Eesti Maaülikool
Estonian University of Life Sciences

DIVERSITY IN BALTIC POPULATIONS OF POTATO LATE BLIGHT PATHOGEN *PHYTOPHTHORA INFESTANS*

KARTULI-LEHEMÄDANIKU TEKITAJA *PHYTOPHTHORA INFESTANS* BALTIMAADE POPULATSIOONIDE MITMEKESISUS

RIINU KIIKER

A Thesis
for applying for the degree of Doctor of Philosophy in Agriculture

Väitekiri
filosoofiadoktori kraadi taotlemiseks põllumajanduse erialal

Tartu 2020

Eesti Maaülikooli doktoritööd

Doctoral Theses of the
Estonian University of Life Sciences

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Institute of Agricultural and Environmental Sciences Estonian University of Life Sciences

According to verdict No 6-14/14-2, 03.07.2020, the Doctoral Committee of the Agricultural and Natural Sciences of the Estonian University of Life Sciences has accepted this thesis for the defence of the degree of Doctor of Philosophy in Agriculture.

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Defence of the thesis:
Estonian University of Life Sciences, Fr. R. Kreutzwaldi 5, Tartu
on September 10, 2020 at 12.15 (web-based).

The English language was edited by Hannah Joy Kennedy.
The Estonian language was edited by Dr. Liina Jakobson.
Publication of this thesis is supported by the Estonian University of Life Sciences.

ISSN 2382-7076
ISBN 978-9949-698-42-4 (pdf)

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LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following papers, which are referred to by Roman numerals in the text. The papers are reproduced by kind permission from the copyright owners.

Kiiker, R., Hansen, M., Williams, I.H., Cooke, D.E.L., Runno-Paurson, E. 2018. Outcome of sexual reproduction in the *Phytophthora infestans* population in Estonian potato fields. European Journal of Plant Pathology, 152, 395–407. doi: 10.1007/s10658-018-1483-y.

Kiiker, R., Skrabule, I., Ronis, A., Cooke, D.E.L., Hansen, J.G., Williams, I.H., Mänd, M., Runno-Paurson, E. 2019. Diversity of populations of *Phytophthora infestans* in relation to patterns of potato crop management in Latvia and Lithuania. Plant Pathology, 68, 1207–1214. doi: 10.1111/ppa.13030.

Runno-Paurson, E., **Kiiker, R.,** Joutsjoki, T., Hannukkala, A. 2016. High genotypic diversity found among population of *Phytophthora infestans* collected in Estonia. Fungal Biology, 120, 385–392. doi: 10.1016/j.funbio.2015.11.008.

Table 1. Authors´ contribution to each paper.

Paper	Idea and study design	Experiments	Data analysis	Manuscript writing
I	ERP, **RK**	**RK**, ERP, MH, DELC	**RK**, ERP, DELC	**RK**, ERP, IHW, MH, DELC
II	**RK,** ERP	**RK**, ERP, DELC	**RK**, DELC, JGH, ERP	**RK**, ERP, IS, AR, DELC, JGH, IHW, MM
III	ERP	ERP, **RK**, AH, TJ	ERP, **RK**	ERP, **RK**, TJ, AH

RK – Riinu Kiiker; AH – Asko Hannukkala; AR – Antanas Ronis; DELC – David E. L. Cooke; ERP – Eve Runno-Paurson; IHW – Ingrid H. Williams; IS – Ilze Skrabule; JGH – Jens G. Hansen; MH – Merili Hansen; MM – Marika Mänd; TJ – Tiina Joutsjoki

ABBREVIATIONS

Avr	avirulence
E_5	evenness
F_{ST}	Wright's fixation index
H	gene diversity
Hs	Shannon diversity index corrected for sample size
HWE	Hardy-Weinberg equilibrium
I_A	index of association
MLG	multilocus genotype
MSN	minimum-spanning network
mtDNA	mitochondrial DNA
PCoA	principal coordinate analysis
PCR	polymerase chain reaction
$\bar{r}_d$	standardised index of association
R gene	resistance gene
RXLR	Arg-Xxx-Leu-Arg (Arginine-Any aminoacid-Leucine-Arginine)
SSR	simple-sequence repeat

1. INTRODUCTION

Late blight, caused by the oomycete *Phytophthora infestans* (Mont. de Bary 1876), is one of the most economically damaging plant diseases and certainly the most devastating disease in potato. *P. infestans* caused the Irish Potato Famine in the 19th century, which resulted in the death of over one million people and extensive emigration from Ireland. Charles Darwin wrote in 1845 "What a painfully interesting subject it is," referring to potato late blight. More than 170 years after the disaster, the pathogen still poses a significant threat to global food security and is one of the most studied plant pathogens.

The estimated total costs and losses of the blight disease are close to € 5 billion per year globally (Haverkort et al., 2009, 2016). The application of fungicides is currently the most widely used method to control the disease, however, this involves high costs, about 10% of the total production cost in developed countries, as well as negative environmental effects (Haverkort et al., 2008, 2009). More applications of fungicides are needed because most potato cultivars grown commercially are early maturing cultivars, which have higher susceptibility to late blight (Cooke et al., 2011; Runno-Paurson et al., 2013a). There are indications of earlier outbreaks of late blight in northern Europe which require more frequent fungicide treatments throughout the growing season to control the disease. Part of the cause is likely because primary inoculum source oospores are present year-round in the fields in recent decades (Hannukkala et al., 2007; Runno-Paurson et al., 2013b; Yuen and Andersson, 2013). Contrary to the current state of high fungicide input in potato production, the EU Commission has clearly stated in the Communication on the European Green Deal (2019) the necessity to significantly reduce the use and risk of chemical pesticides in agriculture and increase the area under organic farming in Europe.

There are clear indications that genetic changes have occurred in *P. infestans* populations worldwide in the last few decades, which coincide with severe late blight outbreaks on potato and tomato crops in Europe, USA and Asia (Chowdappa et al., 2015; Cooke et al., 2012; Fry et al., 2013; Li et al., 2013b; Montarry et al., 2010). Changes in the pathogen populations most likely occur due to adaptation to environmental conditions, overcoming host resistance, or becoming resistant to pesticides used for controlling the disease. According to the Fungicide Resistance Action

Committee (FRAC), *P. infestans* is classified as a medium risk pathogen to developing resistance to pesticides, particularly to phenylamide fungicides (FRAC, 2020). Therefore, the effectiveness of integrated disease control strategies and the evolution of future pathogen populations needs to be considered (Ritchie et al., 2019).

The changes in pathogen populations have a direct effect on the breeding and deployment of resistant cultivars, the performance of disease warning systems, and the efficacy of pesticides. Production sustainability requires efficient control of late blight which should be adapted with up-to-date knowledge about the pathogen population. In case of emergence of new clonal lineages of *P. infestans* which are insensitive to certain fungicides or which break down host resistance, countermeasures can be taken to prevent further spread of these populations. The transfer of up-to-date knowledge of pathogen populations for optimization of practical late blight control is already done through national and international networks, for example EuroBlight in Europe (EuroBlight, 2019) and USABlight in USA (USABlight, 2019). Dissemination of research findings on the current pathogen situation (emergence and spread of *P. infestans* clones and genetically diverse genotypes) to growers, advisors, breeders, and agrochemical companies has already improved awareness and helped to slow the spread of fungicide insensitive clonal lineages.

Results of genotyping have revealed that most of the populations in Great Britain, France, Switzerland, Netherlands, Belgium, as well as in North America, India, and China are clonal with a few dominating genotypes in the populations, with sexual reproduction events being rare (Chowdappa et al., 2015; Cooke et al., 2012; Fry et al., 2013; Gisi, et al., 2011; Hu et al., 2012; Li et al., 2012, 2013b; Montarry et al., 2010). The clonal populations in western Europe are thriving because of large areas of commercial potato production with a few dominating host cultivars and mild winters, which enable the pathogen to survive from one season to another through asexual structures in the plant debris, volunteer plants, and potato tubers (Cooke et al., 2011). However, changes in the population genetic structure and new emerging clones (for example, *EU_36_A2, EU_37_A2,* and *EU_41_A2*) are identified in Europe every season. Each clone has its own combination of characteristics (virulence, aggressiveness, fungicide insensitivity, etc.), which make it successful (EuroBlight, 2019).

Conversely, recent studies in the Nordic countries (Finland, Sweden, Norway, Denmark), Russia, and Poland have shown high genotypic diversity in *P. infestans* populations with many different genotypes present, indicative of the relevance of sexual reproduction in these populations (Brurberg et al., 2011; Brylińska et al., 2016; Chmielarz et al., 2014; Elansky et al., 2015; Sjöholm et al., 2013). In northern Europe, during the winter the temperature drops below 0°C and snow usually covers the ground which limits the asexual survival of the pathogen from one season to another. Fortunately for the pathogen survival, during the sexual phase of the pathogen life cycle, two isolates with opposite mating types (A1 and A2) mate and produce oospores, which can survive unfavourable conditions. Sexual reproduction increases pathogen fitness via higher genetic diversity and production of oospores as a source of primary inoculum in the soil for the next growing season (Barton and Charlesworth, 1998; Goodwin, 1997; Widmark et al., 2011).

Potato is one of the least vulnerable food crop to the unfavourable weather conditions and climatic change; therefore, it is a key crop for global food security (Kruus et al., 2018; LIFE AgriAdapt, 2020). But still, potato late blight remains a serious threat to potato production in the Baltic countries (Estonia, Latvia, Lithuania) causing considerable yield loss in weather conditions favourable for the disease. Estonian populations collected in 2001–2007 of *P. infestans* have previously been investigated with several phenotypic (mating type, virulence, metalaxyl sensitivity) and molecular markers (RG57 fingerprints, mtDNA haplotypes) (Runno-Paurson et al., 2009, 2010a, 2010b, 2012, 2013b, 2014). However, information on Latvian and Lithuanian populations of *P. infestans* is scarce and limited to only phenotypic characterization (Aav et al., 2015; Bebre et al., 2004; Runno-Paurson et al., 2015; Valskyte et al., 2003). Studies have shown that virulence pathotype structure of *P. infestans* in the Baltic population is highly diverse with several pathotypes present (Aav et al., 2015; Runno-Paurson et al., 2009, 2010a, 2012, 2014, 2015, 2016). Potato fields were threatened by a considerably high proportion of metalaxyl-resistant isolates identified from conventional production fields in Estonia in the 2000s and Lithuania in the 1990s (Runno-Paurson et al., 2009, 2010a, 2012, 2014; Valskyte, 2003). Studies from the 2010s have shown an increase of metalaxyl-sensitive isolates in the Baltic *P. infestans* population due to more sensible use of metalaxyl-based fungicides (Aav et al. 2015; Runno-Paurson et al. 2015; 2016). Additionally, population studies from the 2000s and 2010s show that

the frequency of A1 and A2 mating types is almost equal. The occurrence of both mating types in the same potato field indicates continuously high potential for sexual recombination of the pathogen, which increases the risk of oospore-derived infections (Aav et al. 2015; Runno-Paurson et al. 2009, 2010a, 2011, 2012, 2014, 2015).

The current research results are valuable and comparable with other populations on a larger scale because standardised methods were applied. Until now, the Baltic *P. infestans* population had not been characterised with highly informative simple-sequence repeat (SSR) markers. SSR marker panel and protocols used for genotyping in Europe are harmonised between laboratories and the results are quality checked and curated before uploading to the Euroblight database. Further research is needed to obtain knowledge regarding the role of sexual reproduction in the pathogen population in the region, as well as to assess the spread and survival of asexual clones adapted to local conditions. Thus, there is a continuous need for pathogen population studies in order to stay current with the population's spatio-temporal situation in order to improve disease management strategies and advise potato breeders, advisors, and farmers accordingly.

2. REVIEW OF THE LITERATURE

2.1. Potato production

Potato (*Solanum tuberosum* L.) is a highly attractive crop since it combines high yield potential with a high nutritional value. Potatoes are a good source of energy, minerals, proteins, fats, and vitamins (King and Slavin, 2013). It is ranked as the fourth most cultivated and consumed crop globally, after maize, wheat, and rice, being a considerable food source for more than a billion people worldwide (FAOSTAT, 2019). Nearly 370 Mt of potatoes was produced worldwide on 17,6 Mha in 2018 (FAOSTAT, 2019). However, while countries with high-input agriculture (USA, France, Germany) can achieve average potato yields greater than 45 t ha^{-1}, the average potato yields are 20 t ha^{-1} worldwide (Koch et al., 2020; FAOSTAT, 2019). Potato is a key crop for global food security, as it gives rise to more nutritious food, more quickly, on less land than other cultivated food crops and even in severe conditions and additionally is the least vulnerable food crop to the unfavourable weather conditions and climatic change (Kruus et al., 2018; LIFE Agri-Adapt, 2020).

In the Baltic countries, potato is one of the most important locally grown food crops. The average potato consumption from 2000 to 2013 was 117, 111, and 110 kg per capita per year in Latvia, Lithuania, and Estonia, respectively (FAOSTAT, 2019), which is considerably higher than in most European countries. In the Baltic countries, particularly in Latvia and Lithuania, a large proportion of potato growing farms are small in size and having limited financial resources to purchase certified seed potato or to apply sufficient chemical control against late blight. Furthermore, they do not grow varieties with better resistance to the disease or practice adequate crop rotation. In favourable conditions the potato late blight pathogen *P. infestans* can reproduce and spread freely, causing substantial crop loss (Aav et al., 2015; Ronis et al., 2007; Runno-Paurson et al., 2015). In contrast, large conventional production farms use high-quality certified potato seeds, plant no more frequently than every third year, and apply adequate fungicides 4–7 times per season (Aav et al., 2015; Runno-Paurson et al., 2015).

2.2. Potato late blight

The late blight disease caused by *P. infestans* is regarded as one of the most devastating of plant diseases and certainly the most devastating disease in potato (Agrios, 2005). It has significant effect on agriculture as the estimated total costs and losses of the disease are close to € 5 billion per year globally (Haverkort et al., 2009, 2016). It can totally destroy potato foliage (Figure 1), prevent tuber maturing, and infect potato tubers; it also causes severe losses in tomato (Fry et al., 2015). The genetic flexibility and the coevolution of late blight pathogen populations in Central and South America with host species among wild *Solanum* species have yielded a notably high genetic diversity in populations of this pathogen. Therefore, *P. infestans* is able to react rapidly to selection pressures within agricultural systems and under changing climate conditions, resulting in global late blight epidemics (Fry, 2008).

The first late blight attacks were detected in the early 1840s in the eastern United States (Stevens, 1933). However, the most notorious outbreak of the disease was observed in Europe in the summer of 1845, when the destruction of potatoes led to massive famine, especially in Ireland where farmers were dependent on potatoes (Bourke, 1993). In 1845, epidemics were severe on both continents, farmers and peas-

Figure 1. Potato late blight infection on potato foliage.

ants suffered, and scientists were debating about the cause. Late blight still poses a real threat to agriculture globally; significant changes have occurred in *P. infestans* populations worldwide recently, which concur with serious late blight outbreaks on potato and tomato crops (Chowdappa et al., 2015; Cooke et al., 2012; Fry et al., 2013; Li et al., 2013b; Montarry et al., 2010).

2.3. Potato late blight threat in the Baltic countries

The initial inoculum source from fields which are unprotected against late blight is a serious threat to seed and large production fields especially in the cool and moist conditions favourable for late blight (Ronis et al., 2007; Runno-Paurson et al., 2019). Depending on weather conditions, the disease incidence and severity are highly variable between years, ranging from very low (5%) to up to 100%. The local *P. infestans* population can infect and destroy potato foliage of most of the cultivars introduced and grown in the Baltic countries (Valskyte et al., 2003; Runno-Paurson et al., 2019). Average yield losses of 20–25% have been recorded from Estonian potato production fields with even higher losses in fields with inadequate disease control (Runno-Paurson et al., 2010a). In contrast with observations in Finland and Estonia (Hannukkala et al., 2007; Runno-Paurson et al., 2013b), no records have been made of changes in the timing of the first symptoms of late blight in Latvia and Lithuania. Also, in Latvia and Lithuania the symptoms of late blight do not necessarily start from the lower plant leaves (2016 letters from Dr. I. Skrabule and Dr. A. Ronis to me, unreferenced), which is contrary to oospore initiated late blight infections and epidemics recorded in Estonia and the Nordic countries (Brurberg et al., 2011; Hannukkala et al., 2007; Runno-Paurson et al., 2009; 2010a; 2012).

According to the national data from Latvia (State Plant Protection Service, 2019) and Lithuania (State Plant Service, 2019), around 50 potato cultivars are grown in these countries every season, resulting in high virulence race diversity in *P. infestans* populations (Aav et al., 2015; Runno-Paurson et al., 2015). Because the growing season is relatively short and to obtain a higher price for marketable potatoes, growers prefer to plant early maturing potato cultivars, which are unfortunately rather susceptible to late blight (Asakavičiūtė et al., 2009; Runno-Paurson et al., 2013a; 2019). Cultivars bred by the breeding companies from western Europe having commercially favourable features (quality, yield, earliness

etc.) are well distributed to the potato growing farms in the Baltic countries. Studies have shown lower foliar late blight resistance of imported cultivars in the Baltic conditions as claimed by the breeding companies (Runno-Paurson et al., 2013a; 2019). Resistence depends on the regional weather and agrotechnological conditions as well as *P. infestans* population characteristics (Runno-Paurson et al., 2013a; 2019). Therefore, late blight control strategies must be optimized.

Continuous effort is allocated to national potato breeding programs in the Baltic countries for developing potato cultivars suitable for local weather and growing conditions, improved resistance to pests and diseases, and excellent quality traits to satisfy consumer requirements (Asakavičiūtė et al., 2009; Tähtjärv, 2016). Unfortunately, the availability and distribution of local cultivars does not satisfy producers needs because of low multiplication rates (Runno-Paurson et al., 2019; Tähtjärv, 2016). Limited financial resources also preclude use of high-quality seed potato at some potato growing farms thus self-propagated seeds of uncertain quality are often used.

2.4. The success of *Phytophthora infestans*

P. infestans is a heterothallic oomycete, with two mating types (A1 and A2). Oomycetes are eukaryotic organisms that resemble filamentous fungi, but instead are phylogenetically related to diatoms and brown algae in the stramenopiles (Gunderson et al., 1987; Thines, 2014). It has a near-obligate hemibiotrophic life style under natural and agricultural conditions. The ability of sporangia or zoospores to infect and colonise host tissue (aggressiveness) combined with efficient spread and survival from one season to another (fitness) are key adaptive traits, which determine the success of particular *P. infestans* genotypes (Andrivon et al., 2013; Mariette et al., 2016). The ability to overcome host resistance, acquire fungicide resistance, or fast responses to altered environmental conditions are also important determinants of evolutionary success of the pathogen (Andrivon et al., 2011; Cooke et al., 2011; Hannukkala et al., 2007).

For nearly 100 years after initiating the epidemic in Europe in the mid-nineteenth century, *P. infestans* was thought to be an asexual organism (Goodwin et al., 1994). In the 1950s it was discovered that the *P. infestans* population in Toluca Valley in central Mexico was reproducing

sexually (Galindo and Gallegly, 1960; Niederhauser, 1956). It was not until late in the twentieth century that the A2 mating type was detected outside of central Mexico, in Europe (Hohl and Iselin, 1984).

The hypothesis of *P. infestans* originating from central Mexico was supported by the discovery of both mating types (A1 and A2) and high genotypic diversity in the population (Grünwald et al., 2001; Grünwald and Flier, 2005). A study by Gomez-Alpizar et al. (2007) on mitochondrial and nuclear gene genealogies of isolates from several locations worldwide came to conclusion that *P. infestans* had a South American origin instead. However, later on Goss et al. (2014), using a wider set of isolates and including more close relatives of *P. infestans*, again came to the conclusion that the pathogen originates from the highlands of central Mexico.

A single recombination event may give rise to a pathogen genotype with a set of advantageous traits that enables all its asexually derived descendants (with further minor genetic variation due to mutation and mitotic recombination) to spread and become a dominant clone (Goodwin, 1997). Such clonal lineages of *P. infestans* are very successful and cause serious damage globally, for instance in the United States (Fry et al., 2013), India (Chowdappa et al., 2015), China (Li et al., 2013b), and western Europe (Cooke et al., 2012; Li et al., 2012; Schepers et al., 2018).

P. infestans is a successful plant pathogen which continues to threaten world food security, therefore, its genome sequence is studied closely (Cooke et al., 2012; Fry, 2008; Haas et al., 2009). *P. infestans* has a large (240 Mb) genome with alternating gene-dense regions and repeat-rich expanded regions. Gene-dense regions have conserved gene order across *Phytophthora* species and dynamic repeat-rich regions include complex families of effector genes encoding secreted proteins that modify host physiology and facilitate colonisation (Haas et al., 2009). These repeat-rich regions promote the plasticity of effector genes and enhance the genetic variation in *P. infestans*. Most notable among these are numerous RXLR (including avirulence (Avr) factors) and Crinkler (CRN) cytoplasmic effectors (Whisson et al., 2007). As Avr factors are effectors recognized by the plant, if a host plant is carrying the corresponding late blight resistance (R) gene it responds with a hypersensitivity reaction (HR) in a so-called "gene-for-gene" action. For instance, *P. infestans* pathotypes may contain Avr3a gene and then fail to heavily infect potato varieties with the corresponding R3a gene (Armstrong et al., 2005). Re-

sistance breeding offers great potential, but the durability of resistance conferred by R genes has been continually challenged by the evolution of new virulence traits within pathogen populations (Fry, 2008).

2.5. *Phytophthora infestans* life cycle

2.5.1. Asexual life cycle

The asexual life cycle of *P. infestans* enables rapid population growth in susceptible host tissue, which can cause severe epidemics of late blight. Entire fields of potato foliage can be destroyed within a few weeks or even days. Sporangia in free water germinate directly by forming a germ tube at higher temperatures (optimum 20–25°C), or by releasing biflagellated zoospores at lower temperatures (optimum 10–15°C). The biflagellated (tinsel-type and whiplash flagella) zoospores are motile for a short time (often less than 60 min) before encysting to a surface. Encysted zoospores germinate directly via a germ tube to penetrate leaf or stem tissue. Macroscopically, there are generally no visible necrosis symptoms for at least 2 days (trait of a hemibiotroph), depending on host susceptibility and environmental conditions. Within another day or two, under favorable conditions, sporangiophores that grow from infected tissue may produce more than 300 000 sporangia per lesion, which can be aerially dispersed to other plant tissues or to other potato fields many kilometers away (Fry, 2008; Fry et al., 2013; Mariette et al., 2016b).

Pathogen functions essentially as an obligate parasite. Detached sporangia can survive in soil for weeks, depending on environmental conditions (Andrivon, 1995). Sunlight is detrimental to sporangia, but under cloudy conditions, sporangia in the air can survive for several hours (Minogue and Fry, 1981; Mizubuti et al., 2000). In infected plant tissue, survival of vegetative mycelium is dependent on survival of the host. Potato tubers in the temperate zones are often stored overwinter in temperature-controlled (4 to 10°C) conditions, which enables viability of the pathogen in an infected tuber.

There are several mechanisms by which *P. infestans* can be dispersed. Short-distance dispersal is achieved by wind and water-splash of sporangia during irrigation events or during rainy weather. Thus, it is possible for viable sporangia to be deposited meters, or even thousands of meters away from its origin. Dispersal over longer distances across countries

requires human activity; the most efficient method for spreading this pathogen is transportation of infected potato seed tubers, tomato fruit, and infected transplants (Hu et al., 2012). For instance, in 2009 infected tomato transplants shipped in the northeastern United States were involved in a severe epidemic mainly caused by a specific *P. infestans* genotype, US-22, which infects potato and tomato (Hu et al., 2012).

2.5.2. Sexual life cycle

Before the 1980s, the A2 mating type was absent from Europe, where *P. infestans* populations were clonal and reproduced only asexually. The A2 mating type was first reported in Switzerland in 1981 (Spielman et al., 1991) and six years later, in 1987, in Estonia and Latvia (Vorobyeva et al., 1991; Bebre et al., 2004). Unfortunately, previous knowledge of pathogen populations in Lithuania is scarce and the A2 mating type was not reported until 2010 (Runno-Paurson et al., 2015). In the 1990s, sexual reproduction of the pathogen and the presence of soil-borne inoculum was confirmed in the Netherlands and Sweden (Andersson et al., 1998; Drenth et al., 1995). New genotypes were more competitive and adaptable to the host and environment, therefore, they spread quickly across Europe displacing the old clonal lineage (Gisi and Cohen, 1996; Spielman et al., 1991). After the spread of the A2 mating type and subsequent population changes due to sexual reproduction events at the end of 20th century, genetically highly diverse populations have been detected in Europe, for example in the Netherlands (Li et al., 2012), Denmark, Norway, Sweden, Finland (Brurberg et al., 2011; Sjöholm et al., 2013), Estonia (Runno-Paurson et al., 2010), Poland (Brylińska et al., 2016; Chmielarz et al., 2014), and Russia (Elansky et al., 2015).

Sexual reproduction increases pathogen fitness via higher genetic diversity and production of oospores (Figure 2) as a source of primary inoculum in the soil, which can increase the rate of adaptation of a population (Barton and Charlesworth, 1998; Goodwin, 1997; Widmark et al., 2011). In northern regions, it is especially beneficial for *P. infestans* to survive cold winters as oospores which are very resilient to adverse climatic factors and remain viable for many years (Andersson et al., 1998; Drenth et al., 1995; Turkensteen et al., 2000). Oospores can survive dry and cold conditions (Fernandez-Pavia et al. 2004), but moderately high temperatures (>43°C) are lethal (Fay and Fry, 1997). Facultative sexual organisms can switch from asexual to sexual reproduction when

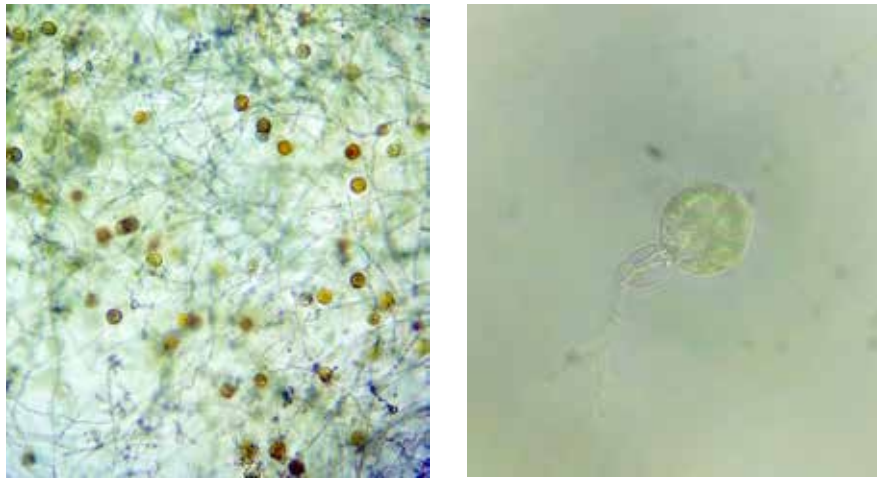


Figure 2. *Phytophthora infestans* oospores.

conditions are poor due to stress caused by environmental changes (for example, temperature instability), nutritive quality of the host tissue, competition, or lack of habitat or host (Hadany & Comeron, 2008).

Sexual reproduction is determined largely by the presence of compatible sexual partners (Chamberlain and Ingram, 1997; Daniele et al., 2003; Hadany and Comeron, 2008; Judelson et al., 2009). To date, few studies have evaluated resistance to the oospore phase of the pathogen cycle when host tissues were co-inoculated with both A1 and A2 isolates. For example, Turkensteen et al. (2000) and Clement et al. (2010) observed a correlation between host resistance and oospore production. Results from Hammi et al. (2001) and Sanchez-Perez et al. (2017) showed that the largest number of oospores were produced in a specific combination of host cultivar with sexually compatible *P. infestans* isolates. Selection for maintaining locally adapted genotypes or heterozygosity in a pathogen population may occur when there is less need for sexual oospores for survival (Gladieux et al., 2015). A recent study revealed that *P. infestans* isolates collected from the Baltic countries had long latent periods and low lesion growth rates relative to isolates from warmer regions of Europe (Mariette et al., 2016b). Such characteristics would likely favour co-infection by other isolates, increasing the probability of sexual reproduction between isolates of opposite mating types (Clement et al., 2012).

2.6. Features used for pathogen population characterization

Pathogen populations are characterized using different phenotypic and genotypic markers. To identify individual genotypes and to explore population diversity, molecular markers have to be used. Several genotypic methods, for instance, analysis of isozymes (Shattock et al., 1986; Tooley et al., 1985), mtDNA and RG57 restriction fragment length polymorphism (RFLP) patterns (Goodwin et al., 1994), as well as amplified fragment length polymorphisms (AFLPs) (Cooke et al., 2003; Flier et al., 2003) have been used. More recently, simple-sequence repeats (SSRs) have been applied (Knapova and Gisi, 2002). SSR markers are neutral, co-dominant, polymorphic, single-locus molecular markers, and are therefore considered to be the most informative and effective markers in pathogen population studies (Cooke and Lees, 2004; Lees et al., 2006). From the beginning of the 21st century SSR markers have been used to characterise *P. infestans* populations all over the world, because the method is commonly available and based on cost-effective, high-throughput, robust and freely available detailed protocols (Chowdappa et al., 2015; Cooke et al., 2012; Cooke and Lees, 2004; Gisi et al., 2011; Knapova and Gisi, 2002; Lees et al., 2006; Li et al. 2013a; Montarry et al., 2010). The SSR marker panel is routinely used for detecting pathogen spread and diversity in Europe and the results are made publicly available via the Euroblight network database (EuroBlight, 2019). The tracking of allele frequencies and distributions over time will advance the understanding of the spatio-temporal dynamics of *P. infestans* populations, as well as help to estimate gene flow and investigate the balance between the forces of natural selection and chance effects of genetic drift and migration (Cooke and Lees, 2004). Combining the up-do-date population genetic data with phenotypic characters, for example, fungicide insensitivity, overcoming host resistance, and aggressiveness, would benefit long-term disease control.

The monitoring of A1 and A2 mating-type ratios is important to aid predictions of the extent of sexual recombination and thus the risk of long-lived oospores serving as primary inoculum sources (Cooke et al., 2011). In addition to its epidemiological impact, sexual recombination is likely to increase the rate of pathogen adaptation (Barton and Charlesworth, 1998), thus reducing the predictability of disease management practices.

Fungicide insensitivity is an essential feature for the pathogen to be successful when fungicides are applied for disease control. Insensitive clonal genotypes may have an advantage to spread and dominate locally or regionally. Since 2004 a very successful metalaxyl-resistant clone, *EU_13_A2,* has spread rapidly across Europe (Cooke et al., 2012; Li et al., 2012; Mariette et al., 2016a) and severe outbreaks have also been reported in India and China (Li et al., 2013b; Chowdappa et al., 2015). Considerably high proportion of metalaxyl-resistant isolates were previously identified from conventional production fields in Estonia in the population studies carried out in the 2000s and in Lithuania in the 1990s (Runno-Paurson et al., 2009, 2010a, 2010b, 2012, 2013b, 2014; Valskyte, 2002). Recently, reduced fluazinam sensitivity has been noted in clonal lineages *EU_33_A2* and *EU_37_A2* in Europe (EuroBlight, 2019; Schepers et al., 2018). Genotype *EU_37_A2* was first detected in the Netherlands in 2013, and occurred infrequently locally in 2014 and 2015. In 2016 *EU_37_A2* was also sampled in United Kingdom, Belgium, and northern France; it further spread to Poland, and in 2017 this genotype reached 14.4% of the total population sampled in Europe (EuroBlight, 2019).

Virulence determination is essential to breeders and plant pathologists willing to use race-specific R genes in breeding programmes and control strategies. Pathogen population monitoring for virulence of the R genes deployed plays a key role in the IPM2.0 control strategy (Kessel et al., 2018). When virulence to a specific R gene is completely absent from the *P. infestans* population, the crops containing this R gene do not need additional protection by fungicides (Kessel et al., 2018). Complex virulence races of *P. infestans* are common to Polish (Sliwka et al., 2006), Nordic (Lehtinen et al., 2007, 2008), Dutch (Van Raaij et al., 2008) and also to Estonian populations (Runno-Paurson et al., 2009, 2010a, 2013b), which differ from other parts of Europe. Two virulence pathotypes, 1.3.4.7.10.11 and 1.2.3.4.6.7.10.11, have recently been common in the Nordic (Lehtinen et al., 2007, 2008), Russian (Statsyuk et al., 2013), Polish (Chmielarz et al., 2014), and Estonian populations (Runno-Paurson et al., 2010a, 2013b, 2014).

2.7. *Phytophthora infestans* populations in Europe

Most of the populations in western Europe are clonal with a few dominating genotypes in the populations each season with sexual reproduction events being rare (Cooke et al., 2012; Gisi, et al., 2011; Li et al., 2012;

Montarry et al., 2010). The clonal populations are thriving because of large areas of commercial potato production with a few dominating host cultivars and mild winters which allow pathogens to survive from one season to another through asexual structures in the plant debris, volunteer plants, and tubers (Cooke et al., 2011). High fungicide input can be selective for insensitive clonal lineages, for instance, metalaxyl-resistant clone *EU_13_A2* has spread across Europe since 2004. More recently, since 2013 clone *EU_37_A2* with reduced sensitivity to fluazinam has persisted in the pathogen populations in western Europe (EuroBlight, 2019; Cooke et al., 2012; Li et al., 2012; Schepers et al., 2018). Populations are evolving and new emerging clones (for example, *EU_36_A2, EU_37_A2* and *EU_41_A2*) are identified in Europe every season, some of them become more threatening to potato production than others with the urgent need to optimise IPM strategies (EuroBlight, 2019).

After the spread of the A2 mating type and subsequent population changes due to sexual reproduction events at the end of 20th century, genetically diverse populations with countless different genotypes have been detected in Europe, for example in the Netherlands (Li et al., 2012), Denmark, Norway, Sweden, Finland (Brurberg et al., 2011; Sjöholm et al., 2013), Estonia (Runno-Paurson et al., 2010), Poland (Brylińska et al., 2016; Chmielarz et al., 2014), and Russia (Elansky et al., 2015). In northern Europe, during the winter the temperature drops below 0°C and snow covers the ground, limiting the asexual survival of the pathogen from one season to another. In these conditions the pathogen has adapted to reproduce sexually, increasing its fitness via greater genetic diversity and production of oospores as a source of primary inoculum in the soil (Barton and Charlesworth, 1998; Goodwin, 1997; Widmark et al., 2011). High genetic diversity in these regions probably increases disease management problems due to evolving virulence against novel host resistance genes and reduced sensitivity to fungicide active ingredients (EuroBlight, 2019).

2.8. Pathogen control measures

Currently potato late blight remains the most significant disease in potato cultivation and is traditionally controlled by frequent fungicide applications supported by preventative cultural measures such as crop rotation, the use of disease resistant cultivars, healthy seeds, and the timely destruction of primary sources of inoculum (Cooke et al., 2011). In spite of these measures, potato late blight remains responsible for an estimat-

ed annual economic loss of € 1 billion on the 6 Mha of potatoes grown in the EU (Haverkort et al., 2008). This includes the costs of control and losses. Potato, thus, is one of the largest consumers of pesticides among the agricultural crops grown in the EU (Cooke et al., 2011). Using pesticides has environmental costs which are related to the pollution of groundwater, energy costs for application, and negative effects on human health (Haverkort et al., 2008). The EU Commission has clearly stated, in the Communication on the European Green Deal (2019), the necessity to significantly reduce the use and risk of chemical pesticides in agriculture, consequently, the number of pesticide products in the market is decreasing. Over time efficacy of fungicides may reduce as pathogens become insensitive to the active ingredient and insensitive clonal lineages spread causing substantial damage to the crop (Chowdappa et al., 2015; Cooke et al., 2012; Fry et al., 2013, 2015).

One of the important strategies that could lead to more sustainable disease management is the use of late blight-resistant potato cultivars, which significantly reduces the average fungicide input, negative impact on the environment, and production costs (Kessel et al., 2018). Genes that have resistance against late blight (R genes) were first discovered in the closely related species *Solanum demissum* in the beginning of the twentieth century (Fry, 2008). When potato cultivars have these single or multiple R genes (stacked genes) in their genome they cannot get infected by pathogens which do not have a matching virulence gene (gene-for-gene interactions) (Flor, 1971). These R genes from wild *Solanum* species and potato cultivars have been used in classical breeding programs to develop resistant cultivars (Haverkort et al., 2016; Lammerts Van Bueren et al., 2008). Recently introduced late blight-resistant cultivars are not attractive to all farmers as they lack some of the preferred market characteristics and current resistant cultivars are not as high yielding as some of the established cultivars (Pacilly et al., 2016). Moreover, when they became more widely used, resistance breakdown occurred due to the adaptive ability of the pathogen to overcome R gene mediated host resistance, resulting in the emergence of new virulent pathotypes (Fry, 2008; Haas et al., 2009). More complex virulence pathotypes are found in the cultivars with greater resistance to late blight (Flier et al., 2007; Young et al., 2009). For example, *P. infestans* clonal genotype *EU_13_A2* successfully infect late blight resistant potato cultivars ´Sarpo Mira´ and ´Bionica´ in Ireland and ´Stirling´ in UK (Cooke et al., 2012; Lees et al., 2012; Stellingwerf et al., 2018).

Despite these virulent pathotypes, the studies of spatial epidemiology of *P. infestans* have shown that an increasing proportion of fields of late blight-resistant cultivars resulted in a strong decrease in disease incidence and limited the pathogen spread across the landscape and thereby reduced yield losses in susceptible cultivars without fungicide application (Pacilly et al., 2018; Skelsey et al., 2010). There are two main ways disease resistant cultivars influence the pathogen in the environment. First, a greater fraction of resistant cultivars in the landscape prolongs the initial infection and reduces the primary inoculum at the beginning of the growing season. Secondly, the potato fields with the resistant cultivars act as a barrier for disease dispersal to potato fields with susceptible cultivars (Pacilly et al., 2018; Skelsey et al., 2010).

An important preventive measure to avoid late blight epidemics is lengthening crop rotations. It would not let pathogens specific to a host accumulate in the soil, this is necessary in preventing oospore-driven epidemics, especially in the northern Europe. Cold winters significantly reduce survival of clones in plant debris, volunteer potatoes, and weed hosts between growing seasons (Brurberg et al., 1999; Grönberg et al., 2012). Conversely, oospores can survive in the soil for several years (Drenth et al., 1995) despite of low temperatures, which may even conserve their viability (Lehtinen and Hannukkala, 2004). The difference in the late blight pathogen population structure between the Nordic region and most other parts of Europe may be due to climatic conditions (Brurberg et al., 1999).

3. AIMS AND HYPOTHESES OF THE STUDY

The potato late blight causing pathogen, *P. infestans,* population structure and variation studies are needed to make knowledge-based disease management decisions and advise potato breeders, advisors, and farmers accordingly. Current research results are valuable and permit comparison with other populations on a larger scale, because standardised methods are applied. This is the first attempt to characterize the Baltic *P. infestans* population with highly informative SSR markers. This research is needed to obtain knowledge regarding the role of sexual reproduction, as well as to assess the structure and variation in the pathogen population in the Baltic countries.

Specific aims (A) and hypotheses (H) of the study were:

A1 To reveal the late blight pathogen genotypes, population structure, and diversity in the three Baltic countries – Estonia, Latvia, and Lithuania (**I, II, III**).

H1.1: *P. infestans* populations in the Baltic countries are genetically diverse.

H1.2: Locally emerged *P. infestans* clones adapted to the conditions in the region would predominate rather than invasive clonal lineages from other European potato growing regions.

A2 To determine the role of sexual reproduction in *P. infestans* survival and diversity in Estonia (**I, III**).

H2: Both mating types are present in fields, enabling sexual reproduction which contributes to the pathogen survival and diversity in the region.

4. MATERIAL AND METHODS

4.1. Collection of *Phytophthora infestans* isolates

Potato leaves infected by *P. infestans* were collected from 8 sites in Estonia in 2004 (**III**) and over the three-year period 2010–2012 from 23, 21 and 15 sites within the main potato-growing areas of Estonia (**I**), Latvia and Lithuania (**II**), respectively (Table 2). The samples were collected from conventional production fields, organic fields, and potato field trials. Conventional producers were divided into two groups. Most of the potato growers were small-scale conventional farmers who used potato seeds of uncertain quality and applied chemical late blight treatments occasionally, varying from no sprays to 1–4 sprays per season. Large areas of potato are thus insufficiently protected by fungicides against late blight. Crop rotation by small growers varies widely, some not rotating, others after three to four years. In large conventional production fields, high-quality certified potato seeds were used, planted no more frequently than every third year, and fungicides were applied 4–7 times or even as many as 11 times per season. Potato field trials were located at the Estonian Crop Research Institute in Jõgeva, at the Lithuanian Research Centre for Agriculture and Forestry in Akademija, and at the Priekuli Research Centre of Institute of Agricultural Resources and Economics in Priekuli, Latvia. The sampled plants were randomly selected across the field, from each plant a blighted leaf with a single lesion was selected at random. From each field, 2–15 isolates were collected. Isolations were carried out as described in Runno-Paurson et al. (2009). The pure cultures were preserved on rye B agar at 5°C.

Table 2. Origin of *Phythophthora infestans* isolates collected from the Baltic countries analysed in the studies **I, II** and **III**.

Country	Years	Sites	Number of isolates analysed				
			LSC[a]	SSC[a]	O[a]	T[a]	Total
Estonia	2004 (III)	8	27	34	-	9	70
	2010-2012 (I)	23	72	16	32	21	141
Latvia	2010-2012 (II)	21	70	22	12	21	125
Lithuania	2010-2012 (II)	15	8	47	-	14	69
Total		67	177	119	44	65	405

[a] LSC- large scale conventional farms; SSC- small scale conventional farms; O- organic farms; T- trial fields.

4.2. Genotyping

Pure-culture *P. infestans* isolates were grown on rye B agar plates for 2–4 weeks at 17–18°C (**I, II, III**). Mycelium was subsequently harvested and frozen. DNA extractions were carried out with the DNeasy Plant Mini Kit (QIAGEN) according to the manufacturer`s instructions (**I, II, III**). SSR markers used for genotyping the EE population in 2004 were Pi02, Pi04, Pi16, Pi26, Pi33, Pi4B, Pi4G, G11, D13 (**III**) and for 12-plex Pi02, Pi04, Pi4B, Pi63, Pi70, SSR2, SSR4, SSR6, SSR8, SSR11, G11, D13 (**I, II**). Five SSR markers (Pi02, Pi04, Pi4B, G11, D13) were overlapping in every study.

Genotyping of *P. infestans* populations from 2010–2012 (**I, II**) was performed at The James Hutton Institute (Dundee, Scotland), following the Li et al.´s (2013a) SSR marker 12-plex method with some modifications. PCR reactions were performed in a volume of 12.5 µl consisting of 1x Type-it Multiplex PCR Master Mix (QIAGEN Type-it Microsatellite PCR Kit, Cat. No. 206243), primers for each locus in optimal concentration (0.03–0.32 µM) and 1 µl of template DNA (approximately 20 ng μl^{-1}). Amplification reactions were carried out under the following conditions: 95°C for 5 min followed by 28 cycles of 95°C for 30 s, 58°C for 90 s, and 72°C for 20 s, and a final extension at 60°C for 30 min. PCR products were diluted in ultrapure H_2O 0–100 times depending on the initial DNA concentration in the PCR reaction mix. The diluted PCR product (0.6 µl) was added to a mix containing 10.14 µl of deionized formamide (Hi-Di Formamide, Applied Biosystems) and 0.06 µl of GeneScan-500LIZ standard (Applied Biosystems). The fluorescent-labelled PCR products were analysed using an ABI3730 DNA Analyzer (Applied Biosystems) according to the manufacturer's instructions. The size of the alleles was determined using GeneMapper v3.7 (Applied Biosystems) software.

Genotyping of Estonian *P. infestans* population from 2004 (**III**) was performed at MTT Agrifood Research Finland in Natural Resources Institute Finland. Nine polymorphic SSR regions were amplified using PCR with primers labelled with a fluorescent dye (6-FAM, NED, PET, or VIC) for loci Pi02, Pi04, Pi16, Pi26, Pi33 from Lees et al. (2006) and loci 4B, 4G, G11, D13 according to Knapova and Gisi (2002). Single locus amplifications were performed and the thermal cycling was carried out according to Brurberg et al. (2011). PCR products were diluted

12.5 – 71-fold depending on the different markers and pooled together by isolate. 1 µl of diluted PCR products were added to a loading buffer containing 8.8 µl Hi-Di formamide (Applied Biosystems) and 0.2 µl of GeneScan-500LIZ standard (Applied Biosystems). The samples were analysed using an ABI3730 DNA Analyzer (Applied Biosystems) according to the manufacturer's instructions. The fragments were sized according to known fragment lengths of the LIZ-labelled marker peaks and scored using GeneMapper v4.0 (Applied Biosystems) by comparison with ten reference isolates (C1–C10) kindly supplied by Drs Lees and Cooke, from The James Hutton Institute (Dundee, Scotland).

4.3. Mating type analysis

Mating types were determined (**I, III**) by the method described in Runno-Paurson et al. (2009). The A1 and A2 tester isolates were the same as described in Hermansen et al. (2000) (**I**) or Lehtinen et al. (2007) (**III**). Isolates forming oospores on plates with the A1 mating type were registered as A2; isolates that formed oospores with the A2 mating type were registered as A1.

4.4. Data analysis

For this PhD thesis some of the data in the original publication **I** (Kiiker et al., 2018) was reanalysed (**I$_x$**). Data analysis of genotyping results was performed using Microsoft Office Excel 2013 Add-in GenAlEx version 6.501 (Peakall and Smouse, 2006, 2012) (**I, I$_x$, II, III**) and package *poppr* (Kamvar et al., 2014) within R version 3.1.0 (**I, I$_x$, II**). All of the isolates were included in the calculations of single allele (**I, II, III**) frequencies at studied loci. Gene diversity (*H*) was calculated for all the loci according to the formula of $H = 1-\sum xj^2$, where *xj* is the frequency of the *j*th allele at the locus (Nei, 1978) (**I, III**). The Gini-Simpson index ($1-\lambda$) (Simpson, 1949), Nei's unbiased gene diversity ($H_{exp} = \left(\frac{n}{n-1}\right)(1-\sum xj^2)$) (Nei, 1978) and evenness (E_5) (Grünwald et al., 2003) of the alleles at each locus were calculated (**II**). Differences in the index values between Latvian and Lithuanian populations were analysed with one-way ANOVA ($\alpha = 0.05$) (**II**).

Multilocus genotypes (MLGs) were compiled for all the *P. infestans* isolates using 11 (**I$_x$, II**) or 5 (**III**) SSR loci. A single difference in an allele size was considered enough to classify as a unique MLG. If an isolate

had one missing locus it was regarded as identical to another MLG if the other loci were identical. Genotypic diversity was calculated by a Shannon diversity index corrected for sample size (*Hs*) (Sheldon, 1969) (**I_x, II, III**). Values for *Hs* may range from 0 (only single MLG present) to 1 (each isolate with a different MLG). The differences in the Shannon's index values between countries (**I_x**) and collecting years (**I**) were analysed with one-way ANOVA and Tukey HSD test. Other estimates of genetic diversity for the populations included calculating the number of original MLGs, the number of expected MLGs at the smallest sample size based on rarefaction (eMLG) (Hurlbert et al., 1971), Simpson index (λ) (Simpson, 1949), evenness (E_5) (Grünwald et al., 2003), and Nei´s unbiased gene diversity (*Hexp*) (Nei, 1978) (**I_x, II**).

A clone corrected dataset was constructed by including only one isolate of each MLG (**I_x, II**). Deviations from the Hardy-Weinberg equilibrium (*HWE*) at loci were tested with a chi-square test using this data set (**I_x, II**). Random recombination between pairs of SSR loci was assessed by calculating the index of association (I_A) and the standardised index of association ($\bar{r}_d$), which accounts for the number of loci sampled (Agapow and Burt, 2001) (**I_x, II**).

Genotypic differentiation was assessed by calculating the Bruvo's genetic distance between the MLGs (Bruvo et al., 2004) (**I_x, II**) or counting the number of pairwise genetic differences between all isolates (**III**). This difference was used in a principal coordinate analysis (PCoA) (**III**) or minimum-spanning network (MSN) (**I_x, II**) to visualize any clustering based on isolate collecting year (**I_x, II**) or origin (**I_x, II**, **III**). Pairwise population Nei's genetic distance (Nei 1978) and Wright's fixation index (F_{ST}) values were calculated to estimate the genetic differentiation among the countries (**I_x**) or regions within a country (**III**). The significance of pairwise F_{ST} values was tested by 999 permutations. Analysis of molecular variance (AMOVA) calculated with 999 simulations was performed to estimate how much of the genetic variation is explained among and between subpopulations collected from different sites within countries (**II**).

Statistical analyses of phenotypic tests (**I, III**) were performed with SAS/STAT version 9.1 (SAS Institute Inc., Cary, NC, USA). Differences in the prevalence of the two mating types of *P. infestans* isolates between years and study sites were tested using a logistic analysis (GENMOD procedure in SAS) with a multinomial response variable (A1, A2, or both).

5. RESULTS

5.1. Genotyping

Collection of *P. infestans* isolates were genotyped with 9 (**III**) or 12 SSR marker assays (**I, II**). All SSR loci were polymorphic and in studies applying SSR 12-plex, 49, 55 and 51 alleles were detected in Lithuanian, Latvian and Estonian populations, respectively (**I, II**). In the Estonian population from 2004, studied with 9 SSR markers, altogether 35 alleles were identified (**III**). Genotyping with SSR markers revealed that the locus D13 was not amplified for 64% of the EE population in 2004 (**III**), 48% of the EE population in 2010–2012 (**I**), 42% of the LV population and 16% of the LT population (**II**). That demonstrates that locus D13 is not very informative or useful in genotyping the *P. infestans* population in the Baltic countries. Therefore, this marker was excluded from the multilocus genotype calculations and genetic diversity analysis (**I_x, II, III**).

Gene diversities (H) or Nei's unbiased gene diversity (H_{exp}) for each SSR locus were calculated by further processing allele frequencies. The least informative locus in Estonian and Latvian populations was Pi70, H = 0.081 in EE and H_{exp} = 0.047 in LV with only two detected alleles (**I, II**). The most informative locus in the same populations was G11, H=0.804 in EE and H_{exp} = 0.805 in LV with 9 alleles detected in populations collected in 2010–2012 (**I, II**). However, in the Lithuanian population the least informative locus was Pi02 (H_{exp} = 0.151) with four detected alleles and the most informative locus was SSR4 (H_{exp} = 0.753) with 7 alleles (**II**). In the Estonian population collected in 2004, gene diversity was lowest at locus Pi33 (H = *0.322*) with two detected alleles and highest at locus Pi26 (H = *0.713*) with 5 alleles (**III**). Variability was detected in the allele frequencies between collection years 2010–2012 in the EE population (**I**), between the sampling regions in the EE population in 2004 (**III**), and between the LV and LT populations in 2010–2012 (**II**) for nearly all the determined loci. This shows that *P. infestans* populations in the Baltic countries are dynamic and recombining. Analyses showed that differences between the Latvian and Lithuanian populations (**II**) according to the Gini–Simpson index (F(11,12) = 4.695, p = 0.006), Nei's unbiased gene diversity (F(11,12) = 4.793, p = 0.006), and evenness (F(11,12) = 3.272, p = 0.026) values of the alleles were statistically significant.

MLG analysis performed on the isolates collected in 2010–2012 with the combination of 11 SSR markers, excluding locus D13, indicated 94, 77, and 44 MLGs in the EE, LV, and LT populations, respectively (**I$_x$, II**). In the EE, LV, and LT populations 68%, 75%, and 84% of the MLGs were unique (**I$_x$, II**). The EE population of 66 isolates, collected in 2004, consisted of 46 different MLGs as a combination of five SSR loci (Pi02, Pi04, Pi16, Pi26, Pi33) (**III**). 34 (74%) of these MLGs were unique (**III**).

Local clones that shared an MLG were associated with isolates collected from the same field, but some of them were also discovered at separate sites and in continuous years. The most abundant clone in the LT population (*LT_MLG19*) was detected 20 times (Appendix 1), it survived between seasons and was detected in a large production field in Panevėžys in 2011, a trial field in Akademija in 2011, and a small conventional field in Paežeriai in 2012; each of these sites were located over 90 km apart (**II**). The most frequent local clone in LV (*LV_MLG10*) was detected 10 times (Appendix 1) in only in one year (2012), in an organic field and a trial field in Priekuli, as well as a large production field in Dreimani 50 km apart (**II**). The two most frequent clones in EE (*EE_MLG10, EE_MLG11*) were presented by 6 isolates (**I$_x$**, Appendix 1). There were also three MLGs found in both EE and LT in 2010 and one MLG found in LV in 2010 and later in EE in 2012 (**I$_x$,** Appendix 1). From the EE population in 2004 one MLG was represented by a maximum of 4 isolates, but other repeatedly identified MLGs only appeared on two occasions (**III**). Fortunately, invasive clones coming from other parts of Europe were not detected in these studies.

Genotypic richness can be measured as the number of observed MLGs, but because sample sizes from the three Baltic countries in 2010–2012 differed (**I$_x$, II,** Table 3), an approximation of the number of genotypes that would be expected at the largest shared sample size based on rarefaction (eMLG) would be more appropriate. The value of eMLG was highest for the EE population (54.8) and lowest for the LT population (44) (Table 3). In the EE population collected in 2010–2012, genotypic diversity was higher (λ = 0.99; *Hs* = 0.89) compared to the LV population (λ = 0.98; *Hs* = 0.85) and the LT population (λ = 0.9; *Hs* = 0.77) (Table 3). The Shannon diversity index was not statistically significantly different between years in the EE population (p = 0.09) (**I**) and between countries in 2010–2012 (p = 0.99) (**I$_x$**). Gene diversity among these pop-

ulations was highest in LV (*Hexp* = 0.54) and somewhat lower in EE (*Hexp* = 0.52) and LT (*Hexp* = 0.49) (Table 3). Genotypic evenness was closer to equal abundance in the isolates from EE (*E.5* = 0.82) and LV (*E.5* = 0.72), where none of the MLGs dominated in the population. In the LT population, genotypic evenness was lower (*E.5* = 0.38), mostly due to one well-adapted local clone, *LT_MLG19*, which comprised 29% of the sampled LT population (**II**). Genotypic and gene diversity in EE *P. infestans* populations in 2004 (**III**) and 2010–2012 ($\mathbf{I_x}$) were consistent with each other (Table 3). Although, the distribution and number of isolates within the country varied and MLGs in the first study (**III**) were calculated with 5 SSR loci compared to 11 SSR loci in the subsequent study ($\mathbf{I_x}$).

Table 3. Genetic diversity in Estonian ($\mathbf{I_x}$, **III**), Latvian (**II**), and Lithuanian (**II**) *Phytophthora infestans* populations.

Variable*	**Population**			
	Estonia 2004	**Estonia 2010-2012**	**Latvia 2010-2012**	**Lithuania 2010-2012**
N	70	141	125	69
MLG	46	94	77	44
eMLG	NA**	54.8	49.5	44
SE	NA**	2.52	2.59	0
λ	NA**	0.985	0.977	0.903
E_5	NA**	0.819	0.724	0.378
Hs	0.878	0.886	0.845	0.766
H_{exp}	0.577	0.519	0.538	0.490
I_A	NA**	0.037	0.011	0.103
P_{IA}	NA**	0.210	0.395	0.096
$\bar{r}_d$	NA**	0.004	0.001	0.011
$P\bar{r}_d$	NA**	0.201	0.394	0.091

*Statistics given are the number of isolates (N), number of original multilocus genotypes (MLG), number of expected MLG at the smallest sample size based on rarefaction (eMLG), standard error based on eMLG (SE), Simpson index (λ), evenness (E_5), normalized Shannon diversity index (Hs), Nei´s unbiased gene diversity (H_{exp}), the index of association (I_A) with *P*-value, and the standardised index of association ($\bar{r}_d$) with *P*-value.

**NA- not available

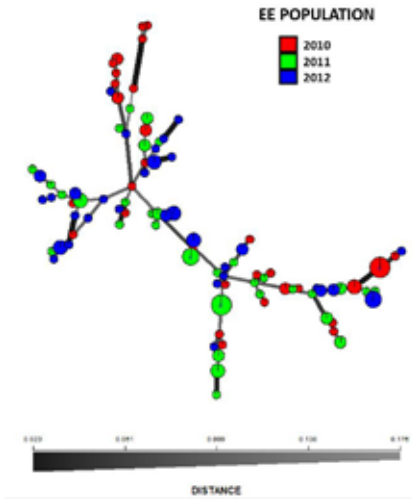
The regional distribution of the *P. infestans* population studied in EE in 2004 showed diversity was highest in the eastern region (*Hs* = 0.952) and somewhat lower in the southern (*Hs* = 0.867) and the northern

regions (*Hs* = 0.883) (**III**). Among fields, the highest genotypic diversity (*Hs* = 1.000) was identified in an experimental field from the eastern region (Jõgeva) because all the isolates collected from this field had unique MLGs (**III**).

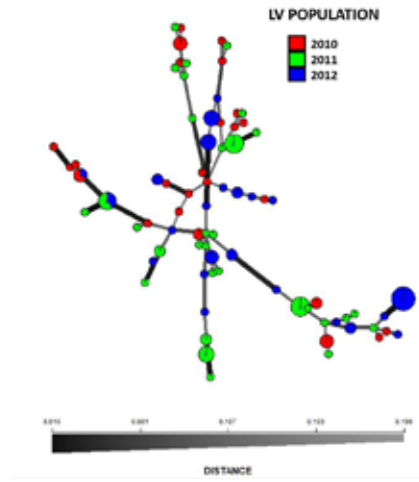
To test whether the population structure was consistent with frequent sexual recombination in the populations, SSR loci were tested for linkage disequilibrium. Sampling during epidemics could lead to over-representation of clones. Therefore, a clone corrected data set was analysed. No significant linkage among markers was seen, suggesting prevailing sexual recombination in the population (**I_x, II**). As expected in naturally evolving non-equilibrium populations, five loci were not in *HWE* in the LV population, and four loci were not in *HWE* in the LT and EE populations (**I_x, II**).

However, in the EE, LV, and LT populations in 2010−2012, the standardized index of association ($\bar{r}_d$) between loci did not differ significantly from zero ($\bar{r}_d$ = 0.001−0.011). The null hypothesis that there would be no linkage among loci failed to be rejected (p = 0.09−0.39), which could be explained by recombination of alleles into new genotypes during sexual recombination (**I_x, II**, Table 3).

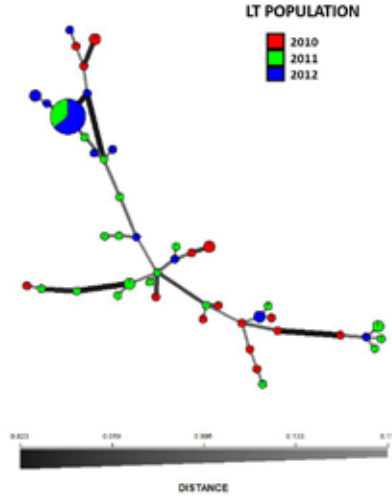
AMOVA analysis of LV and LT populations revealed the most genetic variation (92%) was found within fields and only a small amount of variation was explained between fields or countries (**II**). MSN, based on Bruvo´s genetic distance, visually display the relationships between multiple single-isolate MLGs and minor clones with larger nodes. Minor variants of local clones exist connected by very thick lines. MLGs shared over more than one season are rare in EE, LV, and LT populations in the study years 2010–2012 (**I_x, II,** Figures 3a, 3b, 3c). The absence of country-specific clustering by genetic distance amongst the studied populations is also apparent (**I_x, II,** Figure 4). Pairwise population analysis of Nei's genetic distance (0.012–0.070) and Wright's fixation index (F_{ST}) (0.012–0.061) also concurred with these results and showed limited genetic differentiation between the countries (Table 4).



3a



3b



3c

Figures 3a-c. Minimum-spanning network of Estonian (3a), Latvian (3b), and Lithuanian (3c) populations of *Phytophthora infestans* created from data analysis of simple sequence repeat (SSR) markers. Each multilocus genotype is represented by one node sized in proportion to its frequency in the populations. Line width and shading represent relatedness – darker, thicker lines indicate the nodes are more closely related; line length is arbitrary.

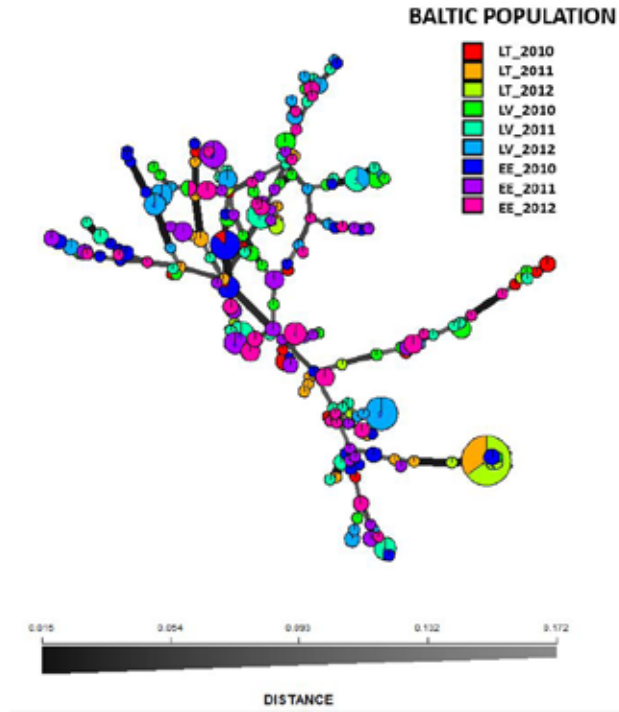


Figure 4. Minimum-spanning network of the Baltic populations of *Phytophthora infestans* created from data analysis of simple sequence repeat (SSR) markers. Each multilocus genotype is represented by one node sized in proportion to its frequency in the populations. Line width and shading represent relatedness – darker, thicker lines indicate the nodes are more closely related; line length is arbitrary.

Table 4. Matrix of population differentiation and genetic distance for *Phythophthora infestans* populations collected in 2010-2012 from Estonia, Latvia and Lithuania (**I_x, II**). Pairwise population F_{ST} values are below the diagonal and Nei's genetic distance values above the diagonal.

Population	Lithuania	Latvia	Estonia
Lithuania	-	0.070	0.033
Latvia	0.061	-	0.012
Estonia	0.031	0.012	-

Genotypic differentiation was assessed by counting the number of pairwise genetic differences between all isolates from the *P. infestans* population in EE in 2004 (**III**). The first two principal components of the PCoA explained a large proportion (45.16%) of the overall genotypic variation in this population (**III**). Subpopulations collected from different geographical regions did not significantly differ, although isolates from the southern region were more closely grouped together than isolates from other regions (**III**). These results were in concordance with the low values of pairwise F_{ST} (0.006–0.010) and Nei's genetic distance (0.024–0.036) calculations (**III**).

5.2. Mating type analysis

Both A1 and A2 mating types were found in every study year from the *P. infestans* population in EE (**I, III**). The overall distribution of A1 and A2 mating type isolates was nearly equal (1:1) in the EE population as 52% of the isolates collected in 2010–2012 were A1 and 48% were A2 mating type isolates (**I**) and in 2004 49% were A1 and 51% were A2 (**III**). Both mating types were present at every field in 2004 (**III**) and at 87% of the collection fields in 2010–2012 (**I**). There were no statistically significant differences in A1 and A2 mating type counts between years 2010–2012 (Chi-square = 1.99, df = 2, $p = 0.37$) (**I**). The sample size in some fields was too small to detect any statistically significant differences in the proportion of A1 and A2 between sampling sites in 2010–2012 (Chi-square = 26.39, df = 22, $p = 0.24$), although the difference between the ratio of mating types in collection fields in 2004 was significant (Chi-square = 14.35, df = 7, $p = 0.045$) (**III**).

6. DISCUSSION

Whereas potato late blight is still a severe threat to potato production in the Baltic countries, continuous monitoring of local highly evolving *P. infestans* population is needed for applying effective late blight management. A large proportion of potato fields in this region are small and close to each other where appropriate crop rotation is not applied. Chemical control against late blight is used insufficiently in an attempt to lower production costs. These factors encourage infections derived from soil-borne oospores and also rapid asexual reproduction and spread of *P. infestans*, which threaten the well managed commercial production fields during the growing season. Therefore, recent changes in *P. infestans* population spatio-temporal variation in the Baltic countries were monitored with genotypic analysis using SSR markers for the first time (**I, II, III**) and phenotypic analysis of mating types (**I, III**).

6.1. Genetically diverse *P. infestans* populations in the Baltic countries

The large diversity in the *P. infestans* population in the Baltic countries is similar to that in Poland, the Nordic countries, and some regions of the Netherlands (Brylinska et al., 2016; Chmielarz et al., 2014; Li et al., 2012; Sjöholm et al., 2013). Moreover, these studies found that 68−75% of the MLGs in the Baltic populations were unique (**I_x, II, III**). These unique *P. infestans* individuals probably have limited spread and survival ability. Similarly, a high level of clonal reproduction within fields but no clonal spread between fields was revealed in the Nordic countries (Montes et al., 2016; Sjöholm et al., 2013). Variation in genotypes among the Baltic populations is different from that in western and central European countries like the UK, France, the Netherlands, and Switzerland, where genotype structure is mostly clonal and only a few genotypes dominate in the growing season (Cooke et al., 2012; Gisi et al., 2011; Li et al., 2012; Montarry et al., 2010).

From Poland to Finland, the values of diversity indices and evenness of *P. infestans* populations increase from south to north, from milder to colder winter periods. In addition, southern populations are more prone to invasive clonal lineages and the spread of local clones than those in the north, which have populations distinct to single fields and only have limited spread of local clones (**I_x, II, III**) (Brurberg et al., 2011; Chmie-

larz et al., 2014; Runno-Paurson et al., 2016). In addition, comparing the results of the Baltic populations of *P. infestans* (**I_x, II, III**) with recent studies carried out in nearby Poland (Brylinska et al., 2016; Chmielarz et al., 2014) and Nordic countries (Brurberg et al., 2011; Sjöholm et al., 2013), most of the SSR marker alleles are shared across all of these populations, with similar patterns of allele frequency at all loci except for the highly variable G11 and SSR4. In general, the populations are genetically similar to each other in these neighbouring countries due to the lack of migration boundaries and the free spread of *P. infestans* isolates, enabling gene flow between populations.

6.2. Locally emerged *P. infestans* clones would predominate in the Baltic countries rather than invasive clonal lineages from other European regions

In the current Baltic *P. infestans* population studies, most of the MLGs were identified from a single field outbreak (**I_x, II, III**), however, a few MLGs were sampled in different years from multiple outbreaks, as in the case of the most common local clones, *LT_MLG19* in Lithuania and *LV_MLG10* in Latvia. Fortunately, invasive clones from outside the Baltic populations were not detected. As potatoes are the primary host for *P. infestans* in Baltic countries, clonal emergence and survival indicates the relative importance of infected seed tubers and volunteer potatoes in the field acting as primary inoculum sources of *P. infestans* (Cooke et al., 2011; Ronis et al., 2007). Dispersal over longer distances across countries occurs via transporting infected potato seed tubers as potato cultivars from western Europe are imported and well distributed to the potato growing farms in the Baltic countries (Hu et al., 2012).

The recently emerged *P. infestans* clonal lineages which are spreading and causing substantial damage to the crop in Europe (*EU_13_A2, EU_37_A2, EU_41_A2*), Asia (*EU_13_A2*) and Northern America (*US8, US22*) are mostly A2 mating types although the reason for that remains elusive (EuroBlight, 2019; Chowdappa et al., 2015; Cooke et al., 2012; Fry et al., 2013; Li et al., 2013b; Montarry et al., 2010). Several clonal lineages (*EU_13_A2, EU_37_A2, EU_41_A2* etc.) have also invaded Poland, which is the neighbouring country to Lithuania (EuroBlight, 2019; Chmielarz et al., 2014). Fortunately, invasive clones from other potato growing regions were not detected in the Baltic countries during the study years 2004 and 2010–2012 (**I_x, II, III**). During *P. infestans*

genotype tracking by the Euroblight network team in the last few years, only occasional findings of *EU_13_A2* have been confirmed in the Baltic countries, including five cases in 2016 and 2017 in Lithuania and one in Estonia in 2015 (EuroBlight, 2019). Generally, the survival and viability of the clonal lineages from western Europe are probably limited in cooler climates due to their local adaptation to temperature conditions prevailing in the area of origin (Mariette et al., 2016b), it is also probably more difficult for a single genotype to invade a population with high diversity, compared to one where only a single or a few genotypes dominate (Chmielarz et al., 2014). Additionally, *P. infestans* is evolving and a recently emerged highly virulent clonal lineage *EU_41_A2* seems to be adapted to the Nordic countries, as it was first detected in Denmark in 2013 and spread to Norway, Sweden, Poland in the next years (EuroBlight, 2019).

6.3. Possible sexual reproduction contributes to the pathogen survival and diversity in the Baltic countries

Possible sexual reproduction and oospore formation in the Baltic *P. infestans* populations are supported by the fact that opposite mating type (A1 and A2) isolates coexist in the potato fields and the A1 and A2 mating type ratio is almost equal in the population (**I, III**) (Aav et al., 2015; Runno-Paurson et al., 2014, 2015). These findings of mating types are generally comparable to the findings from Nordic countries (Hannukkala, 2012; Lehtinen et al., 2008), eastern Europe (the Russian Federation, Moscow region) (Statsyuk et al., 2013), and central Europe (Poland, Czech Republic) (Chmielarz et al., 2014; Mazakova et al., 2006). Recently, it has been suggested that long latent periods and low lesion growing rates of the *P. infestans* individuals originating from northern Europe would favor coinfection of the host tissues by various isolates and therefore increase the probability of sexual reproduction between isolates with opposite mating types (Mariette et al., 2016b). From the farmers´ perspective, short crop rotations between growing potatoes in the same fields facilitate oospore-driven epidemics, which stimulate a genetically diverse *P. infestans* population and make disease management more difficult as the inoculum source is accumulated in the soil and thus is constantly present in the fields. An earlier study in Latvian potato fields found oospores on over 80% of collected leaflets (Bimšteine, 2008).

For last few decades *P. infestans* populations in northern Europe commonly reproduce sexually because oospore production benefits the survival and infectiousness of the pathogen (Brurberg et al., 1999; Grönberg et al., 2012; Lehtinen and Hannukkala, 2004; Yuen and Andersson, 2013). The highly resilient oospores of *P. infestans* present in the fields have a relevant role in initiating late blight infections early in the growing season, which results in increased use of chemical control (Hannukkala et al., 2007; Hannukkala, 2012; Runno-Paurson et al., 2013a; Widmark et al., 2007). Symptoms of soil-borne infection on lower leaves have been noticed in recent years in Estonian potato fields early in the season, especially in fields without rotation or with only a short rotation of potato crops. In contrast with observations in Finland and Estonia (Hannukkala et al., 2007; Runno-Paurson et al., 2013b), no records have been made of changes in the timing of the first symptoms of late blight in Latvia and Lithuania. Also, in Latvia and Lithuania the symptoms of late blight do not necessarily start from the lower plant leaves (2016 letters from Dr. I. Skrabule and Dr. A. Ronis to me, unreferenced). Although oospore presence in the sample fields was not confirmed in current studies, mating type ratio and high genetic diversity indicated the likely role of sexual reproduction in the *P. infestans* population in the Baltic countries (**I, I_x, II, III**).

The difference in the late blight pathogen population structure between the northern and other parts of Europe may be due to climatic conditions (Brurberg et al., 1999). In the Baltic countries in north-eastern Europe, the temperature in the coldest months (December, January, February) drops below 0°C, with Estonia being the coldest (World Weather Information Service, 2020). Before the *P. infestans* population displacement in the 1980s when sexual reproduction became possible, asexual populations needed a living host for survival to the next growing season (Fry et al., 1993). Thus, the build-up of late blight epidemics was usually delayed until the end of the growing season (Hannukkala, 2012), because cold winters in northern Europe significantly reduce survival of clones between growing seasons in plant debris, volunteer potatoes and weed hosts (Brurberg et al., 1999; Grönberg et al., 2012).

6.4. Fungicide effect on *P. infestans* populations

Fungicides play a crucial role in the integrated control of late blight worldwide, however, it increases production costs and negatively impacts the environment (Haverkort et al., 2008, 2009). For instance, fungicide

Ridomil Gold MZ 68 WG (Syngenta Crop Protection AG), which contains metalaxyl-M and mancozeb is still in common use in the Baltic countries as a protective fungicide at early potato growth stages. Large conventional production farms in the Baltic countries apply fungicides 4–7 times per season (Aav et al., 2015; Runno-Paurson et al., 2015), which is considerably less than in the Netherlands and Belgium (14 sprays on average) and UK (10 sprays on average) (Cooke et al., 2011; Hansen et al., 2015). In order to evaluate the efficacy of the active ingredient metalaxyl-M in late blight control, metalaxyl sensitivity of the *P. infestans* populations have been studied. For instance, in the Estonian *P. infestans* population in 2010–2012, metalaxyl-sensitive isolates prevail (69%) (Kiiker et al., 2018). In Latvia and Lithuania, metalaxyl-sensitive isolates also constitute most of the populations, 54% and 69%, respectively (Aav et al., 2015; Runno-Paurson et al., 2015). Dominance of metalaxyl-sensitive isolates were also noted in recent studies in Finland, Sweden, Denmark and Norway (Lehtinen et al., 2008; Montes et al., 2016; Runno-Paurson et al., 2014), Russia (Moscow region, Statsyuk et al., 2013), Belarus (Pobedinskaya et al., 2011), and Poland (Brylinska et al., 2016; Chmielarz et al., 2014). These results contrast with those of previous studies carried out in 2001–2007 in Estonia, where the majority of the tested isolates were resistant or tolerant to metalaxyl (Runno-Paurson et al., 2009, 2010a, 2012, 2013b, 2014). The trend of increasingly finding metalaxyl-sensitive isolates has continued since then because of reductions in the use of metalaxyl containing fungicides. Overall, fungicide applications are recommended to start at the beginning of crop development when very low levels of pathogen inoculum are present to limit the spread of the pathogen and protect the crop (Cooke et al., 2011). The downside of intense commercial potato production with high fungicide inputs is selectiving for insensitive *P. infestans* clonal strains. For example, the notorious metalaxyl-resistant clone *EU_13_A2,* and more recently *EU_37_A2,* which have reduced sensitivity to fluazinam have become dominant in western Europe and beyond (EuroBlight, 2019; Cooke et al., 2012; Li et al., 2012; Schepers et al., 2018). Late blight control strategies should be adapted according to up-to-date knowledge about the pathogen population. The adoption of new knowledge into practical late blight control through national and international networks, for example EuroBlight in Europe (EuroBlight, 2019) and USABlight in USA (USABlight, 2019) has been very efficient.

7. CONCLUSIONS

The evidence demonstrates that in the Baltic countries the introduction of a diverse population has established residential sexual populations in agricultural fields and the soil has become a source of inoculum. The *P. infestans* populations of EE, LV, and LT from 2004 and 2010–2012 analysed with molecular SSR markers showed that frequent sexual recombination events have resulted in high genetic diversity (**I_x, II, III**). The study representing the first genetic analysis of LV and LT populations of *P. infestans* (**II**), revealed high genetic diversity, a large number of unique genotypes, and some local clones adapted to regional conditions. However, limited spread and survival of clones was noted in the LT pathogen population (**II**) compared to the LV (**II**) and EE populations (**I_x, III**). On the positive side, there was no evidence of invasive clonal lineages originating from other parts of Europe spreading or dominating in this region in the studied years (**I_x, II, III**). It would appear that local populations established from soilborne oospores early in the season are well adapted to the conditions in the region. *P. infestans* as a heterothallic oomycete needs two mating types, A1 and A2, to interact for sexual reproduction. Both mating types A1 and A2 were present at every studied potato field in 2004 (**III**) and at 87% of the fields in 2010−2012 (**I**). The overall distribution of A1 and A2 mating type isolates was nearly equal (1:1) in the EE population (**I, III**).

For sustainable potato production current disease management strategies should be adjusted according to the pathogen population status. Changes in pathogen populations directly affect the breeding and deployment of disease resistant cultivars, the performance of disease warning systems, and the efficacy of fungicides. Potato growers should be advised to implement late blight preventive measures, such as longer field rotation, to minimise oospore infections, especially in LV and EE, and to use more disease resistant cultivars and high-quality seed potatoes. Timely fungicide treatments, using different active ingredients, in conventional fields are necessary to prevent foliage and tuber infection, stop late blight from spreading, and avoid resistance in pathogen populations. Additionally, farmer awareness of *P. infestans* biology and survival including the infection threat caused by oospores should be raised.

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SUMMARY IN ESTONIAN

KARTULI-LEHEMÄDANIKU TEKITAJA *PHYTOPHTHORA INFESTANS* BALTIMAADE POPULATSIOONIDE MITMEKESISUS

Ülemaailmselt kartulikasvatuses suurt majanduslikku kahju ja saagikadusid põhjustav haigus on kartuli-lehemädanik, mida põhjustab munasseen *Phytophthora infestans* (Mont.) de Bary. Patogeeni geneetiline muutlikkus ja populatsioonide koevolutsioon metsikute peremeestaime *Solanum* liikidega Kesk- ja Lõuna-Ameerikas on viinud suure geneetilise mitmekesisuseni patogeeni populatsioonides. Seetõttu on patogeen saavutanud hea kohastumisvõime erinevates klimaatilistes ja põllumajanduslikes ökosüsteemides, põhjustades laiaulatuslikke lehemädaniku epideemiaid (Fry, 2008).

Kartuli-lehemädanikust põhjustatud kahju võib globaalselt ulatuda 5 miljardi euroni aastas ja patogeeni tõrjeks kasutatavad fungitsiidid moodustavad arenenud maades umbes 10% tootmiskuludest (Haverkort et al., 2008; 2009; 2016). Kartul on üks suurema pestitsiidide tarbega põllukultuur. Näiteks Madalmaades tehakse põllul regulaarset keemilist tõrjet keskmiselt 14 korda hooajal ja Suurbritannias 10 korda hooajal (Cooke et al., 2011; Hansen et al., 2015). Sellel on omakorda oluline energiakulu ja negatiivne mõju loodusele, reostades põhjavett, häirides mullaelustikku ja mõjutades inimeste ning loomade tervist (Haverkort et al., 2008). Ajapikku fungitsiidide efektiivsus võib väheneda, kui patogeeni populatsioonis hakkavad levima pestitsiidile resistentsed tüved, mis omakorda suurendab saagikadusid, kui tõrjet ei kohaldata vastavalt (Chowdappa et al., 2015; Cooke et al., 2012; Fry et al., 2013, 2015).

Lehemädanikust tingitud saagikaod võivad tavatootmispõldudel olla keskmiselt 20–25%, aga veelgi suuremad kui taimekaitsevõtteid rakendatakse ebaefektiivselt (Runno-Paurson et al., 2010a). Problemaatilisemad põllud on sellised, kus viljavaheldus on liiga lühike, seemet ei uuendata ega kasutata sertifitseeritud seemnekartulit, kasvatatakse haigusele vastuvõtlikke sorte ega tehta efektiivset haigustõrjet. Arvestuslikult võib sellised kartulipõlde Lätis ja Leedus olla üle 90% kõikidest põldudest (Ilze Skrabule ja Antanas Ronis´e kogutud vaatlusandmed), Eestis vähem. Seega patogeenile soodsates oludes võib *P. infestans* paljuneda ja levida vabalt, põhjustades ulatuslikku kahju nii seemnekar-

tuli- kui ka suurtootmispõldudel, kus integreeritud taimekaitsevõtteid rakendatakse (Aav et al., 2015; Ronis et al., 2007; Runno-Paurson et al., 2015). Samas, suurtootmispõldudel uuendatakse seemet ja kasvatatakse sertifitseeritud seemnekartulit, peetakse viljavaheldust vähemalt 3 aastat ja tehakse lehemädanikutõrjet fungitsiididega 4–7 korda hooajal, et kindlustada saaki (Aav et al., 2015; Runno-Paurson et al., 2015). Lehemädanikutõrje vajadust suurendab seegi, et vastavalt turunõudlusele kasvatatakse nii Baltimaades kui ka mujal Euroopas peamiselt lühema kasvuajaga varajasi sorte, mis on kahjuks patogeenile vastuvõtlikumad (Cooke et al., 2011; Runno-Paurson et al., 2013a). On näidatud, et kartuli-lehemädaniku tekitaja *P. infestansi* kohalik populatsioon on võimeline nakatama enamikke Baltimaades kasvatatavatest kartulisortidest, ka neid, mis on haigusele resistentseks aretatud (Runno-Paurson et al., 2013a; 2019).

Põhjapoolsemates riikides (Eesti ja Soome) on täheldatud lehemädaniku varasemat lööbimist kasvuperioodil (Hannukkala et al., 2007; Runno-Paurson et al., 2013b) ja oospooridest põhjustatud lehemädaniku nakkust (Brurberg et al., 2011; Hannukkala et al., 2007; Runno-Paurson et al., 2009; 2010a; 2012). Lätis ja Leedus lehemädanik ei lööbi oluliselt varem ega ole tingimata märgatav ka lehemädaniku lööbimine taime alumistel lehtedel (Ronis et al., 2007; Ilze Skrabule ja Antanas Ronis´e kogutud vaatlusandmed).

Lehemädaniku tõrjestrateegiad vajavad optimeerimist, võttes arvesse patogeeni populatsiooni omadusi: paljunemist, levimist, talvitumist, geneetilist mitmekesisust. Tundes paremini patogeeni omadusi, saab teha mõistlikuma valiku sortide kasvatamisel ja keemilise haigustõrje osas, selleks et virulentsed ja fungitsiididele resistentsed patogeenitüved ei saaks paljuneda ja edasi levida. Kuna patogeenide levikut ei takista teatavasti riigipiirid, siis on üliolulline patogeeni uuringutest saadud tulemusi levitada põllumajandustootjatele, sordiaretajatele, konsulentidele ja agrokeemiaettevõtetele nii kohalikul tasandil kui ka läbi rahvusvaheliste võrgustike, näiteks EuroBlight Euroopas (EuroBlight, 2019) ja USABlight Ameerika Ühendriikides (USABlight, 2019).

Patogeeni genotüüpide määramine on näidanud, et Suurbritannias, Prantsusmaal, Šveitsis, Madalmaades, Ameerika Ühendriikides, Indias ja Hiinas domineerivad populatsioonides üksikud *P. infestans* kloonid ja geneetiline mitmekesisus on madal (Chowdappa et al., 2015; Cooke

et al., 2012; Fry et al., 2013; Gisi, et al., 2011; Hu et al., 2012; Li et al., 2012, 2013b; Montarry et al., 2010). Nendes riikides on suured kartulipõllud, kus kasvatatakse vastavalt turunõudlusele väheseid domineerivaid sorte ja saagi kindlustamiseks tehakse lehemädaniku leviku ajal iganädalast keemilist tõrjet (Cooke et al., 2011). Tänu soojemale kliimale talvituvad *P. infestans* kloonid nendes piirkondades edukalt taimejäänustel ja mugulates (Cooke et al., 2011). Põhjamaades (Taani, Rootsi, Norra, Soome), Venemaal, Poolas ja Eestis, kus talved on külmemad ja seetõttu kloonide ellujäämisvõime väiksem, on patogeeni elutsüklis oluline roll sugulisel paljunemisel, mille tulemusel on populatsiooni geneetiline mitmekesisus suur ja patogeen on kohastunud talvituma oospooridena mullas (Brurberg et al., 2011; Brylińska et al., 2016; Chmielarz et al., 2014; Elansky et al., 2015; Runno-Paurson et al., 2010a; Sjöholm et al., 2013). Kuni viimaste aastateni peeti Põhjamaades *P. infestans* kloonide levimist ja talvitumist vähetõenäoliseks, kuid alates 2013. aastast on nendes riikides levimas üks väga virulentne kloon *EU_41_A2*, mis on kohastunud külmemates keskkonnatingimustes ellu jääma (EuroBlight, 2019). Kuna patogeenil on väga hea kohastumisvõime, on väga oluline patogeeni populatsioonides toimuvate muutuste ja kloonide leviku jälgimine.

Varasemalt on kartuli-lehemädanikutekitaja *P. infestans* populatsioone Eestis, Lätis ja Leedus uuritud kasutades fenotüüpilisi tunnuseid (paarumistüüp, virulentsus, metalaksüülitundlikkus) ja 2000ndatel aastatel Eesti populatsioone ka molekulaarsete markeritega (RG57 sõrmejälg, mtDNA haplotüübid) (Aav et al., 2015; Bebre et al., 2004; Runno-Paurson et al., 2009, 2010a, 2010b, 2012, 2013b, 2014, 2015; Valskyte et al., 2003). Praegu kasutatakse enamike Euroopa kartuli-lehemädanikutekitaja populatsioonide uurimiseks ja genotüüpide määramiseks 12 SSR markeri *multiplex* analüüsimetoodikat (Li et al., 2013a; EuroBlight, 2019). Seni ei ole Baltimaade populatsioone SSR markeritega analüüsitud ega kirjeldatud. Seni on teadmata ka, milline on patogeeni genotüüpide ja kloonide levik ning ellujäämine kohalikes tingimustes Balti riikides. Kasutades ühtset SSR markerite metoodikat, on võimalik Baltimaade populatsioone võrrelda nii omavahel kui ka teistes Euroopa piirkondades levivate populatsioonidega.

Kartuli-lehemädanikutekitaja *P. infestans* populatsiooniuuringud on vajalikud jälgimaks populatsioonides toimuvaid muutusi, et asjakohaste ennetus- ja tõrjevõtetega reageerida ja nõustada põllumajandustootjaid,

sordiaretajaid ja konsulente. Käesolevas doktoritöös analüüsiti esmakordselt *P. infestans* Baltimaade populatsioonide struktuuri ja mitmekesisust SSR markerite abil.

Sellest tulenevalt oli käesoleva doktoritöö peamine eesmärk välja selgitada, millised kartuli-lehemädanikutekitaja *P. infestans* genotüübid on levinud ja milline on populatsiooni struktuur ja mitmekesisus Eestis, Lätis ja Leedus (**I, II, III**). Lisaks oli eesmärgiks hinnata sugulise paljunemise rolli patogeeni ellujäämisel ja mitmekesisuse suurendamisel Balti riikides (**I, III**). Töös püstitati järgnevad hüpoteesid:

1. Baltimaade *P. infestans* populatsioonid on geneetiliselt mitmekesised;
2. Baltimaade *P. infestans* populatsioonides leidub pigem kohalikke *P. infestans* kloone kui teistest Euroopa piirkondadest pärit invasiivseid kloone;
3. Mõlema paarumistüübiga isolaate leidub samadel põldudel, võimaldades patogeenil suguliselt paljuneda.

Uurimistöö läbiviimiseks koguti 2004. aasta (**III**) ja 2010–2012. aasta (**I, II**) kasvuhooajal põldudelt haigustunnustega kartulilehed, millelt viidi laboris rukkiagarsöötmel puhaskultuuri kartuli-lehemädanikutekitaja *P. infestans* isolaadid. Proovid koguti tavatootmis-, mahe- ja kartulikatsepõldudelt, igalt põllult 2–15 isolaati, kokku 405 isolaati (Tabel 2). *P. infestans* isolaadid genotüpiseeriti 9 SSR markeriga (**III**) või 12 SSR markeri *multiplex* analüüsimetoodika järgi (**I, II**). Paarumistüübid A1 ja A2 määrati Eestist kogutud *P. infestans* isolaatidele (**I, III**).

Töö tulemustest selgus, et kõikides analüüsimiseks kasutatud SSR markerite lookustes oli uuritavates Baltimaade populatsioonides levinud mitu erinevat alleeli. Kokku tuvastati Eesti populatsioonis 35 (**III**) ja 51 (**I**) erinevat alleeli, Lätis 55 ja Leedus 49 erinevat alleeli (**II**). SSR lookuste alleelide sagedused varieerusid Eesti populatsioonis kogumisaastate (**I**) ja piirkondade vahel (**III**) ning Läti ja Leedu populatsioonide vahel (**II**). Lookus D13 puudus paljudel isolaatidel Eesti ja Läti populatsioonides (42–64%), seega ei ole see piisavalt informatiivne lookus Baltimaade populatsioonide kirjeldamisel ja jäeti seetõttu multilookusgenotüüpide (MLG) analüüsist välja. Eestist, Lätist ja Leedust 2010–2012. aastatel kogutud populatsioonides määrati 11 SSR markeri kombinatsiooni põhjal vastavalt 94, 77 ja 44 MLGd ($\mathbf{I_x}$**, II**). Nendes populatsioonides oli suur hulk (68–84%) unikaalseid MLGsid, mis esinesid vaid korra ($\mathbf{I_x}$**,**

II). Eesti 2004. aasta populatsioonis määrati 5 SSR markeri põhjal 46 MLGd, millest 74% olid unikaalsed (**III**). Genotüübiline mitmekesisus Simpsoni ja Shannoni mitmekesisusindeksite järgi oli kõrgeim Eestis ja veidi madalam Lätis ja Leedus (**I_x, II, III,** Tabel 3).

Korduvalt tuvastatud MLGd ehk kloonid olid enamasti pärit samalt kartulipõllult, kuid leidus ka selliseid, mida tuvastati erinevatelt põldudelt ja järjestikustel aastatel. Hea uudisena ei tuvastatud Baltimaade populatsioonidest ühtegi mujal Euroopas laialt levinud invasiivset klooni. Kõige enam esindatud kohalikest kloonidest tuvastati 2010–2012. aastatel Leedus *LT_MLG19* kahekümnel korral, Lätis *LV_MLG10* kümnel korral ja Eestis *EE_MLG10* ja *EE_MLG11* kumbagi kuuel korral (**I_x, II,** Lisa 1). Samuti leidus ka ühiseid kloone Eestis ja Leedus 2010. aastal ja üks kloon, mis tuvastati 2010. aastal Lätis ja 2012. aastal Eestis (**I_x,** Lisa 1**)**. Genotüübid olid ühtlasemalt jaotunud Eesti ja Läti populatsioonides (**I_x, II,** Tabel 3). Leedus aga ei olnud genotüüpide jaotus kuigi ühtlane, kuna üks domineeriv kloon moodustas 29% kogu populatsioonist (**II,** Tabel 3).

Geenide ahelduse tasakaalutusanalüüsid toetasid sugulise rekombinatsiooni toimumist Balti riikide *P. infestans* populatsioonides (**I_x, II)**. *Bruvo* geneetilise kauguse põhjal arvutatud minimaalsete kauguste võrgustikul on näha, et mitmel järjestikusel aastal esinevad kloonid on Baltimaade populatsioonides haruldased (**I_x, II,** Joonised 3a, 3b, 3c) ja MLGd ei klasterdu riikide kaupa (**I_x, II,** Joonis 4). *Nei* geneetilise kauguse ja *Wright'*i fikseerumisindeksi tulemused kinnitasid eelnevat ja näitasid väga madalat Baltimaade populatsioonide vahelist eristumist (**I_x,** Tabel 4). Eesti populatsioonis 2004. aastal MLGd regioonide järgi ei grupeerunud, kuid mitmekesisus oli kõrgeim Ida-Eestis ja madalaim Põhja-Eestis (**III**).

Töö tulemused näitasid, et Eesti *P. infestans* populatsioonides leidub mõlema paarumistüübiga (A1 ja A2) isolaate, 2004. aasta uuringus kõikidel uuritavatel põldudel (**III**) ja 2010–2012. aastatel 87% põldudel (**I**). Paarumistüüpide A1 ja A2 esinemine on Eesti populatsioonis enam-vähem võrdne (**I, III**). Need tulemused toetavad sugulise paljunemise toimumist populatsioonis ja seeläbi patogeeni geneetilise mitmekesisuse suurenemist ja oospooride moodustumist, mille abil organismile ebasoodsad tingimused üle elada ja mullas talvituda. Mõlema paarumistüübi esinemist on ka varasemalt leitud *P. infestans* populatsioonides Lätis (Aav et al., 2015), Leedus (Runno-Paurson et al., 2015) ja Eestis (Runno-

Paurson et al., 2009, 2010a, 2010b, 2011, 2012, 2013b, 2014). Selline olukord teeb patogeeni tõrje keerukamakas, kuna patogeen on kohastunud püsima oospooridena mullas. Soodsate keskkonnatingimuste ja peremeestaime olemasolul on oospoorid idanemisvõimelised veel isegi 4 aastat pärast mulda sattumist (Turkensteen et al., 2000).

Käesoleva uurimistöö tulemused näitasid, et Eesti, Läti ja Leedu kartuli-lehemädaniku tekitaja *P. infestans* populatsioonid on sagedase sugulise paljunemise tulemusel geneetiliselt väga mitmekesised (**I, II, III**). Nendes populatsioonides esineb suur hulk unikaalseid genotüüpe, kuid leidub ka kohalikke kloone, mis on kohastunud piirkonnas paljunema, levima ja ellu jääma (**I, II, III**). Baltimaade *P. infestans* populatsioonides levinud genotüüpide ja geneetilise mitmekesisuse uurimine kasutades geneetilisi SSR markereid oli esmakordne (**I, II, III**). Teistes Euroopa riikides laialt levinud invasiivseid kloone Balti riikides veel ei tuvastatud (**I, II, III**), kuid arvestades patogeeni väga hea kohastumisvõime ja muutuvate kliimatingimustega, on see tõenäoline. *P. infestans* isolaatide paarumistüüpide A1 ja A2 esinemine Eestis on peaaegu võrdne ja paarumistüübid esinevad enamasti koos samadel põldudel, mis suurendavad sugulise paljunemise toimumise tõenäosust (**I, III**).

Tulenevalt sugulise paljunemise võimekusest ja suurest geneetilisest mitmekesisusest Baltimaade *P. infestans* populatsioonides, tuleks kartulikasvatajatel lehemädaniku nakkusohu vähendamiseks hoida põldudel pikemat viljavaheldust (vähemalt 4 aastat), kasvatada lehemädanikule resistentsemaid sorte ja kasutada haigusvaba sertifitseeritud seemnekartulit. Kartulipõldude õigeaegne ja mõistlik töötlemine fungitsiididega, kasutades erineva toimeainega preparaate, on vajalik taimiku ja mugulate nakatumise vältimiseks, lehemädaniku leviku pidurdamiseks ja resistentsete patogeenipopulatsioonide tekke vältimiseks. Patogeeni genotüüpide ja kloonide levikut ning ellujäämist Balti riikides tuleb järjepidevalt jälgida, et populatsiooni muutustele asjakohaste ennetus- ja tõrjevõtetega reageerida.

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my supervisors Dr. Eve Runno-Paurson and Prof. Marika Mänd for their continuous encouragement and belief in me. My sincere thanks to my mentor, Dr. David Cooke from The James Hutton Institute for his contribution, valuable comments, and moral support.

Merili, Alice, Kätlin, and Helina are thanked for technical support out in the fields collecting isolates and all the hard work in the laboratory. I admire Dr. Eva Randall who helped and supported me in The James Hutton Institute. I´m grateful to Hannah Joy Kennedy, Dr. Reet Karise, and Dr. Liina Jakobson for language editing of my PhD thesis.

I´m grateful to scientists from the EuroBlight network for giving my studies an international dimension and introducing the world of *P. infestans* to me. I appreciate the national and international project partners for their input in collecting, managing, sharing and publishing the data included in this thesis. I acknowledge farmers and producers for their common sense and efforts in sustainable agriculture.

My dear friends and fellow fighters (Kaire, Liina, Britt, Gerit, Kalev, Jon) in the journey of doctoral studies, there has been highs and lows, but finally we achieve our goal. Thank you Reet for being on my side. Special thanks to all my colleagues for helpful discussions and support through my doctoral studies. I have learned a lot from you, especially that a key to success is co-operation, open mindedness, and inspiring each other. You always have to challenge yourself, otherwise you will get bored.

I appreciate my family and friends for sustaining, inspiration, and toleration. I love you.

This study has received funding from, Institutional Research Funding project IUT36-2, Coordinated Integrated Pest Management (C-IPM) in Europe project IPMBlight 2.0, Estonian Science Foundation grant 9432, and Archimedes Foundation project RESIST 3.2.0701.11-0003. The Scottish Government is acknowledged for funding at The James Hutton Institute. The study visit to The James Hutton Institute and participation in international conferences and workshops were supported by the European Social Fund's Doctoral Studies and Internationalisation

Programme DoRa and DoRa+, which are carried out by Foundation Archimedes. Doctoral studies were implemented under the Doctoral School in Earth Sciences and Ecology of EULS financed by ASTRA project "*Value-chain based bio-economy*" EU European Regional Development Fund.

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ORIGINAL PUBLICATIONS

Kiiker, R., Hansen, M., Williams, I.H., Cooke, D.E.L., Runno-Paurson, E. 2018.

OUTCOME OF SEXUAL REPRODUCTION IN THE PHYTOPHTHORA INFESTANS POPULATION IN ESTONIAN POTATO FIELDS.

European Journal of Plant Pathology, 152, 395–407.

Eur J Plant Pathol
https://doi.org/10.1007/s10658-018-1483-y

Outcome of sexual reproduction in the *Phytophthora infestans* population in Estonian potato fields

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Accepted: 9 April 2018

Abstract In this study, the Estonian population of *Phytophthora infestans* was characterized with mating type, sensitivity to metalaxyl, virulence on 11 potato R-gene differentials and 12 SSR markers to show the outcome of potential sexual reproduction in the population. During the three years 2010–2012, 141 *P. infestans* isolates, collected from 23 potato fields, showed quite a high and stable frequency of the A2 mating type, 48% of the total population. In 87% of all sampled potato fields, both mating types were recorded, suggesting continuous sexual reproduction of *P. infestans* and possible oospore production. Metalaxyl-sensitive isolates prevailed in all three years (68 out of 99 isolates). Amongst the 95 isolates tested, 51 virulence races were found. The race structure was diverse, and most pathotypes were unique, appearing only once; the two most common pathotypes, 1.2.3.4.6.7.10.11 and 1.2.3.4.7.10.11, comprised 35% of the population. The *P. infestans* population was genetically highly diverse and most of the multilocus genotypes (MLGs) appeared only once. Furthermore, all of the MLGs appeared in only one of the three sampling years. Our results confirm that the high diversity in the Estonian *P. infestans* population is most likely the result of frequent sexual reproduction, which benefits the survival, adaptability and diversity of the pathogen in the climate of North-Eastern Europe.

Keywords SSR markers · Genetic diversity · Mating type · Virulence · Metalaxyl resistance · Late blight

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10658-018-1483-y) contains supplementary material, which is available to authorized users.

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Introduction

Late blight, caused by the oomycete pathogen *Phytophthora infestans*, which made its first appearance in Europe in the mid-1840s, remains a major threat to European potato crops. Despite recent active research and progress, late blight still requires vigilance and often numerous applications of fungicide for effective control (Cooke et al. 2011). The disease is a serious problem for Estonian potato production, particularly under favourable conditions, when it can destroy the whole potato haulm and can cause 20–25% or even more loss of yield in untreated fields (Runno-Paurson et al. 2010). Fungicides are used routinely in conventional potato production, but under favourable conditions for the disease, with heavy pressure from the pathogen, timing the first preventive spraying and later protection of large areas is complicated without using information from decision support systems (DSS) (Runno-Paurson et al. 2010).

The oomycete *P. infestans* is heterothallic with two mating types, A1 and A2, enabling the pathogen to

Published online: 17 April 2018

reproduce both sexually and asexually (Fry et al. 1993). Before 1980, the population of the late blight pathogen, worldwide except in Mexico, appeared to be asexual and to consist of a single clonal lineage with only the A1 mating type. Nevertheless, moderately complex pathogen races dominated among the European populations (Shattock et al. 1977; Goodwin et al. 1994; Gisi and Cohen 1996). After the migration of new genotypes and the A2 mating type, apparently via potato imports from Mexico into Europe in the late 1970s, sexual reproduction of the pathogen became possible (Fry et al. 1993). In Europe, the A2 mating type was first reported in Switzerland in 1981 (Spielman et al. 1991) and six years later in 1987 in Estonia (Vorobyeva et al. 1991). New genotypes were more competitive, adaptable to the host and environment and therefore spread quickly across Europe displacing the old clonal lineage (Spielman et al. 1991; Gisi and Cohen 1996), which latterly has been found only rarely (Cooke et al. 2011). Sexual reproduction results in oospores which can survive in the soil for several years (Drenth et al. 1995); they may also survive cold temperatures which may even conserve their viability (Turkensteen et al. 2000; Lehtinen and Hannukkala 2004). The presence of sexual reproduction changes the epidemiology (Mayton et al. 2000) and increases the adaptability of the pathogen, and thus changes the way in which disease control must be approached (Cooke et al. 2011; Yuen and Andersson 2013).

There are clear indications that changes have occurred in *P. infestans* populations worldwide in the last decade, which concur with the increasing severity of late blight outbreaks on potato and tomato crops (Montarry et al. 2010; Cooke et al. 2012; Fry et al. 2013; Li et al. 2013b; Chowdappa et al. 2015). In the Nordic region, there are indications of earlier outbreaks of late blight, requiring more frequent fungicide treatments per season to control the disease, probably partly because of oospore-derived infections (Hannukkala et al. 2007; Hannukkala 2012; Yuen and Andersson 2013). Long-term observation data from Finland and Estonia show that the first findings of blight now occur one month earlier than 20 years ago and blight outbreaks are more severe (Hannukkala et al. 2007; Runno-Paurson et al. 2013a). In 2014, late blight infection was registered earlier than ever before, already on 19 June in several large potato production fields in southern Estonia (monitored by E. Runno-Paurson).

In addition to oospore-caused infections, increasing severity of late blight outbreaks could also be associated with specific pathogen genotypes. To identify individual genotypes and to explore population diversity, molecular markers have to be used. Simple-sequence repeat (SSR) markers are neutral, co-dominant, polymorphic, single-locus molecular markers, and therefore are considered to be the most informative and effective in pathogen population studies (Cooke and Lees 2004; Lees et al. 2006). From the beginning of the twenty-first century SSR markers have been used to characterise *P. infestans* populations all over the world and at present this is the preferred genotyping method (Knapova and Gisi 2002; Lees et al. 2006; Montarry et al. 2010; Gisi et al. 2011; Cooke et al. 2012; Li et al. 2013a; Chowdappa et al. 2015). Genotyping results have revealed that most of the populations in Great Britain, France, Switzerland, Netherlands, Belgium as well as in North America, India and China are clonal with few dominating genotypes in the populations probably with rare sexual reproduction events (Montarry et al. 2010; Gisi et al. 2011; Cooke et al. 2012; Hu et al. 2012; Li et al. 2012, 2013b; Fry et al. 2013; Chowdappa et al. 2015). Conversely, recent studies in the Nordic countries (Finland, Sweden, Norway, Denmark), Russia and Poland have shown high genotypic diversity in *P. infestans* populations with many different genotypes present indicative of the relevance of sexual reproduction in these populations (Widmark et al. 2007; Brurberg et al. 2011; Sjöholm et al. 2013; Chmielarz et al. 2014; Statsyuk et al. 2014; Brylinska et al. 2016).

Estonian populations of *P. infestans* have previously been investigated with several phenotypic and molecular markers in 2001–2007. The *P. infestans* pathotype structure was highly diverse and complex and also a high number of fingerprint genotypes and considerable number of metalaxyl-resistant isolates were identified in Estonia (Runno-Paurson et al. 2009, 2010, 2012, 2013b, 2014). Previous studies have shown a steady almost equal ratio of A1 and A2 mating types in the population and furthermore, both mating types were found in most of the studied potato fields (more than 80%). All this indicates the high potential for sexual recombination in the pathogen population (Runno-Paurson et al. 2009, 2010, 2012, 2013b, 2014). Due to sexual reproduction, there is an ongoing change in genotypes and continuous diversification occurring in the Estonian *P. infestans* population, and this should be continuously monitored. Whereas late blight is still a serious threat to Estonian

potato production, pathogen population studies are needed to efficiently control the pathogen population and manage late blight. Passing data on *P. infestans* genotypes through national and international networks to growers, advisors, breeders and agrochemical companies has already improved awareness and has had success in transfer of knowledge of population structure for adoption in practical late blight control (EuroBlight 2017; USAblight 2017).

In a previous study, nine polymorphic SSR markers were used to genotype Estonian *P. infestans* isolates from 2004 (Runno-Paurson et al. 2016), and a very high genotypic diversity was detected. In this more detailed study, more isolates from the main potato-growing areas in Estonia during a three-year period (2010–2012) were isolated and characterized phenotypically with mating type, resistance to metalaxyl, virulence assays and genotypically with a novel standardised SSR marker 12-plex approved and used by the EuroBlight network for monitoring *P. infestans* populations since 2013. Therefore, *P. infestans* genotypes and population structure in Estonia can be compared to the pathogen population situation in the whole of Europe. The main aim was to monitor recent changes in spatio-temporal variation in the population of *P. infestans* in Estonia. The following hypotheses were tested: 1) sexual reproduction is frequent in the population; 2) sensitivity to metalaxyl has increased; 3) a high number of virulence pathotypes are present; 4) no invasive clonal lineages are spreading or dominating in the population.

Materials and methods

Collection and isolation of *P. infestans* strains

Potato leaves infected by *P. infestans* were collected from 23 sites within the main potato-growing areas of Estonia (seven sites in 2010, eight sites in both 2011 and 2012) (Fig. 1, Table 1). The samples were collected from conventional production fields, organic fields and potato field trials, and at the beginning of late blight infection, in mid-outbreak (1–2 weeks later) and at the end of the growing season (>3 weeks later) each year. In the early stages of the outbreak, approximately 10–15% of the leaf area of the infected plants and less than 10% of plants were infected with late blight. In the later stages, about 20–30% of the leaf area and more than 50% of the plants were infected. Several fungicides with active ingredients such as metalaxyl + mancozeb, fluopicolide + propamocarb hydrochloride, cyazofamid, fenamidone + propamocarb hydrochloride, fluazinam, and mandipropamid were applied at different sites.

Two to fifteen isolates were cultured from each sampling site (Table 1). The plants from which samples were collected were located randomly across the field. From each plant, only single-lesion leaves were taken at random, excluding any with several or no lesions. Isolations were carried out and maintained using methods described by Runno-Paurson et al. (2009). All phenotypic tests were carried out every year of the study immediately after the isolations were finished (October to January). *P. infestans* isolates from this study are preserved at the Tartu Fungal Collection (TFC) in Estonia.

Phenotypic assays

Mating types were determined by the method described in Runno-Paurson et al. (2009). The tester isolates were 90,209 (A1) and 88,055 (A2) as described in Hermansen et al. (2000). Isolates forming oospores on plates with the A1 mating type were registered as A2; isolates that formed oospores with the A2 mating type were registered as A1.

Resistance to metalaxyl was tested using a modification of the floating leaflet method (Hermansen et al. 2000). Leaf disks (14 mm diameter) were cut with a cork borer from leaves of five-week-old greenhouse-grown plants. The susceptible cultivar 'Berber' was used. Six leaf disks were floated abaxial side up in Petri plates (50 mm diameter) each containing 7 mL of distilled water or metalaxyl in concentrations of 10.0 or 100.0 mg L^{-1} prepared from technical grade mefenoxam (Syngenta experimental compound (metalaxyl-M), CGA 329351A). The inoculation and trial incubation was done as described by Runno-Paurson et al. (2009). The isolates were rated resistant if they sporulated on leaf disks in 100 mg L^{-1} metalaxyl (Hermansen et al. 2000). Those sporulating on leaf disks in a metalaxyl concentration of 10 mg L^{-1}, but not on leaves floating on 100 mg L^{-1} were rated intermediate, and those sporulating only in water were rated sensitive.

The virulence pathotype was determined in 2011 and 2012 with a detached-leaflet set of Black's differentials of potato genotypes containing resistance genes R1-R11 from *Solanum demissum* (Malcolmson and Black 1966) (provided by the Scottish Agricultural

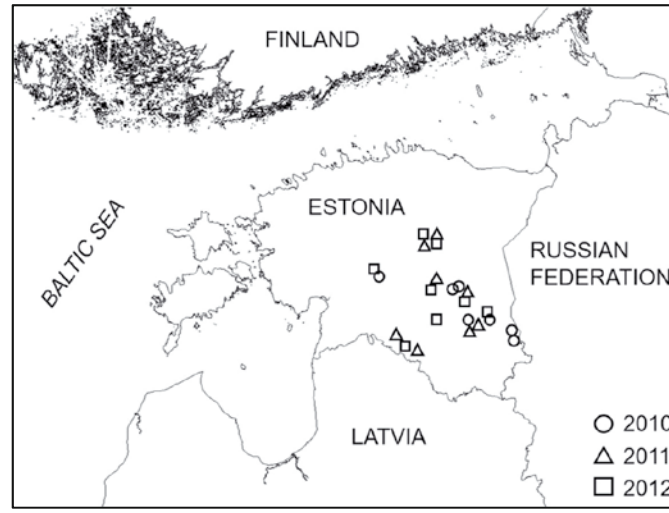

Fig. 1 Field sites in Estonia from where late blight samples were collected during 2010–2012

Science Agency). Laboratory procedures were as described in Runno-Paurson et al. (2009). Pathotypes were not tested in 2010 for logistic reasons.

Genotyping

Pure-culture *P. infestans* isolates were grown on rye B agar plates for 2–4 weeks at 17 °C. Mycelium was harvested and frozen. DNA extractions were carried out with the DNeasy Plant Mini Kit (QIAGEN), according to the manufacturer's instructions. Genotyping was performed at The James Hutton Institute (Scotland, UK), following the Li et al. (2013a) SSR marker 12-plex method with some modifications. One primer for each locus was labelled with different fluorescent labels (6FAM; NED; VIC; PET, Applied Biosystems) ensuring that no two markers with the same fluorescent dye had overlapping allele size ranges. PCR reactions were performed in a volume of 12.5 μl consisting of 1× Type-it Multiplex PCR Master Mix (QIAGEN Type-it Microsatellite PCR Kit, Cat. No. 206243), primers for each locus in optimal concentration (Online Resource 1) and 1 μl of template DNA (approximately 20 ng μl^{-1}). Amplification reactions were carried out under the following conditions: 95 °C for 5 min followed by 28 cycles of 95 °C for 30 s, 58 °C for 90 s, and 72 °C for 20 s, and a final extension at 60 °C for 30 min.

PCR products were diluted 0–100 times depending on the initial DNA concentration in the PCR reaction mix. The diluted PCR product (0.6 μl) was added to a mix containing 10.14 μl of deionized formamide (Hi-Di Formamide, Applied Biosystems) and 0.06 μl of GeneScan-500LIZ standard (Applied Biosystems). The fluorescent-labelled PCR products were analysed using an ABI3730 DNA Analyzer (Applied Biosystems) according to the manufacturer's instructions. The size of the alleles was determined using GeneMapper v3.7 (Applied Biosystems) software.

Data analysis

Statistical analyses were performed with the SAS/STAT version 9.1 (SAS Institute Inc., Cary, NC, USA). Differences in the prevalence of the two mating types of *P. infestans* isolates between years and study sites were tested using a logistic analysis (GENMOD procedure in SAS) with a multinomial response variable (A1, A2, or both). Analogous logistic procedures were used to examine the differences in the resistance to metalaxyl (a multinomial response variable: resistant, intermediate or sensitive) between years, sites and also between different mating types. The dependence of specific virulence (percent of isolates that show virulence against particular R-genes) on years, sites and R-genes was analysed with type III ANOVA and Tukey HSD post-hoc tests ($\alpha = 0.05$). In all analyses, "year" and "site" were treated as categorical variables. Race diversity was calculated with the normalized Shannon's diversity index (Sheldon 1969). The dependence of race complexity on isolation time was analysed with one-way

Table 1 The results of mating type (A1 and A2) and metalaxyl resistance (R–resistant; I–intermediate; S–sensitive) testing of *Phytophthora infestans* isolates collected in 2010–2012 from different sites in Estonia

Site	Year	Mating type		Response to metalaxyl		
		A1	A2	R	I	S
Kabila	2010	4	2	0	0	4
Kurista (I)	2010	2	2	–	–	–
Meeksi	2010	4	2	1	1	2
Naha	2010	3	3	0	2	2
Reola (I)	2010	3	3	0	0	2
Reola (II)	2010	2	6	0	0	5
Võnnu (I)	2010	3	2	–	–	–
Aardla	2011	1	6	0	0	4
Jõgeva (I)	2011	5	1	1	1	1
Jõgeva (II)	2011	9	6	1	3	9
Kiinimäe	2011	0	2	0	1	0
Kurista (II)	2011	1	1	0	0	1
Kurista (III)	2011	2	0	0	0	2
Pihle	2011	0	4	0	1	1
Reola (III)	2011	5	8	1	0	7
Eerika	2012	4	1	0	0	5
Holdre	2012	5	1	2	0	4
Jõgeva (III)	2012	3	3	1	3	2
Jõgeva (IV)	2012	5	1	0	2	4
Reola (IV)	2012	4	4	0	1	4
Sürgavere	2012	3	4	1	1	5
Tilga	2012	3	3	2	2	2
Võnnu (II)	2012	2	3	2	1	2
Total		73	68	12	19	68

ANOVA and Tukey HSD test, as were the differences in the Shannon's index values between collecting years and sites.

For genetic analysis, *P. infestans* subpopulations were defined according to sampling years and collecting sites. Data analysis was performed using Microsoft Office Excel 2013 Add-in GenAlEx version 6.501 (Peakall and Smouse 2006, 2012) and package *poppr* (Kamvar et al. 2014) within R version 3.1.0. All of the isolates were included in the calculations of single allele and allele pair (genotype) frequencies at all studied loci. Gene diversity (H) was calculated for all the loci according to the formula of $H = 1\text{-}\sum xj^2$, where xj is the frequency of the jth allele at the locus (Nei 1978). An H value equal to 0 implies no diversity at that locus and the limit of H is 1, which corresponds to the highest possible diversity. Inbreeding coefficient (F_{IS}) and fixation index (F_{ST}) were calculated over all collecting sites for 12 SSR loci.

Multilocus genotypes (MLGs) were compiled for all the isolates using all 12 SSR loci. A single difference in an allele size was considered enough to discriminate a unique MLG. If an isolate had one missing locus it was regarded as identical to another MLG if the other eleven loci were identical. This could possibly lead to a lower estimation of genotypic diversity but not substantially because high diversity is expected in the population. Genotypic diversity was calculated by a normalized Shannon's diversity index (*Hs*) (Sheldon 1969). Values for *Hs* may range from 0 (only single MLG present) to 1 (each isolate with a different MLG). The differences in the Shannon's index values between collecting years and sites were analysed with one-way ANOVA and Tukey HSD test.

A clone-corrected data set was constructed by including only one isolate of each MLG. Deviations from the Hardy-Weinberg equilibrium at loci were tested with a chi-square test using this data set. Random recombination between pairs of SSR loci was assessed with the standardized index of association ($\bar{r}_d$) which was calculated with 999 simulations (Agapow and Burt 2001). It is less biased than the index of association (I_A) as it accounts for the number of loci used for analysis.

Genotypic differentiation was assessed by calculating the Bruvo's genetic distance between the MLGs (Bruvo et al. 2004). This difference was used in a principal coordinate analysis (PCoA) to visualize any clustering based on isolate collecting year. Pairwise population Nei's genetic distance (Nei 1978) and Wright's fixation index (F_{ST}) values were calculated to estimate the genetic differentiation among the three years. The significance of pairwise F_{ST} values was tested by 999 permutations.

Results

P. infestans isolates collected from 23 sites in 2010–2012 were analysed for mating type and SSR marker genotype (141 isolates), a subset of 99 isolates for metalaxyl response and 95 for virulence (isolates only from 2011 and 2012).

Both A1 and A2 mating types were found in all three study years. Among 141 isolates, 73 were A1 mating

type and 68 were A2 mating type (Table 1). Both mating types were present at 20 of 23 sites (87%). Although the number of A1 and A2 mating types varied with year, there were no statistically significant differences between years (Chi-square = 1.99, df = 2, $p = 0.37$). Sample size was too small to detect any statistically significant differences in the proportion of A1 and A2 between sampling sites (Chi-square = 26.39, df = 22, $p = 0.24$). No statistically significant association between response to metalaxyl and mating type was found (Chi-square = 0.08, df = 2, $p = 0.96$).

Of the 99 isolates analysed for metalaxyl response, 12 were resistant, 19 were intermediate and 68 were sensitive (Table 1). No statistically significant differences were found between sampling years (Chi-square = 3.23, df = 4, $p = 0.52$) and sampling sites (Chi-square = 41.22, df = 40, $p = 0.42$).

All 11 known virulence factors were found among the 95 tested isolates (Fig. 2). A statistically significant difference in the prevalence of virulence factors (R1–R11) was observed in the two sampling years ($F_{(10,\ 154)} = 69.81$, $p < 0.001$). Virulence factors 9 (2011: 10.3 ± 4.7%; 2012: 4.2 ± 2.7%), 5 (2011: 13.4 ± 5.0%; 2012: 4.2 ± 4.2%) and 8 (2011: 17.0 ± 7.2%; 2012: 1.8 ± 1.8%) were relatively rare (Fig. 2). No statistically significant differences in virulence factors were found between years ($F_{(1,150)} = 2.64$, $p = 0.11$). However, there were statistically significant differences between study sites ($F_{(14,139)} = 3.60$, $p < 0.001$).

There was a high level of race diversity with 52 pathotypes identified among the 95 isolates tested (Table 2). The average number of virulence factors per isolate was 6.3 and ranged among sites from 4.3 to 8.5 and among years from 6.0 in 2012 to 6.6 in 2011. The most common races were 1.2.3.4.7.10.11 and 1.2.3.4.6.7.10.11, represented by 33 (35%) isolates. In 2011, 24 (60%) of the pathotypes were found only once, and in 2012, 18 (45%) of the detected pathotypes were unique (Table 2). The overall normalized Shannon's diversity index was very high, 0.74, but with no statistically significant differences between years ($F_{(1,10)} = 0.05$, $p = 0.83$).

Among all the 141 isolates characterised with SSR markers, 51 alleles were detected at 12 loci, which were all polymorphic (Online Resource 2). The number of alleles differed among the loci, ranging from two at loci SSR2 and Pi70 to nine at locus G11. In addition, at locus D13 a null allele was observed meaning that this locus was not amplified for as many as 48% of the isolates. Variability was detected in the allele frequencies between collecting years for nearly all the determined loci. 18 alleles out of all 51 had an overall frequency lower than 0.05 and were considered as rare alleles, which mainly appeared in the isolates collected in the same year. Two loci, D13 and G11, stood out with a high proportion of rare alleles in contrast to most of the loci where only one rare allele was detected. Gene diversities (H) for each SSR locus were calculated by further processing allele frequencies (Online Resource 2). The H value ranged between 0.08 (locus Pi70) to 0.8 (locus G11) with the mean of 0.53 over all loci. As the H value estimates the mean expected heterozygosity at the locus, the lowest value $H = 0.08$ at locus Pi70 makes it the least informative locus, because of one frequent dominant homozygous genotype 192/192 ($f = 0.92$) (Online Resource 3). By contrast, at locus G11 with the highest diversity nine alleles were observed and 13 heterozygous and only six homozygous genotypes were detected. However, the most frequent genotypes at locus G11 were homozygous 156/156 ($f = 0.13$), 154/154 ($f = 0.1$) and 162/162 ($f = 0.1$) (Online Resource 3). At the second-highest-diversity locus SSR4 ($H = 0.74$), 11 out of 14 genotypes were heterozygous and even five genotypes had a frequency $f \geq 0.1$. The lowest number of genotypes were observed at loci Pi70 (genotypes 192/192, 192/195) and SSR2 (173/173, 173/175, 175/175). Overall, 90 genotypes were detected and genotype frequencies varied between collecting years for all the determined loci (Online Resource 3). The rarest genotypes at each locus were detected mainly in one collecting year, for example genotypes 142/156, 152/208, 154/208, 164/164 at locus G11. At locus D13 as many as 8 out of 12 genotypes were rare and observed in one collecting year (Online Resource 3).

Four loci (D13, G11, Pi04 and Pi4B) were not in Hardy-Weinberg equilibrium. The inbreeding coefficient (F_{IS}) was negative for all but two loci indicating an excess of heterozygosity at most of the loci and asexual reproduction within sites (Table 3). The fixation index (F_{ST}) at all SSR loci varied between 0.101 and 0.394, with a mean of 0.220, which shows some degree of genetic differentiation between subpopulations collected from different sites (Table 3). However, the standardized index of association ($\bar{r}_d$) between loci did not differ significantly from zero ($\bar{r}_d = 0.003$) and also the null hypothesis of no linkage among loci failed to be rejected ($p = 0.26$), which could be explained by

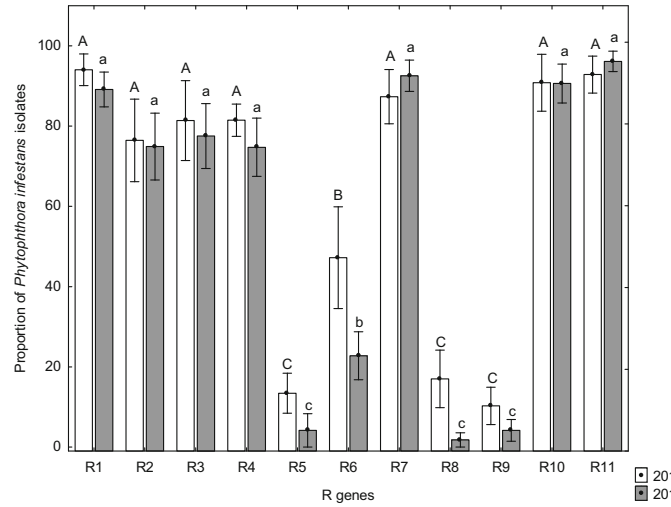

Fig. 2 Estimated mean frequency of virulence to potato R-genes in the Estonian population of *Phytophthora infestans* over all study sites during 2011–2012. Data are presented as means ± *SE* of the values for each location. Different letters upon the bars indicate significant differences at $\alpha = 0.05$ (ANOVA, Tukey HSD test), whereas uppercase and lowercase letters are for samples from 2011 and 2012, respectively

recombination of alleles into new genotypes during sexual recombination.

Multilocus genotypes (MLGs) were the result of the combination of alleles from all 12 SSR loci. In total, 96 MLGs were identified among the studied isolates, including 69 (72%) MLGs which appeared only once so were unique. Most of the MLGs that were identified more than once were associated with isolates collected from the same field and only seven (26%) repeated MLGs were discovered from separate sites. Moreover, all of the MLGs appeared in only one sampling year and not in other years. Genotypic diversity calculated by the normalized Shannon's diversity index was altogether very high ($Hs = 0.89$), being highest in 2010 ($Hs = 0.90$) and somewhat lower in 2012 ($Hs = 0.86$) and 2011 ($Hs = 0.84$), but with no statistically significant differences between years ($F_{(2,20)} = 2.78$, $p = 0.09$). Among sites, the highest genotypic diversity ($Hs = 1$) was identified in six fields out of 23 as all the isolates collected from these sites had unique MLGs.

Principal coordinate analysis (PCoA) did not reveal any clustering based on isolate collecting year (Fig. 3). These results were in concordance with the low values

Table 2 Number of different pathotypes among isolates of *Phytophthora infestans* from Estonia in 2011–2012

Pathotype	Number of isolates		Total
	2011	2012	
1.2.3.4.7.10.11	6	15	21
1.2.3.4.6.7.10.11	7	5	12
1.3.4.7.10.11	3	1	4
1.2.4.7.10.11	1	2	3
1.4.7.10.11	1	2	3
1.2.3.4.5.6.7.10.11	1	1	2
1.2.3.4.6.7.8.10.11	2	0	2
1.2.4.6.7.10.11	1	1	2
1.3.4.7.8.10.11	2	0	2
1.3.7.10.11	0	2	2
Pathotypes found once	24	18	42
Total number of isolates			95
Total number of pathotypes			52

Table 3 Inbreeding coefficient (F_{IS}) and fixation index (F_{ST}) values over all sampling sites for 12 SSR loci

SSR locus	F_{IS}	F_{ST}
G11	0.002	0.332
SSR4	−0.182	0.245
Pi4B	−0.322	0.145
Pi04	−0.480	0.101
Pi63	−0.324	0.214
D13	0.890	0.394
SSR8	−0.283	0.201
SSR6	−0.239	0.143
SSR2	−0.153	0.234
SSR11	−0.199	0.187
Pi02	−0.120	0.189
Pi70	−0.417	0.255
Mean	−0.152	0.220

of pairwise F_{ST} (0.007–0.020) and Nei's genetic distance (0.021–0.035) calculations, which additionally showed no differentiation between subpopulations collected in different years (Table 4).

Discussion

Whereas late blight is still a severe threat to Estonian potato production, continuous monitoring of local highly evolving *P. infestans* population is needed for effective late blight management. Therefore, recent changes in spatio-temporal variation were monitored with analysis of mating type, sensitivity to metalaxyl, virulence on 11 potato R-gene differentials and 12 SSR markers to show the outcome of possible sexual reproduction in the population. Increase in sensitivity to metalaxyl was presumed so metalaxyl-based fungicides could be efficiently used for late blight control. Frequent sexual reproduction was expected in the pathogen population as well as a high number of virulence pathotypes and high genetic diversity with no spread of clonal lineages.

In this study, both A1 and A2 mating types were found in Estonian *P. infestans* populations in all study years. The frequency of the A2 mating type remained quite stable over the three years, varying from 41% (20 out of 49) in 2012 to 55% (28 out of 51) in 2011, with an average of 48% over all isolates. These findings of mating types in Estonia are generally comparable to those found previously in Estonia (Runno-Paurson et al. 2014) and recently in Finland (Hannukkala 2012), other Nordic countries (Denmark, Norway, Sweden) (Lehtinen et al. 2008), Latvia (Aav et al. 2015), Lithuania (Runno-Paurson et al. 2015), Poland (Chmielarz et al. 2014), the Russian Federation (Moscow region) (Statsyuk et al. 2013) and the Czech Republic (Mazakova et al. 2006).

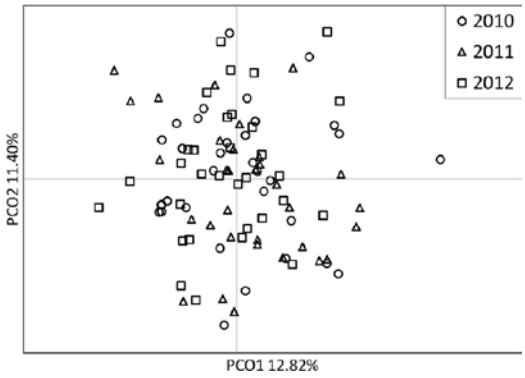

Fig. 3 Principal coordinate analysis using Bruvo's genetic distances of Estonian *Phytophthora infestans* isolates labelled according to the collecting year

Table 4 Matrix of population differentiation and genetic distance for *Phythophthora infestans* subpopulations collected in different years. Pairwise population F_{ST} values are below the diagonal and Nei's genetic distance values above the diagonal

Collecting year	2010	2011	2012
2010	–	0.021	0.035
2011	0.007	–	0.022
2012	0.020	0.009	–

There are several *P. infestans* populations in Europe, in the UK (2006–07), France (2006–07), Switzerland (2006–07) and Belgium (2007), where A2 mating type frequency has increased to high levels compared to previous studies (Gisi et al. 2011). These findings are related to the significant changes that have occurred in *P. infestans* European populations, including the UK. From 2004 to 2008, the extent of the A2 mating type increased from very low (<5%) up to 80% among the UK population mainly due to one specific invasive *P. infestans* genotype *13_A2* (also known as "Blue_13") (Cooke et al. 2012). The same genotype dominated the Dutch population from its first appearance in 2004 until 2009 (Li et al. 2012). It has also been found in other European countries including Poland (Chmielarz et al. 2014), which indicates the possibility of *13_A2* spreading to nearby populations, although as yet we found none in Estonia. Furthermore, no other clonal lineage appeared either. It is suggested that it is more difficult for a single genotype to invade a population with high diversity, compared to one where only a few genotypes dominate (Chmielarz et al. 2014).

Behind the high level of diversity in the Estonian *P. infestans* population is the presence of both mating types in the same fields enabling potential sexual reproduction and diverse MLGs together with a limited spread of these genotypes through the asexual cycle. In addition to the overall high genetic diversity, in six sampling fields all of the isolates had unique MLGs. Furthermore, all of the MLGs were detected in only one sampling year and none of them dominated in the whole population. These results are in accordance with the previous *P. infestans* population genotyping studies from Estonia in 2004 (Runno-Paurson et al. 2016), the

Nordic countries (Brurberg et al. 2011; Sjöholm et al. 2013), Poland (Chmielarz et al. 2014; Brylinska et al. 2016) and Russia (Statsyuk et al. 2014). Variation in genotypes among Estonian populations is so vast it totally differentiates from that in Western and Central European countries like the UK, France, the Netherlands and Switzerland (Montarry et al. 2010; Gisi et al. 2011; Cooke et al. 2012; Li et al. 2012), where genotype structure is clonal and only a few genotypes dominate.

Isolates with the same MLG collected from the same field at the same time referred to clonal (asexual) reproduction occurred within 74% of sampled sites. Also negative F_{IS} values in SSR loci (Table 3) are expected in asexually reproducing populations (Balloux et al. 2003). In contrast to the clonal reproduction within fields, only minimal clonal spread was observed between fields nearby (seven MLGs out of 96 were found in two separate fields). Additionally, F_{ST} values (0.101–0.394) support some differentiation of local field populations. Besides, none of the invasive clonal lineages common to Western Europe were detected. Similarly, studies in the Nordic countries showed a high level of clonal reproduction within fields, but no clonal spread between fields and the majority of genotypic variation within collecting sites (Sjöholm et al. 2013; Montes et al. 2016).

Comparing Estonian *P. infestans* population SSR marker results (Online Resource 2) with those from other Northern (Finland, Sweden, Norway, Denmark) and Eastern European (Poland) countries revealed both similarities and dissimilarities in allele frequencies and occurrence, although each study had slightly different sample collecting strategy. Dominating alleles were the same at all loci in Estonian and Polish populations, but allele frequencies were variable and the number of different alleles was higher in every studied locus within the Polish population probably due to extensive sampling from more collecting sites. But there were also alleles that were detected in the Estonian population that were missing in Poland, for example, allele 215 at locus Pi4B, 208 at G11, 138 at D13 (Chmielarz et al. 2014; Brylinska et al. 2016). Although the Nordic populations have been characterized with a different SSR marker set, some of the loci (Pi04, Pi4B, D13, G11) were the same (Brurberg et al. 2011; Sjöholm et al. 2013). Allele frequencies of the most common alleles at these loci were similar between Estonian and the Nordic populations, besides some of the alleles appeared in the Estonian population, but not in the Nordic countries, for example, allele 215 at locus Pi4B, 206 at G11, 142 at D13 (Brurberg et al. 2011; Sjöholm et al. 2013). Altogether, *P. infestans* isolates from these countries are likely to be genetically different, but all referred populations share the most common alleles at SSR loci although reported with variable frequencies.

The present study also showed that metalaxyl-sensitive isolates prevailed (69%) in the population. The frequency of metalaxyl sensitivity was quite homogeneous between different study sites and years, except for a small decrease in 2012, when the frequency of metalaxyl-sensitive isolates decreased from 78.9% in 2010 and 73.5% in 2011 to 60.9% in 2012. The product Ridomil Gold MZ 68WG, which contains metalaxyl-M and mancozeb is still in common use as an effective fungicide at the beginning of a late blight control strategy by potato growers in Estonia. Moreover, in Estonia, sales of Ridomil Gold increased steadily from 2011 to 2015 (producer's information, 2015). The increase in metalaxyl resistance coincides with increased use of metalaxyl-based fungicides in potato fields in 2012 when late blight occurred earlier and disease pressure was extra high. In contrast, weather conditions in 2010 and 2011 were unfavourable for late blight and disease appeared in the fields quite late in both seasons (Runno-Paurson et al. 2013a). These results contrast with those of previous studies carried out in 2001–2007 in Estonia, where the majority of the tested isolates were resistant or tolerant to metalaxyl (Runno-Paurson et al. 2009, 2010, 2012, 2013b, 2014). However, the increase in the frequency of metalaxyl-sensitive isolates was noticed in 2006 and 2007 (Runno-Paurson et al. 2012, 2014). This increasing trend has continued, and perhaps can be explained by more moderate use of metalaxyl fungicide than in the early 2000s. Our research findings about the dominance of metalaxyl-sensitive isolates in the Estonian population of *P. infestans* corroborate results from recent studies from other Northern and Eastern European countries, such as Finland, Sweden, Denmark and Norway (Lehtinen et al. 2008; Runno-Paurson et al. 2014; Montes et al. 2016), Latvia (Aav et al. 2015), Lithuania (Runno-Paurson et al. 2015), Russia (Moscow region, Statsyuk et al. 2013), Belarus (Pobedinskaya et al. 2011) and Poland (Chmielarz et al. 2014; Brylinska et al. 2016). Indeed, they differ completely from the findings in France in 2006–07 and the UK in 2006–07 (Gisi et al. 2011), where the frequency of metalaxyl-resistant isolates was very high probably due to high incidence of genotype *13_A2*.

The Estonian *P. infestans* virulence race structure found in this study was diverse and complex, affected by a high diversity of potato genotypes from which the isolates were obtained. On average more than half of the races were unique, and the two most common pathotypes 1.2.3.4.6.7.10.11 and 1.2.3.4.7.10.11 comprised only 35% of the population. These findings are similar to those of previous studies in Estonia (Runno-Paurson et al. 2009, 2010, 2012). The prevailing race of *P. infestans* (1.3.4.7.10.11) in most European populations (Hermansen et al. 2000; Knapova and Gisi 2002; Lehtinen et al. 2008; Hannukkala 2012; Chmielarz et al. 2014; Runno-Paurson et al. 2014) was found only three times in 2011 and once in 2012 in Estonia. In addition, the average number of virulence factors (infected Black's differentials) per isolate was 6.3, which is lower than those found in other populations from Eastern Europe (Śliwka et al. 2006; Statsyuk et al. 2013; Aav et al. 2015; Runno-Paurson et al. 2015; Michalska et al. 2016) and also from Estonia in previous long-term studies (Runno-Paurson et al. 2012, 2014). It has been shown that *P. infestans* isolates collected from more resistant varieties have more complex virulence races (Flier et al. 2007; Blandón-Díaz et al. 2012). Although locally bred potato varieties with undetermined R-genes in the genome are quite resistant to late blight in field conditions, these are not grown extensively in Estonia due to limited seed multiplication. However, conventional producers prefer to grow early maturity varieties mostly imported from the Netherlands and Germany, which are quite susceptible to late blight (Runno-Paurson et al. 2013a).

It has earlier been suggested that the difference in the population structure of the late blight pathogen between the Nordic region and most other parts of Europe is caused by climatic conditions (Brurberg et al. 1999). Estonia is located in North-Eastern Europe, where the temperature in the coldest months (December, January, February) is below 0 °C (−4.5 °C to −2.0 °C in average) with absolute minimum below −30 °C (Estonian Weather Service 2016). Before the *P. infestans* population displacement in the 1980s when sexual reproduction became possible, asexual populations needed a living host for survival to the next growing season (Fry et al. 1993). Because climatic conditions, especially cold winters in this region significantly reduce survival of clones between growing seasons in plant debris, volunteer potatoes and weed hosts (Brurberg et al. 1999; Grönberg et al. 2012), the build-up of blight epidemics was usually delayed until the end of the growing season in Northern Europe (Hannukkala 2012). For last few decades *P. infestans* populations in Northern Europe commonly reproduce sexually because oospore production benefits the survival and infectiousness of the pathogen (Brurberg et al. 1999; Lehtinen and Hannukkala 2004; Grönberg et al. 2012; Yuen and Andersson 2013). Additionally, highly resilient oospores of *P. infestans* present in the fields have a relevant role in initiating late blight infections early in the growing season which results in increased use of chemical control (Hannukkala et al. 2007; Widmark et al. 2007; Hannukkala 2012; Runno-Paurson et al. 2013a). Symptoms of soil-borne infection on lower leaves have been noticed in recent years in Estonian potato fields early in the season, especially in fields without or with short rotation of potato crops (personal observation by E. Runno-Paurson). Although oospore presence in the sample fields was not confirmed, mating type ratio, no predominant clonal lineages and high genetic diversity indicated the likely role of sexual reproduction in the *P. infestans* population in Estonia.

In Estonia, potato is grown under different field management practices. It is still usual to grow potatoes with a low seed quality in small field plots (<1 ha), with limited late blight control and irregular crop rotation (none to three years) (Runno-Paurson et al. 2013b). On the other hand, large conventional producers use certified seed potatoes, apply fungicide as many times as needed and grow potatoes in the same field only every 2–3 years. Study results show that the *P. infestans* population in Estonia is highly diverse in virulence races and MLGs. Additionally, no genotypes prevailed or persisted through the winter until the next growing season. The complexity of the pathogen population makes late blight management in Estonian potato fields more difficult and, for better results, fungicide treatments should be adjusted according to the population situation. In addition to high population diversity, sexual reproduction also results in oospores which can survive in the soil for several years and with a suitable host in range can induce late blight infection (Drenth et al. 1995). To efficiently control late blight in Estonia, the main aim should be to minimise oospore-derived infections. Therefore, longer crop rotations between growing potatoes in the same field and effective fungicide treatments in conventional fields to prevent infection and stop the late blight from spreading are necessary. Additionally, farmer awareness of *P. infestans* biology and

survival including the infection threat caused by oospores should be raised.

To conclude, our study indicates that in north-eastern Europe, where Estonia is located, the late blight pathogen *P. infestans* is reproducing sexually, which results in higher population diversity and production of oospores that contribute to better survival through the cold winters. Metalaxyl-sensitive isolates dominate in the pathogen population indicating a sensible and moderate use of metalaxyl-based fungicides. Altogether, the *P. infestans* population in Estonia is characterised by great virulence race diversity with a high number of virulence pathotypes present. Likewise, the genetic diversity is very high and no clonal lineages are spreading or dominating in the population.

Acknowledgments Alice Aav, Gerit Dreyersdorff, Kätlin Jõgi, Liis Laane, Helina Nassar, Terje Tähtjärv and Grete Zahkna are thanked for technical support. We are grateful to Asko Hannukkala from the Natural Resources Institute Finland (Luke) for supplying tester isolates for mating type determination. Many thanks too to Dr. Eva Randall at the James Hutton Institute for technical assistance.

Funding This study was supported by Estonian Foundation grant no 9432, Institutional research funding IUT36–2 of the Estonian Ministry of Education and Research, projects RESIST 3.2.0701.11–0003 and IPMBlight 2.0 8T150054PKTK. The Scottish Government is acknowledged for funding at the James Hutton Institute. The study visit to The James Hutton Institute was supported by the European Social Fund's Doctoral Studies and Internationalisation Programme DoRa, which is carried out by Foundation Archimedes.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Kiiker, R., Skrabule, I., Ronis, A., Cooke, D.E.L., Hansen, J.G., Williams, I.H., Mänd, M., Runno-Paurson, E. 2019.

DIVERSITY OF POPULATIONS OF *PHYTOPHTHORA INFESTANS* IN RELATION TO PATTERNS OF POTATO CROP MANAGEMENT IN LATVIA AND LITHUANIA.

Plant Pathology, 68, 1207–1214.

Plant Pathology (2019) Doi: 10.1111/ppa.13030

Diversity of populations of *Phytophthora infestans* in relation to patterns of potato crop management in Latvia and Lithuania

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Potato crop losses can be substantial when conditions for late blight (*Phytophthora infestans*) development and spread are favourable. In this study, drivers of differences between the *P. infestans* population structures in Latvia and Lithuania, two neighbouring countries with similar potato-growing traditions, were investigated. Genotypes of *P. infestans* and population genetic diversity were analysed using a 12-plex simple sequence repeat (SSR) marker assay. High genetic diversity was demonstrated in both populations, with population diversity being higher in Latvia. It would appear that local populations established from soilborne oospores early in the season are well adapted to the conditions in the region. However, somewhat greater spread and survival of local clones was detected in Lithuania, suggesting that potato cropping there is more vulnerable to clonal invasion than in Latvia. For effective disease management, current strategies should be adjusted according to the specific pathogen populations in the region, considering the reproduction and survival of the pathogen. Potato growers should implement late blight preventive measures such as longer field rotation to prevent oospore infections, especially in Latvia, and should use more disease resistant cultivars and high-quality seed potatoes.

Keywords: adaptation, late blight, population diversity, sexual reproduction, simple sequence repeat (SSR) markers

Introduction

Late blight, caused by the oomycete *Phytophthora infestans*, is one of the most economically damaging plant diseases, and certainly the most devastating disease, of potato. *Phytophthora infestans* caused the Irish Potato Famine in the 19th century, which resulted in the death of over one million people and extensive emigration from Ireland. This pathogen still poses a real threat to agriculture globally, especially to potato and tomato production, and has become serious in many countries during the last decade (Fry *et al.*, 2013; Chowdappa *et al.*, 2015). Because most potato varieties are susceptible to late blight, more applications of fungicide are needed, increasing production costs (Cooke *et al.*, 2011; Runno-Paurson *et al.*, 2013).

Phytophthora infestans has a heterothallic mating system with two mating types, A1 and A2. Epidemics of late blight are enabled by rapid asexual propagation of *P. infestans*, which, under favourable conditions, dramatically increases the inoculum load. Rapid spread of this pathogen can destroy entire fields of potato foliage within a few weeks or even days. A single recombination event may give rise to a pathogen genotype with a set of advantageous traits that enables all its asexually derived descendants (with further minor genetic variation due to mutation and mitotic recombination) to spread and become a dominant clone (Goodwin, 1997). Such clonal lineages of *P. infestans* are very successful and cause serious damage globally; examples include epidemics in the United States (Fry *et al.*, 2013), India (Chowdappa *et al.*, 2015) and western Europe (Cooke *et al.*, 2012; Li *et al.*, 2012).

Before the 1980s, the A2 mating type was absent from Europe, where only asexual reproduction was possible in the populations. In contrast, in Mexico for example, genetic diversity has always been high, and both mating types A1 and A2 have coexisted, enabling sexual reproduction (Grünwald & Flier, 2005). In the 1990s, sexual reproduction of the pathogen and the presence of soilborne inoculum was confirmed in the Netherlands and Sweden (Drenth *et al.*, 1995; Andersson *et al.*, 1998). After the spread of the A2 mating type and subsequent population changes due to sexual reproduction events at

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the end of 20th century, genetically highly diverse populations have been detected in Europe, for example in the Netherlands (Li *et al.*, 2012), Denmark, Norway, Sweden, Finland (Brurberg *et al.*, 2011; Sjöholm *et al.*, 2013), Estonia (Runno-Paurson *et al.*, 2016; Kiiker *et al.*, 2018) and Poland (Chmielarz *et al.*, 2014; Brylinska *et al.*, 2016). Sexual reproduction increases pathogen fitness via higher genetic diversity and production of oospores as a source of primary inoculum in the soil, which can increase the rate of adaptation of a population (Goodwin, 1997). In northern regions, it is especially beneficial for *P. infestans* to survive cold winters as oospores, which are very resilient to adverse climatic factors and remain viable for many years (Drenth *et al.*, 1995; Andersson *et al.*, 1998). Because of the high cost of sexual reproduction, facultatively sexual organisms can switch to sexual reproduction only when conditions are poor due to stress caused by environmental changes (for example temperature instability), food shortage, competition or lack of habitat or host, and otherwise reproduce asexually (Hadany & Comeron, 2008). Clement *et al.* (2010) observed a strong correlation between host effect on sporangia and oospore production, probably because of the trade-off between growth and reproduction in the condition of limited resources. Also, selection for maintaining locally adapted genotypes or heterozygosity in a pathogen population may occur when there is less need for sexual oospores for survival (Gladieux *et al.*, 2015).

In Latvia and Lithuania, the potato is one of the most important locally grown food crops. Most potato fields are small (≤1 ha) and many people grow their own potatoes (authors' personal observations). The average potato consumption from 2000 to 2013 was 117 and 111 kg per capita per year in Latvia and Lithuania, respectively (http://www.fao.org), which is considerably higher than in most European countries. Late blight is a serious threat to potato production almost every growing season and symptoms are usually first recorded in small potato fields and plots (Ronis *et al.*, 2007). In contrast with observations in Estonia (Runno-Paurson *et al.*, 2013), the first symptoms of late blight in Latvia and Lithuania have not been recorded significantly earlier than previously. However, depending on weather conditions, the disease incidence and severity are highly variable between years, ranging from very low (5%) to up to 100% (Valskyte *et al.*, 2003; I. Skrabule, personal observations). In both Latvia and Lithuania, the agricultural background and potato-growing traditions are similar, most of the small-scale farmers having limited financial resources to purchase certified seed potato and to apply sufficient chemical control against late blight. Furthermore, they do not grow varieties with better resistance to the disease or practise adequate crop rotation. Therefore, in favourable conditions, the pathogen can reproduce and spread freely, causing substantial damage to the crop (Ronis *et al.*, 2007; Aav *et al.*, 2015; Runno-Paurson *et al.*, 2015).

Effective late blight management is aided by up-to-date knowledge of the pathogen population. However, information on Latvian and Lithuanian populations of *P. infestans* is scarce and limited to only a few studies of phenotypic characterization (Valskyte *et al.*, 2003; Bebre *et al.*, 2004; Aav *et al.*, 2015; Runno-Paurson *et al.*, 2015). The mating type ratio of nearly 1:1, with both mating types occurring in the same fields, indicates a high potential for sexual reproduction and oospore formation (Aav *et al.*, 2015; Runno-Paurson *et al.*, 2015). In addition, oospores and late blight symptoms characteristic of oospore infections have previously been recorded in potato fields in Latvia (Bimšteine, 2008). However, further research is needed to obtain knowledge of the role of sexual reproduction in the pathogen population, as well as to assess the spread and survival of asexual clones adapted to Latvian and Lithuanian conditions.

The aim of this study was to determine the late blight pathogen genotypes and population structure in Latvia and Lithuania with a widely used and approved set of SSR markers (Li *et al.*, 2013). The role of sexual reproduction in *P. infestans* populations in these two countries (with similar potato-growing conditions and traditions) was also investigated and the hypothesis that sexual reproduction has led to genetic diversity of the *P. infestans* populations in both countries was tested. In addition, it was ascertained whether locally emerged clones adapted to the conditions in the region predominated over invasive clonal lineages from other European potato-growing regions.

Materials and methods

Collection of *P. infestans* isolates

Isolates of *P. infestans* were collected over the 3-year period 2010–2012 from 15 potato fields in Lithuania (69 isolates) and 21 fields in Latvia (125 isolates), all within the main potato-growing areas (Fig. 1). They were collected from conventional production fields (28 sites), organic fields (three sites), potato field trials at the Lithuanian Research Centre for Agriculture and Forestry in Akademija, Lithuania (three sites), and the Priekuli Plant Breeding Institute in Latvia (two sites). The majority of the potato growers were small-scale conventional farmers (15 sites) who used potato seeds of uncertain quality and applied chemical late blight treatments occasionally, varying from no sprays to four sprays. Large areas of potato were thus insufficiently protected by fungicides against late blight. Crop rotation by the small-scale growers varied widely, some not rotating, others after 3–4 years. In large conventional production fields (13 sites), high-quality certified potato seeds were used, planted no more frequently than every third year, and fungicides were applied four to seven times per season. The sampled plants were randomly selected across the field, and from each plant a blighted leaf with a single lesion was selected at random. From each field, 2–14 isolates were collected. Isolations were carried out as described in Runno-Paurson *et al.* (2009). *Phytophthora infestans* isolates used in this study are preserved in the Tartu Fungal Collection (TFC) in Estonia.

Genotyping with SSR marker 12-plex

Pure cultures of *P. infestans* were grown on rye B agar plates for 2–4 weeks at 17 °C. Mycelium was subsequently harvested

Figure 1 Location of potato fields from which isolates of *Phytophthora infestans* were collected in Latvia and Lithuania (2010–2012).

and frozen. DNA extractions were carried out with a DNeasy Plant Mini kit (QIAGEN) according to the manufacturer's instructions. Genotyping was performed in The James Hutton Institute (UK), following Li *et al.*'s (2013) SSR marker 12-plex method, with some modifications described in Kiiker *et al.* (2018). In short, PCRs were performed in a volume of 12.5 μL consisting of 1× Type-it Multiplex PCR Master Mix (QIAGEN), with fluorescence-labelled primers for each locus in optimal concentration (0.03–0.32 μM) and 1 μL template DNA (approximately 20 ng μL^{-1}). Amplification reactions were carried out under the following conditions: 95 °C for 5 min; followed by 28 cycles of 95 °C for 30 s, 58 °C for 90 s, and 72 °C for 20 s; and a final extension at 60 °C for 30 min. PCR products were diluted in ultrapure H_2O up to 100-fold, depending on the initial DNA concentration in the PCR mix. The diluted PCR product (0.6 μL) was added to 10.2 μL deionized formamide (Hi-Di formamide; Applied Biosystems) containing GeneScan-500LIZ standard (Applied Biosystems) at the ratio of 6 μL size standard to 1.014 mL deionized formamide. The fluorescent-labelled PCR products were analysed using an ABI3730 DNA Analyzer (Applied Biosystems) according to manufacturer's instructions. The size of the alleles was determined using GeneMapper v. 3.7 (Applied Biosystems) software.

Data analysis

Data analysis was performed using Microsoft Excel 2013 add-in GenAlEx v. 6.501 (Peakall & Smouse, 2012) and package poppr v. 2.0.2 (Kamvar *et al.*, 2014, 2015) in R. All of the isolates were included in the calculations of allele frequencies at all studied loci. Also, the Gini–Simpson index ($1 - \lambda$), Nei's unbiased gene diversity (H_{exp}), and evenness (E_5) of the alleles at each locus were calculated using poppr v. 2.0.2 in R. Differences in the index values between countries were analysed with one-way analysis of variance (ANOVA; $\alpha = 0.05$).

Multilocus genotypes (MLGs) were calculated for all isolates using the alleles from 11 SSR loci, excluding locus D13 because this locus was not amplified for as much as 42% of the Latvian and 16% of the Lithuanian isolates. A single difference in an allele size was considered enough to distinguish a unique MLG. If an isolate had one missing locus, it was regarded as identical to another genotype if the other 10 loci were identical. The abundance of isolates with unique MLGs versus local clonal MLGs was tested using a logistic analysis regarding the field type (large and small conventional, organic and trial fields) they were collected from. Estimates of genetic diversity for the populations included calculating the number of original MLGs, the number of expected MLGs at the smallest sample size based on rarefaction (eMLG), Simpson index (λ), evenness (E_5), Shannon diversity index corrected for sample size (H_S), and Nei's unbiased gene diversity (H_{exp}) using poppr v. 2.0.2 in R. Different measures were used for estimating genotypic diversity in populations. The Simpson index (λ) estimates the chance that two randomly selected genotypes in a population are different (Simpson, 1949). Shannon diversity index (H_S) accounts for both abundance and evenness of the genotypes present in a population (Shannon, 1948). Nei's genetic distance (H_{exp}) accounts for the genetic differences per locus within populations (Nei, 1978).

A clone-corrected dataset was used to test for linkage disequilibrium by calculating the index of association (I_A) and the standardized index of association ($\bar{r}_d$), which accounts for the number of loci sampled (Agapow & Burt, 2001). The same dataset was also used to test for Hardy–Weinberg equilibrium (HWE) in the populations.

Analysis of molecular variance (AMOVA), calculated with 999 simulations, was performed to estimate how much of the genetic variation was explained among and between subpopulations collected from different sites. Genetic relatedness among MLGs was assessed by calculating Bruvo's genetic distance between the isolates and was then visualized using a minimum-spanning network using poppr v. 2.0.2 in R.

Results

Altogether, 125 isolates from 21 sites in Latvia and 69 isolates from 15 sites in Lithuania were collected during 2010–12 and genotyped with 12 SSR markers. All 12 loci were polymorphic and 60 alleles were detected in total, varying from two at loci SSR2 and Pi70 to 11 at locus D13 (Table 1; Table S1). Although many alleles were detected at locus D13, only allele 136 dominated in the populations, and other alleles appeared in few isolates. Furthermore, a null allele appeared at locus D13 in 64 isolates, indicating that this locus was not amplified for as much as 42% of the Latvian and 16% of the Lithuanian isolates. Overall, variability was detected in the allele frequencies between the countries for all the determined loci (Table S1). Analyses showed that differences between the Latvian and Lithuanian populations according to the Gini–Simpson index ($F_{(11,12)} = 4.695$, $P = 0.006$), Nei's unbiased gene diversity ($F_{(11,12)} = 4.793$, $P = 0.006$), and evenness ($F_{(11,12)} = 3.272$, $P = 0.026$) values of the alleles were statistically significant (Table 1).

Multilocus genotypes analysis indicated 78 and 44 MLGs in the Latvian and Lithuanian isolates, respectively. The most frequent local clone in Lithuania, LT_MLG19 (comprising 29% of the population), survived between seasons and was detected in a large production field in Panevėžys in 2011, a trial field in Akademija in 2011, and a small conventional field in

Table 1 Summary of statistics from analysis of simple sequence repeat (SSR) loci in *Phytophthora infestans* populations from Latvia and Lithuania.

	Latvia				Lithuania			
SSR locus	N_a	$1-\lambda$	H_{exp}	E_5	N_a	$1-\lambda$	H_{exp}	E_5
D13	11	0.49	0.49	0.4	7	0.35	0.35	0.43
G11	8	0.8	0.8	0.88	8	0.68	0.69	0.65
Pi04	4	0.63	0.64	0.92	4	0.67	0.68	0.95
Pi4B	4	0.67	0.67	0.98	4	0.65	0.66	0.93
Pi63	3	0.63	0.63	0.93	3	0.39	0.39	0.62
Pi70	2	0.05	0.05	0.41	2	0.36	0.36	0.77
SSR2	2	0.47	0.47	0.95	2	0.49	0.49	0.98
Pi02	5	0.32	0.33	0.51	4	0.15	0.15	0.42
SSR4	7	0.74	0.75	0.82	7	0.75	0.75	0.81
SSR6	4	0.49	0.49	0.85	2	0.49	0.5	0.99
SSR8	2	0.5	0.5	0.99	3	0.41	0.42	0.69
SSR11	3	0.59	0.59	0.88	3	0.3	0.3	0.55

Number (N_a), Gini–Simpson index ($1-\lambda$), Nei's unbiased gene diversity (H_{exp}), and evenness (E_5) of the alleles at each locus.

Paežeriai in 2012; these sites were located over 90 km apart. The most frequent local clone in Latvia, LV_MLG10 (comprising 8% of the population), was detected only in 1 year (2012) in an organic field and a trial field in Priekuli, as well as a large production field in Dreimani 50 km apart. Other local clones repeatedly found were sampled two to six times, mostly in 1 year of sampling and from a single field outbreak. Some MLGs are closely related to each other and could be considered as minor variants of local clones, due to allelic variation mainly at loci G11 and SSR4. Some spread of local clones was observed but no MLGs were common to both countries despite the fact that sampling sites were located throughout both countries and also near the Latvian-Lithuanian border.

There were also 11 fields where every detected MLG was unique. In total, 75% of Latvian MLGs and 84% of Lithuanian MLGs were sampled only once in these populations. More unique MLGs than local clones were detected from small conventional fields. Also, the subpopulations from large conventional fields were marginally more clonal. However, there were no statistically significant differences in abundance of unique MLGs and local clonal MLGs between field types in the Latvian ($\chi^2 = 6.893$, df = 3, $P = 0.075$) or Lithuanian populations ($\chi^2 = 0.262$, df = 2, $P = 0.877$).

Genotypic richness can be measured as the number of observed MLGs, but because sample sizes from the two populations differed, an approximation of the number of genotypes that would be expected at the largest shared sample size based on rarefaction (eMLG) would be more appropriate. The value of eMLG was still higher for the Latvian population (49.5) than for the Lithuanian population (44; Table 2). In the Latvian population, genotypic and gene diversity were higher ($\lambda = 0.98$; $H_S = 0.85$; $H_{exp} = 0.54$) than in the Lithuanian population ($\lambda = 0.9$; $H_S = 0.77$; $H_{exp} = 0.49$; Table 2). Genotypic evenness was closer to equal abundance in the isolates from Latvia ($E_5 = 0.72$), where 78 MLGs were identified, none being dominant in the population. In the Lithuanian population, genotypic evenness was lower ($E_5 = 0.38$), mostly due to one well-adapted local clone, LT_MLG19, which comprised 29% of the Lithuanian population (Table 2).

To test whether the population structure was consistent with frequent sexual recombination in the populations, SSR loci were tested for linkage disequilibrium. Sampling during epidemics could lead to over-representation of clones. Therefore, a clone-corrected dataset was analysed. No significant linkage among markers was seen, suggesting prevailing sexual recombination in the population (Table 2). As expected in naturally evolving non-equilibrium populations, five loci were not in HWE in the Latvian population, and four loci were not in HWE in the Lithuanian population (Table S2).

AMOVA revealed that most genetic variation (92%) was found within fields and only a small amount of variation was explained between fields and countries (Table 3). Minimum-spanning networks (MSN) based on Bruvo's genetic distance displayed visually the relationships between multiple single-isolate MLGs and minor clones with larger nodes (Fig. 2). Minor variants of local clones also existed, shown connected by very thick lines (indicating close relatedness). MLGs shared over more than one season were uncommon (Fig. 2a, b). The absence of shared MLGs or country-specific clustering by genetic distance amongst the populations sampled in Latvia and Lithuania from 2010 to 2012 was also apparent (Fig. 2c).

Discussion

In Latvia and Lithuania, many people still grow potatoes for their own consumption. Most of the potato fields are

Table 2 Genetic diversity in Latvian and Lithuanian populations of *Phytophthora infestans* based on analysis of simple sequence repeat (SSR) markers.

Population	*N*	MLG	eMLG	SE	λ	E_5	H_S	H_{exp}	I_A	*P*	$\bar{r}_d$	*P*
Latvia	125	78	49.5	2.59	0.977	0.724	0.845	0.538	0.011	0.395	0.001	0.394
Lithuania	69	44	44.0	0	0.903	0.378	0.766	0.490	0.103	0.096	0.011	0.091

Number of isolates (*N*), number of original multilocus genotypes (MLG), number of expected MLG at the smallest sample size based on rarefaction (eMLG), standard error based on eMLG (SE), Simpson index (λ), evenness (E_5), normalized Shannon diversity index (H_S) and Nei's unbiased gene diversity (H_{exp}) for the population. The index of association (I_A) and the standardized index of association ($\bar{r}_d$) with *P*-values from 999 permutations were calculated based on a clone-corrected dataset.

Table 3 AMOVA results of Latvian and Lithuanian *Phytophthora infestans* populations based on analysis of simple sequence repeat (SSR) markers.

Source of variation	df	Sum of squares	Mean squares	Estimated variation	%
Among countries	1	46.69	46.69	0.22	5
Among fields	22	128.37	5.84	0.12	3
Within fields	364	1507.84	4.14	4.14	92

small and close to each other and also appropriate crop rotation is not applied. To achieve lower production costs, chemical control against late blight is used insufficiently in these countries. These factors encourage infections derived from soilborne oospores and also rapid reproduction and spread of *P. infestans* in the potato-growing season. This study, representing the first genetic analysis of Latvian and Lithuanian populations of *P. infestans*, revealed high genetic diversity, a large number of unique genotypes and some local clones adapted to regional conditions. There was no evidence of invasive clonal lineages originating from other parts of Europe spreading or dominating in this region in the years 2010–12.

Possible sexual reproduction and oospore formation in the Latvian and Lithuanian *P. infestans* populations are supported by the fact that A1 and A2 mating type isolates coexist in the potato fields and the mating type ratio is close to 1:1 in the population (Aav *et al.*, 2015; Runno-Paurson *et al.*, 2015). Also, oospores have been found in Latvian potato fields with a frequency of over 80% from collected leaflets, which makes disease management more difficult as the inoculum source is constantly present in the fields (Bimšteine, 2008). Short crop rotations between growing potatoes in the same fields facilitate oospore-driven epidemics, which stimulate a genetically diverse *P. infestans* population. For instance, in Lithuania and Latvia, 98% and 86%, respectively, of the MLGs are found only once or twice and probably have limited spread and survival ability. The high diversity in the *P. infestans* population in these regions is similar to that in Poland, the Nordic countries and some regions of the Netherlands (Li *et al.*, 2012; Sjöholm *et al.*, 2013; Chmielarz *et al.*, 2014; Brylinska *et al.*, 2016). A recent study revealed that *P. infestans* isolates collected from northeastern Europe (including Latvia and Lithuania) had long latent periods and low lesion growing rates relative to isolates from other warmer regions of Europe (Mariette *et al.*, 2016b). The authors hypothesized that such characteristics would favour coinfection by other isolates and therefore increase the probability of sexual reproduction between isolates with opposite mating types. Furthermore, these populations would benefit from increased overwintering survival, primarily with sexual oospores (Mariette *et al.*, 2016b).

In northern and eastern Europe, the values of diversity indices and evenness of *P. infestans* populations increase from south to north, from milder to colder winter periods, from Poland to Finland. In addition, southern populations are more prone to invasive clonal lineages and spread of local clones than those in the north, which have populations distinct to single fields and only limited spread of local clones (Brurberg *et al.*, 2011; Chmielarz *et al.*, 2014; Runno-Paurson *et al.*, 2016; Kiiker *et al.*, 2018). The difference in the late blight pathogen population structure between the Nordic region and most other parts of Europe may be due to climatic conditions (Brurberg *et al.*, 1999). Cold winters significantly reduce survival of clones in plant debris, volunteer potatoes and weed hosts between growing seasons (Brurberg *et al.*, 1999; Grönberg *et al.*, 2012). Conversely, oospores can survive in the soil for several years despite low temperatures, which may even conserve their viability (Drenth *et al.*, 1995). Therefore, genetic diversity in each of these populations is further enhanced by sexual reproduction and recombination. In addition, comparing the results of the present study of Latvian and Lithuanian populations of *P. infestans* with recent studies carried out in nearby Poland (Chmielarz *et al.*, 2014) and Estonia (Kiiker *et al.*, 2018), most of the SSR marker alleles are shared across all of these populations, with similar patterns of allele frequency at all loci except for the highly variable G11 and SSR4. In general, the populations are genetically similar to each other in these neighbouring countries due to the lack of migration boundaries and free spread of *P. infestans* isolates, enabling gene flow between populations. Even in the presence of a functional sexual reproduction system in the Lithuanian *P. infestans* population, selection for maintaining locally adapted genotypes or heterozygosity in the pathogen population may occur when there is less need of sexual oospores for survival (Gladieux *et al.*, 2015).

More fungicide applications can give rise to selection of insensitive clonal strains that may dominate locally or regionally (e.g. 13_A2, 37_A2; http://www.euroblight.net). However, the low fungicide application rates in Lithuania and Latvia reduce the likelihood of such selection pressure and may therefore act to maintain the diversity that comes from the oospore population in the soil (Aav *et al.*, 2015; Runno-Paurson *et al.*, 2015). This may explain why more unique MLGs, rather than local clones, were detected from small conventional fields where chemical late blight control is usually inadequate, varying from no sprays to up to four sprays per season. Consistently, large conventional production fields where fungicides were applied four to seven times per season were slightly more clonal. The situation is comparable to that of Estonia and Finland where four to six sprays are used on average, considerably less than in the Netherlands and Belgium (14 sprays on average) and UK (10 sprays on average; Cooke *et al.*, 2011; Hansen *et al.*, 2015).

Fungicides play a crucial role in the integrated control of late blight. Higher fungicide applications at an early stage of crop development in the presence of very low levels of pathogen inoculum are recommended (Cooke *et al.*, 2011). In areas of commercial potato production

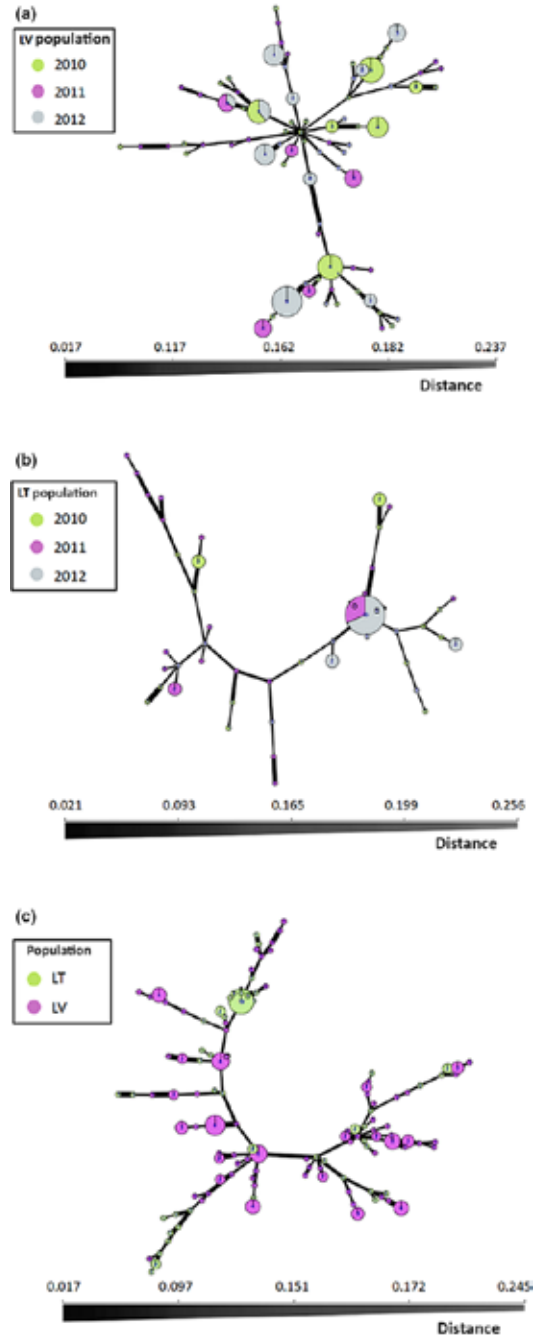

Figure 2 Minimum-spanning network of (a) Latvian (LV), (b) Lithuanian (LT) and (c) Latvian and Lithuanian populations of *Phytophthora infestans* created from analysis of data from simple sequence repeat (SSR) markers. Each multilocus genotype is represented by one node sized in proportion to its frequency in the populations. Line width and shading represent relatedness; line length is arbitrary.

in western Europe, *P. infestans* populations are primarily clonal with a few genotypes dominating in the populations. For example, in Great Britain in 2006–8, France in 2007–8, and the Netherlands in 2004–9, one of the dominant pathogen genotypes was metalaxyl-resistant 13_A2 (Cooke *et al.*, 2012; Li *et al.*, 2012; Mariette *et al.*, 2016a). Changes in population genetic structure occur every season and, over time, newer genotypes including 6_A1 and 1_A1 may displace older genotypes (http://www.euroblight.net). However, none of the clonal lineages found in western Europe (e.g. 13_A2, 6_A1) were detected in the present study of *P. infestans* isolates sampled from Latvia and Lithuania in 2010–12. In the last few years, only occasional findings of 13_A2 and 6_A1 genotypes have been confirmed in northern and northeastern Europe, including five cases of 13_A2 in 2016 and 2017 in Lithuania (http://www.euroblight.net). Generally, the survival and viability of the clonal lineages from western Europe are probably limited in cooler climates due to their local adaptation to temperature conditions prevailing in the area of origin (Mariette *et al.*, 2016b). In other European countries, sampling more isolates with the same clonal genotype has been associated with intense commercial potato production with selection pressure from high fungicide input (Brylinska *et al.*, 2016).

Although most of the MLGs were identified from a single field outbreak, a few MLGs were sampled in different years from multiple outbreaks, as in the case of the most common local clones, LT_MLG19 in Lithuania and LV_MLG10 in Latvia. Clonal emergence and survival indicates the relative importance of infected seed tubers and volunteer potatoes in the field acting as primary inoculum sources of *P. infestans* (Ronis *et al.*, 2007; Cooke *et al.*, 2011). Also, alternative hosts, such as *Solanum nigrum* (black nightshade) and *Solanum dulcamara* (woody nightshade), may interfere with current disease control strategies because weeds infected with *P. infestans* could serve as inoculum reservoirs throughout the growing season as well as between seasons (Flier *et al.*, 2003; Grönberg *et al.*, 2012).

Potatoes are the primary host for *P. infestans* in Baltic countries. Every season, around 50 potato cultivars are grown in each country according to the national data (http://www.vaad.gov.lv; http://www.vatzum.lt), resulting in high virulence race diversity in *P. infestans* populations in Latvia and Lithuania (Aav *et al.*, 2015; Runno-Paurson *et al.*, 2015). Because the growing season is relatively short, and to obtain a higher price for marketable potato, growers prefer to plant early-maturing potato cultivars, which are unfortunately rather susceptible to late blight (Asakavičiūtė *et al.*, 2009; Runno-Paurson *et al.*, 2013). Early maturity potato cultivars Laura, Vineta, Riviera and Solist, which are imported from the Netherlands and Germany, are among the most popular table potato cultivars in Latvia and Lithuania, despite the Latvian cultivars (for example Madara, Lenora, Prelma) being more suitable to the local conditions (http://www.vaad.gov.lv). Much effort is given to national potato breeding programmes in Latvia and Lithuania for developing potato cultivars suitable for local growing conditions and farming systems, with higher resistance to pathogens and excellent quality traits to satisfy consumer requirements (Asakavičiūtė *et al.*, 2009). But still, high-quality seed potato is not available for all potato-growing farms due to limited financial resources and, therefore, self-propagated seeds of uncertain quality are often used.

This first investigation of *P. infestans* genotypes in Latvia and Lithuania has revealed high genetic diversity. Analyses support frequent sexual recombination events in both populations, with oospores generating ephemeral local populations of genotypes that do not spread widely within that growing season or reappear the following year. Limited spread and survival of clones was more prevalent in Lithuanian than Latvian crops. Samples from 2010–12 showed no evidence of invasive clonal lineages from other parts of Europe spreading or dominating in this region. It would appear that local populations established from soilborne oospores early in the season are well adapted to the conditions in the region. Therefore, the two main hypotheses of this study were proven to be valid for the potato late blight population in the Baltic region. For effective disease management, current strategies should be adjusted according to the pathogen population status. Potato growers should be advised to implement late blight preventive measures such as longer field rotation to prevent oospore infections, especially in Latvia, and to use more disease resistant cultivars and high-quality seed potatoes.

Acknowledgements

This study was supported by Coordinated Integrated Pest Management (C-IPM) in Europe project IPMBlight 2.0, the European Regional Development Fund (Centre of Excellence EcolChange), Institutional Research Funding project IUT36-2, Development Fund of Estonian University of Life Sciences funded project PM170156PKTK, Estonian Science Foundation grant 9432, and Archimedes Foundation project RESIST 3.2.0701.11-0003. The Scottish Government is acknowledged for funding at The James Hutton Institute. The study visit to The James Hutton Institute was supported by the European Social Fund's Doctoral Studies and Internationalisation Programme DoRa, which is carried out by Foundation Archimedes. Merili Hansen, Alice Aav, Kätlin Jõgi and Helina Nassar from Estonian University of Life Sciences are thanked for technical support. Dr Eva Randall from The James Hutton Institute is highly acknowledged for help and support on genotyping. The authors are grateful to Dr Reet Karise and Jonathan Willow from Estonian University of Life Sciences and also the anonymous reviewers for critical reviewing of the manuscript.

The authors declare that they have no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Table S1. Allele frequencies of simple sequence repeat (SSR) markers in *Phythophthora infestans* isolates collected in 2010–2012 from Latvia and Lithuania.

Table S2. Summary of chi-square tests for Hardy–Weinberg equilibrium in clone-corrected dataset from simple sequence repeat (SSR) analysis of Latvian and Lithuanian populations of *Phythophthora infestans*.

III

Runno-Paurson, E., **Kiiker, R.,** Joutsjoki, T., Hannukkala, A. 2016.

HIGH GENOTYPIC DIVERSITY FOUND AMONG POPULATION OF *PHYTOPHTHORA INFESTANS* COLLECTED IN ESTONIA.

Fungal Biology, 120, 385–392.

FUNGAL BIOLOGY 120 (2016) 385–392

journal homepage: www.elsevier.com/locate/funbio

High genotypic diversity found among population of *Phytophthora infestans* collected in Estonia

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ARTICLE INFO

Article history:
Received 8 June 2015
Received in revised form
31 October 2015
Accepted 18 November 2015
Available online 2 December 2015
Corresponding Editor:
Hermann Voglmayr

Keywords:
Population variation
Potato late blight
Simple-sequence repeat (SSR)

ABSTRACT

Potato late blight, caused by the oomycete pathogen *Phytophthora infestans*, is one of the most important diseases of potato worldwide. This is the first study characterising Estonian *P. infestans* population using the SSR marker genotyping method. 70 *P. infestans* isolates collected during the growing season in 2004 from eight potato fields in three different regions of Estonia were characterised with nine polymorphic SSR markers. A1 and A2 mating type isolates were detected from every studied field indicating the high potential for sexual reproduction, which raises the genotypic diversity in *P. infestans* population. Results revealed highly diverse *P. infestans* population in Estonia resembling the Northern European populations. Most of the multilocus genotypes were detected only once among the collected isolates. Subpopulations collected from different geographical regions of Estonia showed no differentiation from each other but instead formed one highly diverse group.

Introduction

Oomycete *Phytophthora infestans* (Mont.) de Bary is one of the longest studied pathogens, causing late blight on potatoes and tomatoes. Since the 1840s, late blight has been the most destructive and feared potato disease in the world. Even now about 170 y later late blight is still an ongoing threat to potato production globally requiring effective fungicides despite the efforts of potato breeders and researchers (Cooke *et al.* 2011). *P. infestans* is an heterothallic organism with two mating types A1 and A2 having the ability to reproduce sexually and asexually. Until the mid-1970s, the worldwide population of late blight pathogen, except in Mexico, appeared to be asexual and to consist of a single US-1 clonal lineage with only A1 mating type. But still moderately complex pathogen virulence races dominated the European populations (Goodwin *et al.* 1994; Gisi & Cohen 1996). During the 1980s *P. infestans* populations containing both mating types migrated from Mexico apparently in the 1976/77 into Europe giving rise to sexually reproducing populations (Fry & Goodwin 1997). New genotypes were very adaptable and spread quickly throughout Europe displacing the old clonal lineage which is now found only rarely (Carlisle *et al.* 2002; Cooke *et al.* 2011).

Sexual reproduction results in long-lived oospores which can withstand unfavourable conditions and overwinter in the soil (Drenth *et al.* 1995; Andersson *et al.* 1998;

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http://dx.doi.org/10.1016/j.funbio.2015.11.008

Turkensteen et al. 2000; Lehtinen & Hannukkala 2004). The presence of sexual reproduction in *P. infestans* populations changed the epidemiology of potato late blight (Mayton et al. 2000) and thus changed the way that disease control must be approached (Cooke et al. 2011; Yuen & Andersson 2013). The effects of this change have been clearly noted in potato fields, where epidemics have started earlier and the number of fungicide treatments needed to control late blight has increased, probably partly because of oospore-derived infections (Hannukkala et al. 2007; Hannukkala 2012).

There are clear indications of changes in *P. infestans* populations in the last decade, which correspond with the increasing severity of the late blight outbreaks on potato and tomato crops (Montarry et al. 2010; Cooke et al. 2012; Chowdappa et al. 2013, 2015; Fry et al. 2013; Li et al. 2013). Most of the populations in Great Britain, France, Switzerland, the Netherlands, Belgium as well as in North-America, India and China are clonal with few dominating genotypes in the populations probably with rare sexual reproduction events (Guo et al. 2009; Montarry et al. 2010; Gisi et al. 2011; Cooke et al. 2012; Hu et al. 2012; Li et al. 2012, 2013; Wu et al. 2012; Chowdappa et al. 2013, 2015; Fry et al. 2013). In contrast, recent studies on *P. infestans* populations in the Nordic countries (Finland, Sweden, Norway, Denmark), Russia and Poland have shown high genotypic diversity with many different genotypes present indicating sexual reproduction in these pathogen populations (Widmark et al. 2007, 2011; Statsyuk et al. 2010, 2014; Brurberg et al. 2011; Sjöholm et al. 2013; Chmielarz et al. 2014).

Despite the long history of late blight monitoring and scientific studies, late blight remains a serious threat to Estonian potato production causing considerable yield loss in weather conditions favourable for the disease. Several studies since the early 2000s have shown that pathotype structure of *P. infestans* in the Estonian population is highly diverse and complex. Additionally, the almost equal frequency of A1 and A2 mating types and the occurrence of both mating types on the same potato field indicate the high potential for sexual recombination of the pathogen and increase the risk of oospore-derived infections (Runno-Paurson et al. 2009, 2010a, 2010b, 2011, 2012, 2013b, 2014). Likewise, previous studies on genetic characterisation of *P. infestans* Estonian isolates collected in 2002–2005 using RG57 fingerprints and mtDNA haplotypes have shown considerably high genetic diversity (Runno-Paurson et al. 2009, 2010a, 2010b). As the late blight pathogen population is diversified and variable in Estonia, changes in the population should be monitored regularly. Thus there is a need to continue with pathogen population studies to be up to date with the population situation in order to advise potato breeders and growers accordingly.

Molecular markers allow us to track and trace individual genotypes and to explore population diversity. Simple sequence repeats (SSRs) are neutral, co-dominant, polymorphic, single-locus molecular markers, and therefore they are considered to be the most informative and effective in pathogen population studies (Cooke & Lees 2004; Lees et al. 2006). From the beginning of the 21st century SSR markers have been widely applied for characterising *P. infestans* populations all over the world (Knapova & Gisi 2002; Lees et al. 2006; Montarry et al. 2010; Gisi et al. 2011; Cooke et al. 2012; Li et al. 2012, 2013; Chowdappa et al. 2013, 2015).

The current study provides valuable information on population genetic structure as this is the first study applying SSR assay on the Estonian *P. infestans* population. The main aim of this study was to confirm the existence of high genetic population diversity as a consequence of frequent sexual reproduction of *P. infestans*. We hypothesize that Estonian *P. infestans* population structures based on the collecting region.

Materials and methods

Collection and isolation of Phytophthora infestans *strains*

During the 2004 growing season, *Phytophthora infestans* isolates were collected from eight potato fields (four small scale conventional, three large-scale conventional productions and one untreated experimental field plot) in different regions of Estonia (Fig 1, Table 1) (Runno-Paurson et al. 2010a). Conventional producers were divided into two groups. The small scale conventional farmers used seed potatoes of uncertain quality and did not follow the rotation rules. Chemical late blight treatments were applied occasionally varying from no sprays to 1–4 sprays. In large-scale conventional fields high-quality certified seed potatoes were used, planted no more frequently than every 3rd y (with some exceptions) and applied fungicide 6–7 times or even as many as 11 times per season. In experimental plot at Jõgeva Plant Breeding Institute, diverse cultivars and breeding lines were used, and the quality of seed potatoes was also diverse. In all cases, isolates were collected at the beginning of the late blight outbreak. The sampled plants were randomly selected and from each plant a blighted leaf with a single lesion was selected at random. From each field six to ten isolates were collected (Table 1).

Isolations were carried out by placing a fragment of infected leaf tissue between ethanol and flame-sterilized

Fig 1 – Map of Estonian potato fields where the isolates of *Phytophthora infestans* were collected in 2004.

Table 1 – Origin and characteristics of *Phytophthora infestans* isolates collected from different regions in Estonia in 2004.

Region	Location	Number of isolates analysed for		
		Mating type	SSR alleles	MLGs
Northern	Laheotsa	9	9	9
	Ingliste	9	9	9
Eastern	Jõgeva	9	9	7
	Kambja	9	9	9
	Paalimäe	6	6	6
	Võnnu	9	9	7
Southern	Enge	9	9	9
	Antsla	10	10	10
Total		70	70	66

tuber slices of late blight susceptible cultivar 'Berber'. The slices were kept in sterile Petri dishes with a moist filter paper disk on top and incubated for 6–7 d at 16 °C in a growth chamber until the mycelia had grown through the slice. A small amount of mycelia was transferred with a sterile needle to rye B agar (Caten & Jinks 1968). The pure cultures were preserved at 5 °C and transferred to rye B agar after every 2 m.

Phenotypic analysis

Mating types (Table 1) were determined using the method described in Runno-Paurson *et al.* (2009). Isolates forming oospores on plates with the A1 mating type were classified as A2 and isolates that formed oospores with the A2 mating type were registered as A1. The tester isolates were the same as described in Lehtinen *et al.* (2007). More detailed description of other phenotypic characters (metalaxyl resistance, virulence) has been presented in Runno-Paurson *et al.* (2010a).

DNA extraction and SSR analysis

A representative selection of 70 isolates was chosen from 136 *Phytophthora infestans* isolates collected in 2004 for SSR marker genotyping, keeping the A1 and A2 mating type frequencies within fields and in the whole population about the same as previously published by Runno-Paurson *et al.* (2010a). DNA was extracted from the mycelia of *P. infestans* isolates that were grown on Petri plates of rye B agar (Caten & Jinks 1968) for 2–4 weeks at 18 °C in the dark. Mycelium was scraped off from Petri plates with a sterile scalpel blade. DNA was extracted using the DNeasy Plant Mini Kit (Qiagen), according to the producer's instructions.

SSR analyses were performed at MTT Agrifood Research, Finland. Nine polymorphic SSR regions were amplified using PCR with primers from previous studies: Pi02, Pi04, Pi16, Pi26, Pi33 (Lees *et al.* 2006), 4B, 4G, G11, D13 (Knapova & Gisi 2002). The primers were obtained from Applied Biosystems. For automated fragment analysis, one primer of each locus was labelled with a fluorescent dye (6-FAM, NED, PET, or VIC). Dyes were assigned to loci in such a way that loci with the same dye had non-overlapping ranges of allele sizes. This allowed simultaneous loading of several loci amplification reactions from one isolate, onto the capillary system (see below). Single locus amplifications were performed and the thermal cycling was carried out according to Brurberg *et al.* (2011). All PCR reactions were performed with a GenAmp PCR System 9700 (PE Applied Biosystems).

The fluorescently labelled PCR products of a single isolate were pooled and analysed using an ABI3730 DNA Analyzer with 48 36 cm capillaries, using Performance Optimized Polymer for 3730 DNA Analyzers (POP-7; Applied Biosystems). 1 µl of 12.5–71-fold (depending on the different markers) diluted PCR products were added to a loading buffer containing 8.8 µl Hi-Di™ formamide (Applied Biosystems), and 0.2 µl of GeneScan 500 LIZ size standard (Applied Biosystems). Electrophoresis of the samples was carried out at 66 °C and at 15 kV, for 20 min. The data was collected using the software Data Collection v 2.0 (Applied Biosystems), while GeneMapper v 4.0 (Applied Biosystems) was used to derive the fragment length of the labelled DNA-fragments using the known fragment lengths of the LIZ-labelled marker peaks. Allele size was determined by using the marker and by comparison with ten reference isolates (C1–10) kindly supplied by Drs Lees and Cooke, from The James Hutton Institute (Lees *et al.* 2006).

All the isolates were diploids, except for one isolate which had three identified alleles in locus Pi26. This phenomenon has also been reported by Lees *et al.* (2006), including five of the ten reference isolates that were used in this study. Following the strategy described by Holmes *et al.* (2009), the individual with more than two alleles at a locus was included in the present analysis after systematically excluding the largest allele.

Data analysis

Data analysis was performed using Microsoft Office Excel 2013 Add-in GenAlEx version 6.501 (Peakall & Smouse 2006, 2012). All of the 70 isolates were included in the calculations of allele frequencies in all studied loci. Gene diversity (H) was calculated for all the loci according to the formula $H = 1 - \sum xj^2$, where xj is the frequency of the jth allele at the locus (Nei 1978). An H value equal to 0 implies no diversity at that locus whereas one corresponds to the highest possible diversity.

Multilocus genotypes (MLGs) were compiled for all the isolates using the following five SSR loci: Pi02, Pi04, Pi16, Pi26, Pi33. A single difference in an allele size was considered enough to discriminate a unique MLG. Loci 4B, 4G, G11, and D13 were omitted from the MLG calculations since they failed to give results for more than 20 % of the isolates. If an isolate had one missing locus it was regarded as identical to another genotype if the other five loci were identical. This could possibly lead to a lower estimation of genotypic diversity. For four isolates, the data were missing in more than one of the six loci that were used for composing MLGs. These isolates were discarded from the genotype calculations, all of them were isolated from the Eastern region.

Genotypic diversity was calculated by a normalized Shannon's diversity index (Hs): $Hs = -1^{*}\sum Pi^{*}lnPi/lnN$, where Pi is the frequency of the ith MLG and N is the sample size. This diversity index corrects for differences in sample size (Sheldon

1969). Values for *Hs* may range from 0 (only single genotype present) to 1 (each isolate with a different genotype).

Genotypic differentiation was assessed by counting the number of pairwise genetic differences between all isolates. This difference was used in a principal component analysis (PCA) to visualize any clustering based on isolate origin (region). Nei's genetic distance (Nei 1978) and Wright's fixation index (F_{ST}) values were calculated to estimate the genetic differentiation among the three regions. The significance of F_{ST} values was tested by 999 permutations.

Results

Phytophthora infestans isolates characterised in this study were collected in 2004 from eight potato fields in Estonia at the beginning of the late blight outbreak. Six to ten isolates (mean = 9) from one field were characterised with mating type and SSR marker analysis (Table 1). Both mating types A1 and A2 appeared in every studied field indicating the high potential of sexual reproduction. The ratio of mating types was somewhat variable in different fields (Chi-square = 14.35; $p = 0.045$), and the frequency of A2 mating type varied from 17 % to 78 %. Still, the overall distribution of A1 and A2 mating type isolates was nearly equal to 1:1 as 36 out of 70 isolates were A2 mating type and 34 with A1 mating type (Table 3).

Among the 70 isolates characterised with SSR markers, 35 alleles were detected in nine loci, which were all polymorphic (Table 2). The number of alleles differentiated between the loci, ranging from two at locus Pi33 to five at loci Pi16 and Pi26. Variability was detected in the allele frequencies between the sampling regions for nearly all the determined loci. Ten alleles out of all 35 had an overall allele frequency lower than 0.05 and were considered as rare alleles, which mainly appeared in the isolates collected from the same geographical region. One locus (Pi16) stood out with a high proportion of rare alleles in contrast to most of the loci where only one rare allele was detected.

Gene diversities (*H*) for each SSR locus were calculated by further processing allele frequencies (Table 2). The *H* value ranged between 0.322 (locus Pi33) and 0.713 (locus Pi26) with the mean of 0.553 for all loci. Six out of nine loci (Pi04, Pi26, D13, G11, 4G, 4B) were so diverse that the *H* value was higher than the overall mean. Furthermore, the highest *H* value was calculated in the locus Pi26 and lowest in Pi33, as well as these were the loci were the maximum (5) and minimum (2) number of alleles were detected, respectively.

MLGs were the result of the combination of alleles from five SSR loci (Pi02, Pi04, Pi16, Pi26, Pi33). For genotypic analysis, four isolates were excluded because of the missing data in these loci and 66 isolates remained. In total 46 different MLGs were identified within studied isolates (Table 3), including 34 (74 %) MLGs which appeared only once and were considered as unique. Five of the MLGs that appeared more than once were identified from the same field and seven MLGs were discovered from separate fields. Genotypic diversity calculated by normalized Shannon's diversity index was altogether very high (*Hs* = 0.878) and according to the regional distribution it was highest in the Eastern region (*Hs* = 0.952) and somewhat lower in the Southern (*Hs* = 0.867) and the Northern region (*Hs* = 0.883) (Table 3). Among fields, the highest genotypic diversity (*Hs* = 1.000) was identified in an experimental field (Jõgeva) from the Eastern region because all the isolates collected from this field had unique MLGs.

The first two principal components of the PCA explained over 45 % of the overall genotypic variation in the *P. infestans* population in Estonia. PCA revealed no genetic differentiation within subpopulations collected from different geographical regions, but isolates from the Southern region were more closely grouped together than isolates from other regions (Fig 2). These results were in concordance with the low values of pairwise F_{ST} (0.006–0.010) and Nei's genetic distance (0.024–0.036) calculations, which additionally showed no differentiation between subpopulations collected from different regions (Table 4).

Discussion

In the present study, SSR marker assay was applied for the first time on *Phytophthora infestans* population collected from potato fields in Estonia. Although previous studies from Estonia have shown high genotypic diversity, which indicate a high frequency of sexual reproduction in the population, comparable results with other European studies were missing. Therefore, this study was carried out on a subset of the *P. infestans* population from Estonia using polymorphic co-dominant SSR markers in order to reveal regional population structure, evaluate genotypic diversity and also the relevance of sexual recombination in the population. Due to the location of Estonia and therefore the geographical and climatic similarities to the countries in Northern and North-Eastern Europe we expected a somewhat similar structure in pathogen populations as described in the countries nearby (Widmark *et al.* 2007; Brurberg *et al.* 2011; Sjöholm *et al.* 2013).

In this study, 36 out of 70 isolates were A2 mating type and 34 with A1 mating type. It has been shown in the present and several studies on Estonian *P. infestans* population that the potential for sexual reproduction is high as A1 and A2 mating type isolates have been found from the same potato fields (Runno-Paurson *et al.* 2010a, 2010b, 2011, 2012). Sexual reproduction in *P. infestans* populations in Northern Europe is common, because it benefits the survival and infectiousness of the pathogen (Brurberg *et al.* 1999; Lehtinen & Hannukala 2004; Grönberg *et al.* 2012; Yuen & Andersson 2013). Because climatic conditions, especially cold winters in this region will significantly reduce the survival rate of *P. infestans* mycelia between the seasons in plant debris, volunteer potatoes and weed hosts (Brurberg *et al.* 1999; Grönberg *et al.* 2012), the pathogen needs an alternative way to overcome these conditions (Yuen & Andersson 2013). For this, *P. infestans* benefits from sexual reproduction, which likely increases the adaptability of the pathogen and produces oospores that can survive in the soil for several years. Cold temperatures could even conserve the viability of the oospores (Turkensteen *et al.* 2000). *P. infestans* oospores present on the fields have a relevant role in initiating late blight infections early in the growing season (Hannukkala *et al.* 2007; Widmark *et al.* 2007; Hannukkala 2012; Runno-Paurson *et al.* 2013a). The risk of oospore-derived

Table 2 – Allele frequencies for SSR markers in 70 *Phytophthora infestans* isolates collected in 2004 from Estonia analysed by three geographical regions.

SSR locus	Allele	Allele frequencies				Gene diversity (H)[a]
		Northern	Eastern	Southern	Overall	
		N = 18	N = 33	N = 19	N = 70	
Pi02	156	0.056	0.063	0.000	0.044	0.371
	160	0.194	0.141	0.222	0.176	
	162	0.750	0.781	0.778	0.772	
	164	0.000	0.016	0.000	0.007	
Pi16	158	0.000	0.063	0.000	0.029	0.477
	174	0.412	0.234	0.211	0.272	
	176	0.559	0.656	0.789	0.669	
	178	0.029	0.031	0.000	0.022	
	180	0.000	0.016	0.000	0.007	
Pi04	166	0.000	0.000	0.088	0.026	0.628
	168	0.382	0.348	0.382	0.368	
	170	0.118	0.174	0.118	0.140	
	172	0.500	0.478	0.412	0.465	
G11	142	0.000	0.042	0.000	0.018	0.660
	158	0.438	0.104	0.344	0.268	
	160	0.438	0.333	0.594	0.438	
	162	0.125	0.521	0.063	0.277	
4G	159	0.111	0.310	0.147	0.196	0.579
	161	0.639	0.500	0.618	0.580	
	163	0.250	0.190	0.206	0.214	
	171	0.000	0.000	0.029	0.009	
Pi33	203	0.806	0.867	0.684	0.799	0.322
	206	0.194	0.133	0.316	0.201	
4B	205	0.433	0.333	0.389	0.382	0.669
	209	0.033	0.000	0.000	0.010	
	213	0.300	0.194	0.389	0.294	
	217	0.233	0.472	0.222	0.314	
D13	132	0.000	0.083	0.167	0.080	0.558
	134	0.500	0.500	0.667	0.540	
	136	0.500	0.417	0.167	0.380	
Pi26	177	0.444	0.323	0.395	0.375	0.713
	179	0.056	0.274	0.184	0.191	
	181	0.389	0.306	0.263	0.316	
	183	0.111	0.048	0.158	0.096	
	185	0.000	0.048	0.000	0.022	

a $H = 1 - \sum xj^2$, where xj is the frequency of the j-th allele at the locus (Nei 1978).

infections in Estonia is high, because small scale farmers do not follow rotation rules and may grow potatoes every year on the same field with inadequate control of late blight. As a result, a genetically diverse inoculum is present in fields, which is confirmed by the current results of high genotypic diversity and several unique MLGs determined from each sampled field. Also long-term observation data from Finland and Estonia show that the first late blight symptoms now occur one month earlier than two decades ago and blight outbreaks are more severe (Hannukkala *et al.* 2007; Hannukkala 2012; Runno-Paurson *et al.* 2013a). Furthermore, in the summer of 2004 when the current study was carried out, the first symptoms of late blight (lesions on the lower leaves) indicated oospore-derived infection and also the outbreak was reported approximately one week earlier in the growing season than in previous years.

The distribution and frequency of alleles distinguish Estonian *P. infestans* population from the Nordic countries analysed with the same SSR marker assay (Brurberg *et al.* 2011). For example, the count of different alleles and also locus diversity in two loci (Pi02 and G11) were much higher in the Nordic countries than in Estonia, but most of the alleles present in the Estonian population were also described in the Nordic dataset. However, the dominating alleles in six out of nine SSR markers (Pi04, G11, 4G, 4B, D13, and Pi26) differentiated between Estonian and Nordic populations (Brurberg *et al.* 2011). The Estonian *P. infestans* population corresponds to populations from Northern Europe and other parts of Europe concerning dominating alleles in loci Pi02 (162) in years 2000–2005 and Pi33 (203) in years 2000–2008, which seem to be quite stable alleles in different populations (Brurberg *et al.* 2011; EucaBlight). In addition, homozygotes (176/176) in locus Pi16 dominate in the Nordic countries (Brurberg *et al.* 2011) and Estonia, which is in contrast with the prevailing heterozygotes in locus Pi16 (176/178) in the populations of other European countries (England, Scotland) in 2003–2004 (EucaBlight).

Table 3 – Genotypic diversity and mating type distribution of *Phytophthora infestans* isolates collected from three regions of Estonia in 2004.

Region	Location	Number of				Hs[c]
		Isolates	A1[a]	A2[a]	MLGs[b]	
Northern	Laheotsa	9	3	6	8	0.930
	Ingliste	9	6	3	6	0.763
	Northern total	18	9	9	14	0.883
Eastern	Jõgeva	9 (7)[d]	3	6	7	1.000
	Kambja	9	2	7	9	1.000
	Paalimäe	6	5	1	5	0.871
	Võnnu	9 (7)[d]	2	7	6	0.898
	Eastern total	33 (29)[d]	12	21	26	0.952
Southern	Enge	9	7	2	7	0.833
	Antsla	10	6	4	7	0.819
	Southern total	19	13	6	14	0.867
Overall		70 (66)[d]	34	36	46	0.878

a Number of A1 and A2 mating type isolates, respectively.
b Number of multilocus genotypes (MLGs) based on 5 loci: Pi02, Pi04, Pi16, Pi26, Pi33.
c Normalized Shannon's diversity index, $Hs = -1*\sum Pi*lnPi/lnN$, where Pi is the frequency of the ith MLG and N is the sample size.
d Number of isolates (in the brackets) remained in the genotypic analysis when some of the isolates were excluded because of the missing data.

The results of SSR genotyping indicated high genotypic diversity in the Estonian *P. infestans* population because the majority of the identified MLGs were unique. Additionally, PCA coupled with the calculations of pairwise F_{ST} and Nei's genetic distance confirm the lack of population structure based on the collecting region. Current sampling strategy (2–4 fields from a region) might not have been sufficient for showing population structuring in this study, and future studies are needed by sampling isolates from more fields and geographical regions. But the total high diversity ($Hs = 0.878$) in the population is consistent with frequent sexual reproduction between A1 and A2 mating type individuals. Still there is some degree of clonal reproduction and spread of inoculum over significant distance (100–200 km). While diversity in each field is high and no MLG has an advantage over others, it is difficult for single genotypes to spread and invade a population. The high genotypic variation in Estonia is in contrast to the low variation in Western and Central European countries like UK, France, the Netherlands and Switzerland (Montarry *et al.* 2010; Gisi *et al.* 2011; Cooke *et al.* 2012; Li *et al.* 2012), where genotype structure is clonal and only a few genotypes have an advantage to dominate in the populations. Estonian high diversity population is more similar to the Northern and North-Eastern Europe, where almost every isolate has a different genotype and clonal lineages are not dominant (Brurberg *et al.* 2011; Sjöholm *et al.* 2013; Chmielarz *et al.* 2014; Statsyuk *et al.* 2014). Our results also agree with the high genotypic diversity in the Estonian population shown previously with RG57 fingerprinting and mtDNA haplotype analysis (Runno-Paurson *et al.* 2009, 2010a).

Genotyping *P. infestans* isolates collected from Estonian potato fields with SSR markers provides evidence that sexual reproduction and recombination are common in this population. Therefore, the genotypic diversity of the late blight pathogen in Estonia is extremely high and population shows no regional structuring. The current study was carried out with a limited dataset, but still gave valuable information on the genetic structure of the *P. infestans* population in Estonia. To improve late blight control and make long-term disease management decisions, furthermore extensive studies are required. Therefore, tracking the spatial and temporal

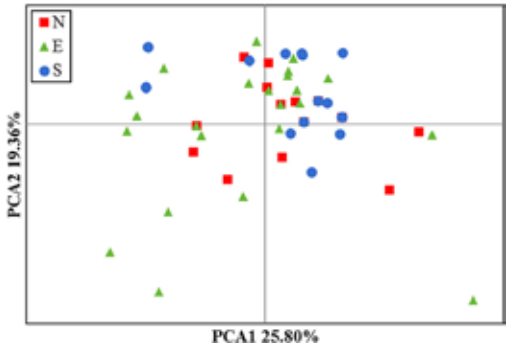

Fig 2 – Principal component analysis of multilocus genotypes of *Phytophthora infestans* isolates collected in 2004 from three Estonian regions (N–Northern, E–Eastern, S–Southern).

Table 4 – Matrix of population differentiation and genetic distance for *Phytophthora infestans* populations from three regions of Estonia (N–Northern, E–Eastern, S–Southern). Pairwise population F_{ST} values are below the diagonal and Nei's genetic distance values above the diagonal.

Region	N	E	S
N	0.000	0.024	0.036
E	0.006	0.000	0.031
S	0.010	0.006	0.000

dynamics of *P. infestans* population is planned for the future to provide a more comprehensive dataset using polymorphic co-dominant SSR marker assay.

Acknowledgements

We thank the anonymous referees for their valuable comments on the manuscript. The study was supported by the Estonian Foundation grant no 9432; Target Financing SF170057s09, Institutional research funding IUT36-2 of the Estonian Ministry of Education and Research and project RESIST 3.2.0701.11–0003. Dr Ingrid H. Williams is highly thanked for language editing. Dr Mati Koppel from the Estonian Crop Research Institute is greatly thanked for applying for Estonian Foundation grant 7391 which covered expenses to sample isolation and transport.

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CERCS speciality B230: Microbiology, bacteriology, virology, mycology

Participation in research projects:

2015-2019	IUT36-2 " Sustainable crop protection: harnessing ecosystem services for plant production ", EULS, other research staff
2016-2019	8T150054PKTK " IPM2.0 for sustainable control of potato late blight – exploitng pathogen population data for optimised Decisions Support Systems", EULS, senior research staff
2017-2018	PM170156PKTK "Molecular phytopathology – diagnostics of plant diseases, identifying and screening of plant pathogens using effective molecular methods", EULS, senior research staff
2013-2015	ETF9432 (ETF9432) "Phenotypic and genotypic characterisation of Baltic and Russian Pskov region populations of *Phytophthora infestans*; the role of oospores as a source of primary inoculum to late blight pathogen epidemiology", EULS, senior research staff
2011-2012	ETF7827 "Metagenomic and genomic approaches in studying of microbial biodegradation: A case study - the Baltic Sea water", University of Tartu, senior research staff

Professional training:

2019	ERASMUS+ Staff mobility for training at the Latvia University of Life Sciences and Technologies in Jelgava, Latvia
2019	Training course "Modern studying and teaching in the University", EULS
2015	Training course in statistics "Principal component analysis", EULS
2014	Professional training at the The James Hutton Institute in Dundee, Scotland for genotyping *P. infestans* populations under the supervision of Dr. David E. L. Cooke.

Research organizational activities:

Since 2015	Member of European Association for Potato Research (EAPR)
Since 2015	Member of EuroBlight network
Since 2013	Member of Estonian Plant Protection Society

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2013-2020	Eesti Maaülikool, doktoriõpe põllumajanduse erialal
2010-2013	Tartu Ülikool, magistriõpe geenitehnoloogia erialal
2007-2010	Tartu Ülikool, bakalaureuseõpe geenitehnoloogia erialal
1996-2007	Tartu Karlova Gümnaasium
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Teenistuskäik:

Alates 2020	Eesti Taimekasvatuse Instituut, Teadur (0,5)
Alates 2019	Eesti Maaülikool, Põllumajandus- ja keskkonnainstituut, Taimetervise õppetool, Nooremteadur (1,00)
2017-2019	Eesti Maaülikool, Põllumajandus- ja keskkonnainstituut, Taimetervise õppetool, Nooremteadur (0,50)
2014-2015	Eesti Maaülikool, Põllumajandus- ja keskkonnainstituut, taimekaitse osakond, Spetsialist (0,50)
2011-2013	Reproduktiivmeditsiini ja -bioloogia Tehnoloogia Arenduskeskuse AS, Laborant (0,75)

Teaduskraad:

2013	Magistrikraad geenitehnoloogia erialal, Tartu Ülikool

Teadustöö põhisuund:

CERCS eriala B390: Taimekasvatus, aiandus, taimekaitsevahendid, taimehaigused; PÕHISUUND: Fütopatoloogia

CERCS eriala B230: Mikrobioloogia, bakterioloogia, viroloogia, mükoloogia

Teadusprojektid:

2015-2019	IUT36-2 "Jätkusuutlik taimekaitse: ökosüsteemi teenuste rakendamine taimekasvatuses", Eesti Maaülikool, täitja
2016-2019	8T150054PKTK "Jätkusuutlik kartuli- lehemädaniku tõrje – patogeeni populatsiooni uuringute andmete alusel nõuandesüsteemi optimeerimine", Eesti Maaülikool, põhitäitja
2017-2018	PM170156PKTK "Molekulaarne fütopatoloogia – taimehaiguste diagnostika ja taimepatogeenide identifitseerimine nende leviku hindamisega kasutades kiireid molekulaarseid metoodikaid ", Eesti Maaülikool, põhitäitja
2013-2015	ETF9432 (ETF9432) "*Phytophthora infestans* Baltikumi ja Venemaa Pihkva regiooni populatsioonide fenotüübiline ja genotüübiline iseloomustamine; oospooride kui esmase nakkusallika roll lehemädanikutekitaja epidemioloogias", Eesti Maaülikool, põhitäitja
2011-2012	ETF7827 "Mikroobse biodegradatsiooni genoomne ja metagenoomne uurimine: Balti mere vee näitel", Tartu Ülikool, põhitäitja

Erialane täiendamine:

2019	Erasmus+ töötatajate õpiränne Läti Põllumajandusülikooli Jelgavas Lätis
2019	Koolitus "Nüüdisaegne õppimine ja õpetamine kõrgkoolis", Eesti Maaülikool
2015	Statistika koolitus "Peakomponentanalüüs", Eesti Maaülikool
2014	Erialane väljaõpe *P. infestans* SSR mikrosatelliitide määramismetoodika õppimiseks, multilookusgenotüüpide tuvastamiseks ja tulemuste analüüsimiseks James Huttoni Instituudis Dundees Šotimaal Dr. David E. L. Cooke juhendamisel.

Teadusorganisatsiooniline tegevus:

Alates 2015	European Association for Potato Research (EAPR) liige
Alates 2015	EuroBlight võrgustiku liige
Alates 2013	Eesti Taimekaitse Seltsi liige

LIST OF PUBLICATIONS

Scholarly articles indexed by Web of Science (1.1.)

Loit, K., Adamson, K., Bahram, M., Puusepp, R., Anslan, S., **Kiiker, R**., Drenkhan, R., Tedersoo, L. (2019). Relative performance of MinION (Oxford Nanopore Technologies) *vs*. Sequel (Pacific Biosciences) third-generation sequencing instruments in identification of agricultural and forest fungal pathogens. Applied and Environmental Microbiology, 85 (21), ARTN e01368-19. DOI: 10.1128/AEM.01368-19.

Kiiker, R., Skrabule, I., Ronis, A., Cooke, D. E. L., Hansen, J. G., Williams, I. H., Mänd, M., Runno-Paurson, E. (2019). Diversity of populations of *Phytophthora infestans* in relation to patterns of potato crop management in Latvia and Lithuania. Plant Pathology, 68, 1207–1214. DOI: https://doi.org/10.1111/ppa.13030.

Kiiker, R., Hansen, M., Williams, I., Cooke, D., Runno-Paurson, E. (2018). Outcome of sexual reproduction in the *Phytophthora infestans* population in Estonian potato fields. European Journal of Plant Pathology, 395–407. DOI: https://doi.org/10.1007/s10658-018-1483-y.

Runno-Paurson, E., **Kiiker, R**., Joutsjoki, T., Hannukkala, A. (2016). High genotypic diversity found among population of *Phytophthora infestans* collected in Estonia. Fungal Biology, 120, 385–392. DOI: https://doi.org/10.1016/j.funbio.2015.11.008.

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Mändar, R., Punab, M., Borovkova, N., Lapp, E., **Kiiker, R.**, Korrovits, P., Metspalu, A., Krjutškov, K., Nõlvak, H., Preem, J.-K., Oopkaup, K., Salumets, A., Truu, J. (2015). Complementary seminovaginal microbiome in couples. Research in Microbiology, 166 (5), 440–447. DOI: https://doi.org/10.1016/j.resmic.2015.03.009.

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methods for vaginal lactobacilli. Beneficial Microbes, 6 (5), 747–751. DOI: https://doi.org/10.3920/BM2014.0154.

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Articles published in local conference proceedings (3.5.)

Runno-Paurson, E., **Kiiker, R.** (2016). Kartuli-lehemädanikutekitaja Eesti populatsioonid on geneetiliselt väga mitmekesised. Luule Metspalu, Katrin Jõgar, Eve Veromann, Marika Mänd (Toim.). Eesti Taimekaitse 95 konverentsi toimetised (61–66). Eesti Maaülikool.

Published conference abstracts (5.1. and 5.2.)

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Viggor, S., Jõesaar, M., **Kiiker, R.**, Pärnpuu, L., Lenskaja, A., Heinaru, A. (2014). Characterization of aliphatic hydrocarbon-degraders community and their alkane hydroxylases (AlkB) in the Baltic Sea bacterioplankton. *Book of Abstracts: 2nd Congress of Baltic Microbiologists, Tartu, 16.10-19.10.2014.* Tartu,.

Kiiker, R., Hansen, M., Randall, E., Cooke, D.E.L., Mänd, M., Runno-Paurson, E. (2014). Genotypic diversity in Estonian populations of *Phytophthora infestans*. *Proceedings of North American Late Blight Symposium: North American Late Blight Symposium, Minneapolis, Minnesota, U.S.A., August 8 and 9, 2014.* 23.

APPENDIX

Appendix 1. The most abundant *Phytophthora infestans* multilocus genotypes in the Baltic populations in 2010–2012 based on 11 SSR markers (**I_x, II**).

MLG	No of isolates	Country	Pi02	Pi04	Pi63	Pi70	Pi4B	G11	SSR2	SSR4	SSR6	SSR8	SSR11
LT_MLG19	20	LT	268/268	166/170	279/279	192/195	205/217	154/154	173/175	292/294	242/244	260/260	341/341
LV_MLG10	10	LV	268/268	166/170	270/273	192/192	205/213	160/160	173/173	284/292	242/244	260/266	341/355
EE_MLG11	7	EE, LT	268/268	166/170	270/279	192/192	213/217	160/206	173/173	292/292	242/244	260/266	341/341
LV_MLG11	6	LV	268/268	166/170	270/273	192/192	205/217	0/0	175/175	284/294	242/244	260/266	341/355
LV_MLG9	6	LV	268/268	166/170	279/279	192/192	213/213	154/156	173/173	284/294	242/244	260/266	341/341
EE_MLG10	6	EE	268/270	166/170	270/279	192/192	205/213	156/162	173/173	294/296	242/242	260/264	341/355
LV_MLG8	5	LV	266/268/0	166/170/0	273/279/0	192/192/0	205/213/217	156/156/0	173/175/0	284/288/296	244/244/0	260/266/0	341/355/0
EE_MLG9	4	EE	268/268	166/170	270/273	192/192	213/217	154/208	173/173	292/294	242/242	260/260	341/341
LV_MLG7	4	LV	266/268	166/170	273/279	192/192	205/217	162/162	173/173	284/284	242/242	266/266	331/331
LV_MLG6	4	LV	268/268	166/170	270/279	192/192	217/217	162/162	173/175	284/284	242/244	266/266	341/341
EE_MLG8	4	EE, LT	268/268	166/170	270/279	192/192	213/217	160/206	173/173	292/294	242/244	260/266	341/341
EE_MLG7	4	EE	268/268	166/170	279/279	192/195	213/213	154/154	175/175	284/294	242/244	266/266	341/341
LV_MLG5	4	LV	268/272	166/170	279/279	192/192	205/213	0/0	173/173	292/292	242/244	266/266	331/341
EE_MLG6	3	EE	258/268	166/170	273/279	192/192	205/205	154/162	173/175	288/292	242/242	260/266	341/355
LV_MLG4	3	LV	258/268	168/168	270/273	192/192	213/217	162/162	173/175	292/292	244/244	260/260	355/355
LV_MLG3	3	LV	266/268	166/170	0/0	192/192	213/213	142/142	173/175	284/294	242/242	260/260	331/331
LV_MLG2	3	LV	266/268/0	166/170/0	273/279/0	192/192/0	205/213/217	156/156/0	175/175/0	284/288/296	244/244/0	260/266/0	341/355/0
EE_MLG5	3	EE	268/268	166/170	273/279	192/192	213/217	154/206	173/175	288/294	244/244	260/260	331/341
EE_MLG4	3	EE	268/268	166/170	273/279	192/195	205/205	154/154	173/175	284/284	244/244	260/266	341/341
EE_MLG3	3	EE	268/268	166/170	279/279	192/192	213/217	156/156	173/173	284/288	244/244	260/266	341/355
LV_MLG1	3	LV	268/268	168/168	270/279	192/192	213/217	154/154	173/173	288/296	240/244	260/266	341/355
EE_MLG2	3	EE	268/268	168/168	273/279	192/192	205/217	156/160	173/175	284/292	244/244	260/260	341/341
EE_MLG1	3	EE	268/268	168/168	279/279	192/192	205/213	156/156	173/175/0	282/284/0	242/244	260/260	341/341
LT_MLG17	3	EE, LT	268/268	168/168	273/279	192/192	205/217	162/162	173/173/0	284/296/0	244/244	260/260	341/341

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ISSN 2382-7076
ISBN 978-9949-698-42-4 (pdf)