacks from oospores is to improve the crop rotation. In a Danish survey, a decrease in early epidemics of *P. infestans* was seen when the crop rotation was improved (Bødker *et al.*, 2006). On Bjäre, potato is grown in monoculture and many farmers also use seed tubers from their own fields for the next season. The results from this study also indicates the importance of good seed quality.

7 Conclusions

There are indications for a mixed reproduction system of *P. infestans* both on Bjäre and in Östergötland. The early infections that were observed on Bjäre could have been derived from oospores or from tuber infections. In Östergötland, late blight symptoms were found much later in the growing season, indicating the primary source of inoculum being caused by asexual sporangia. However, the population in Östergötland showed a higher genotypic diversity than the population on Bjäre, contrary to what was stated in the hypothesis. Weather conditions favouring an asexual spread of a few oospore driven genotypes on Bjäre this year and the sampling methodology could explain the little genotypic variation found on Bjäre. A later sampling date or several samplings throughout the season would probably have increased the genotypic variation on Bjäre. In Östergötland, the observed higher genotypic variation was probably due to several infection sources, where oospore infections might have been involved during the season as a result of both mating types being present in infested seed tubers. The later sampling date in the disease development might have influenced the results.

The isolates from Bjäre showed a higher aggressiveness compared to the isolates from Östergötland when referring to the latency period, but there were no significant differences in lesion growth rate and number of spores per lesion. The relative epidemiological importance of the analysed aggressiveness factors can be discussed.

As seen in this study, there are many factors that will affect the success of *P. infestans*; establishment, development and spread of the pathogen can vary between regions and years. This study shows that a sexual reproduction can also be of importance in fields with good crop rotation practices in an area that is normally dominated by an asexual spread of the pathogen. To gain a full understanding of the development of late blight in the two regions, further studies ideally over several years are needed.

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