Primary inoculum sources and characterization of Norwegian *Phytophthora infestans*

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By

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Abstract

Late blight caused by *Phytophthora infestans* (Mont.) de Barry is a major constrain to potato production in Norway. To characterize present populations of *P. infestans* and to find potential inoculum sources for early epidemics of late blight in Norway, *P. infestans* infected samples were collected from 10 different farms of Norway. 237 plant samples were collected in early epidemic. At least 68 recovered isolates from leaf samples were used for testing of their mating type, metalaxyl and propamocarb resistance. In addition, at least 187 samples were analyzed using simple sequence repeat (SSR) method. The majority (62 of 68) of the isolates were of the A1 mating type. Among 67 tested isolates, 32% of the isolates were found resistant against metalaxyl while about 48% of isolates were sensitive. In Norway, propamocarb resistance in *P.infestans* was detected for the first time in two isolates though most of the isolates (73%) were intermediate resistance. Thirty alleles and 66 multilocous genotypes were detected among 191 samples by using 6 pairs of SSR markers (Pi02, Pi04, Pi4B, PiG11, Pi26, and Pi33). A previously never detected allele 146 of PiG11 was detected. The diversities among farms were quantified by a normalized Shannon's diversity index (H_s). Moderately high variability among 10 farms and clustering pattern of isolates according to their corresponding farms (except some farm) indicates the primary inoculums came through seed tubers carrying inoculums.

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Abbreviations

%	percentage
µl	micro liter
bp	base pairs
BSA	bovine serum albumin
DNA	deoxyribonucleic acid
dNTPs	deoxynucleoside triphosphates
gm	gram
H_2O	water
L	liter
mg	milligram
^oC	degree centigrade
PCR	polymerase chain reaction
rpm	revolution per minute
SSR	simple sequence repeat

1. Introduction

The oomycete, *Phytophthora infestans* (Mont.) de Bary causal organism of potato late blight was one of the first organisms identified to cause plant disease (Talbot, 2004) and is known as *"infectious plant destroyer"* (Greek: phyto = plant, phthora = destruction, infestans = infectious). *P. infestans* is hemibiotrophic, where the pathogen can kill their host and subsequently feed on dead tissue. Asexual phase of pathogen generally survives between crop seasons in potato tubers. The pathogen is heterothallic with two different mating types A1 and A2. Presence of those opposite mating type can induce sexual structure, antheridium or oogonium which ultimately produces oospores by their fusion. Oospores may survive for several years in soil in harsh environmental condition without their host (Andersson *et al*., 1998; Lehtinen & Hannukkala, 2004). Prior to the 1980s, worldwide populations of *P. infestans* were dominated by only A1 mating type (old clonal lineage US-1). A new primary source of inoculum appeared in Europe during the 1980s and the US-1 lineage of *P. infestans* was gradually replaced by new diverse A2 populations (Fry *et al*., 1993, Goodwin, 1997). Presence of both mating types in the Nordic countries (Brurberg *et al*., 1999; Hermansen *et al*., 2000; Lehtinen *et al*., 2008) increases the possibility of genotypic variability of the organism and may result in an increased virulence and/or fungicide resistance/tolerance (Fry *et al*., 1993, Flier *et al*., 2007; Widmark *et al*., 2007; Brurberg *et al*., 2011). Mainly, two possible sources of inoculums could be responsible for disease outbreak in Norway i.e. either soil born inoculums (oospores) or the infected seed tuber (Widmark *et al*., 2007). Finding primary inoculums source and monitoring of their genetic variability is crucial for developing an effective strategy for managing the pathogen. In Norway, extensive study on primary inoculum source for *P. infestans* hasn't been performed until now.

1.1 The Potato (*Solanum tuberosum* L.)

Potato (*Solanum tuberosum*) is an annual herb grown worldwide and belongs to the most important food crop in the Solanaceae family (Friedman *et al*. 1997). In terms of human consumption, it is the third most important food crop in the world after rice and wheat (http://www.cipotato.org/potato). Globally, 20 million hectares of potatoes are grown with the value of 32 billion Euros (Haverkort *et al*., 2008). It is a nutrient dense crop having 17% carbohydrates, 1.6-2.1 % protein and less fat (0.09%) of total fresh weight. In addition, it

contributes vitamins belonging to the B and C groups and supplies a variety of dietary nutrients like potassium, magnesium and phosphorus.

Potato is supposed to be originated in the South American continent. Spooner *et al.* (2005) have provided proof that, *S. tuberosum* is a monophyletic origin from a wild species of *S. brevicaule*. The first time potatoes are believed to be domesticated in the highlands of South America about 10,000 years ago (Hawkes, 1988) and were introduced into Europe in the 1570s. In Norway, potatoes were introduced about 250 years ago and gradually became one of the most important crops and grown all over the country under widely varying climatic environment from north to south. Today, potatoes are grown on around 14,000 ha, resulting in a total yield of 298,200 tons of potatoes annually (http://www.ssb.no/en/) at a wholesale value of approximately NOK 500 million. The crop is susceptible to numerous pathogens which reduce the yield. The pathogen *P. infestans* which causes late blight disease is one of the main reasons to decreased yield of potato in Norway. To control this pathogen, many growers use synthetic fungicides which lead to development of resistant isolates resulting in failures in disease control.

1.2 The Pathogen

The center of origin of *P. infestans* is still in controversy. Grunwald and Flier (2005) claimed the central highlands of Mexico as a center of origin. The recent population studies of *P. infestans* purposed the Andean regions of Peru and Ecuador are considered to be the center of origin (Gómez-Alpizar *et al*., 2007). The main hosts of *P. infestans* are potato and tomato (*Lycopersicum esculentum*). However, it can also infect *S. demissum* (Lindt.), *S. verrucosum* (Schl.), *S. iopetalum* (Bitt.), *S. brachycarpum* (Correll), *Solanum acaule*, *S. stoloniferum* (Schl.) and other species in the genus *Solanum* (Grunwald and Flier, 2005). Other plants like petunia and *Nicotiana benthamiana* were also found to be susceptible for *P. infestans* (Becktell *et al.*, 2006).

The South American staple crop potato was introduced by the Spanish to Europe shortly after their conquest of the New World. Europe stayed free of *P. infestans* for three centuries. In 1844, *P. infestans* finally arrived in Europe and spread rapidly from Belgium (Zadoks, 2008) to other countries of mainland Europe and then to Great Britain and Ireland. This pathogen was

responsible the Irish potato famine in the mid-1840s. During that famine, millions of people were died by extreme hunger and equal amounts of people were migrated to other countries. In addition, this pathogen was also responsible for development of the scientific discipline of plant pathology in the early 1860s when Anton de Bary demonstrated *P. infestans* as the pathogen causing potato late blight. This disease is still among the worst crop diseases of the world despite much research efforts over the years (Fry, 2008). Even when employing resistance potato varieties and effective fungicides, global loss caused by this disease (costs of control and damage) is estimated to 4.8 billion Euros per year (Haverkort *et al*., 2008). In Norway, *P. infestans* was appeared in 1845 (Bourke, 1964).

Phytophthora infestans has been taxonomically classified as a representative of the phylum oomycota and class oomycetes. The phylum oomycota characterized by biflagellate zoospore produced inside sporangia and production of oospores (Thines & Kamoun, 2010). Historically, oomycetes were grouped under kingdom fungi mainly due to their filamentous growth (mycelium) and nutrients via absorption which are the typical characteristic of many true fungi. Fungi and oomycetes show the fundamental differences in physiology biochemistry and genetics (Latijnhouwers *et al*., 2003). The cell wall of oomycete is mainly composed of cellulose unlike the true fungus which is composed of chitin. With the development of different molecular tools like genome sequencing and other DNA finger printing techniques reveled oomycetes are the phototropic origin. Oomycete appeared more close to the heterokont golden brown algae due to presence of 6-phosphogluconate dehydrogenase gene which was most likely inherited from red algae (Andersson & Roger, 2002). (Tyler *et al*., 2006) identified 855 genes of phototrophic origin (red alga or cyanobacterium) and that finding also support a photosynthetic origin for the oomycetes. Due to closeness with the phytotropic organisms, it has been classified into new kingdom Stramenopiles. Current phylogenetic classification of *P. infestans* as described by Thines & Kamoun (2010) is

- Kingdom- Stramenopiles,
- Phylum- Oomycota,
- Order- *Peronosporales*,
- Family- *Peronosporaceae*,
- Genus- *Phytophthora*

1.3 Epidemiology of Late blight

1.3.1 Sources of inoculums

A variety of primary source of inoculums is responsible for the primary outbreaks of potato late blight in different countries (Fry, 2008). Primary inoculums may be oospores, seed tubers, dumps, volunteer and closely related weed hosts. Tubers that are infected by the pathogen may act as an important source of inoculums and able to cause epidemic development for subsequent potato crops (Turkensteen *et al.*, 2000) In the Netherlands and UK, potato dumps were found as a major source of inoculums. However in Norway, the temperature often drops below $0^{o}C$ in winter and tubers under dumps are usually killed by frost. Volunteer plants and weed hosts are also less important in colder climate (Cooke *et al.*, 2011) which increases the possibility of oospore, infected seed tuber or both as primary sources of inoculums. Invasion of sexually reproducing population in Europe, oospores became a major source of inoculums capable of surviving at least five winters in harsh weather conditions (Nordskog *et al.*, Unpublished).

1.3.2 Disease cycle

P. infestans can reproduce both sexually and asexually (Figure 1). The asexual cycle facilitates rapid population growth. Cycle begins with the production of sporangia containing biflagellate zoospores inside. During period of high humidity sporangia are spread over long distances by air blow (Aylor *et al.*, 2001) or short distance through rain water splash. In response to temperature, sporangia germinate forming a germ tube ($18^{o}C$ -$24\ ^{o}C$) release zoospore ($8\ ^{o}C$-$18\ ^{o}C$) and infect leaves, stems, and potato tubers (Goodwin *et al.*, 1998; Vleeshouwers *et al.*, 2000; Judelson and Blanco 2005). Encysted zoospore loses their flagella and produce germ tube to develop appressoria for leaf epidermis penetration (Judelson & Blanco, 2005). The pathogen then establishes a biotrophic (growing on living tissue) phase maximum up to 48 hours without visible symptoms followed by necrotrophic (killing the host and feeding on its content) phase. Necrotrophism leads to formation of lesions on infected tissue. Mycelium emerges through the stomatal openings within 3 to 5 days and produce new sporangia (Kamoun *et al.*, 1998). About 100,000 to 300,000 sporangia can be produced by a single lesion within a day. Sporangia are harvested by rain water and reach the tuber or neighbors healthy plant and establish infection

during wet and cool soil condition. Those infected tubers may act as primary sources of inoculums for next season.

Due to the heterothallic (two compatible mating types A1 and A2) nature of *P. infestans*, the pathogen can reproduce through sexual recombination. In close proximity, both the mating type A1 and A2 can induce their reproductory structure called antheridia and oogonia. Fusion of both structures can produce thick walled dormant oospore which can survive for long period. Germinating oospores produce sporangium in a favorable climatic condition and reestablishes the asexual cycle.

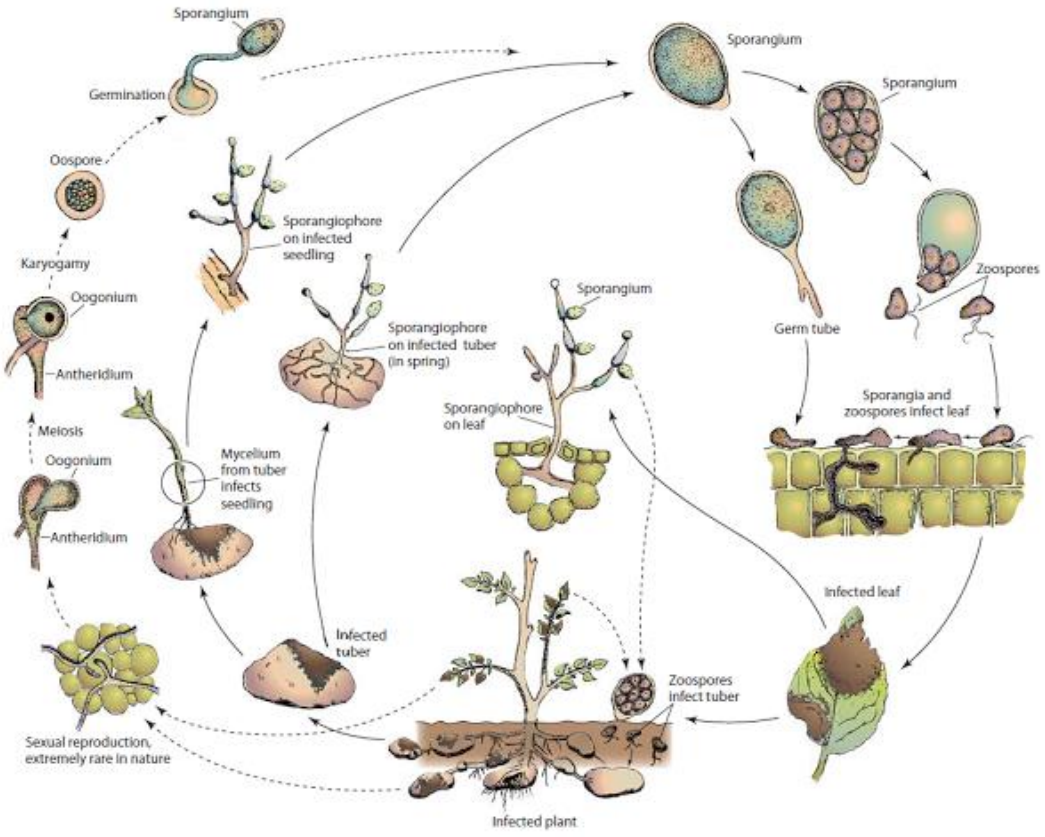


Figure 1: Life cycle of *P. infestans* (Agrios, 2005)

1.3.3 Disease symptoms

Phytophthora infestans can attack on foliage, stems and tubers of the potato plants. Symptoms on infected parts vary with the age of the lesions and climatic conditions. Initial infection appeared as small necrotic lesions on stems and leaves. During cool and wet weather conditions, lesions enlarge rapidly and become water soaked (Figure 2). With the progress of disease production of sporangia get starts, water soaked lesions turns into black-brown color and ultimately gets die. Detached sporangia fall into soil and start to infect tubers. Tubers in the early infection get slightly brown or purple blotches on the skin and later tuber decays rapidly before harvest. Infected tuber may also be attacked by secondary fungal or bacterial infection before and after harvest known as 'wet rot'.

Figure 2: Leaf lesions infected by *P. infestans.*

1.4 Phenotypic and genotypic variation

Knowledge regarding genetic structure of *P. infestans* provides overview of the genetic variation or evolution rate of the pathogen. Genetic variation only deal with genetic differences among individuals of the same species while phenotypic variation comes with interaction between genetic sources and environmental sources. It has been reported repeatedly that the population of *P. infestans* has undergone major changes in Europe during 1980's and afterward (Fry *et al*., 1993; Gisi and Cohen, 1996). In the early '80s, it was discovered that the A2 mating type had been migrated to Europe and many other countries in the world (Fry *et al*., 1993). The A2 mating type was only found in Central Mexico until the early 80's. Introduction of A2 type brought dramatic changes over population structure of *P. infestans* all over the world. In Norway, the A2 mating type was first detected in 1993 (Magnus & Hjønnevåg, 1989) and high level of genotypic variation in local populations of *P. infestans* have been observed afterward by Brurberg *et al*. (1999); Hermansen *et al.* (2000), Flier *et al.* (2007) and Brurberg *et al.* (2011).

High level of genetic diversity may have a significant role in creating new populations due to presence of variation in nucleotides, genes, chromosome or the whole genome of organism

(Wang *et al.,* 2009). *P. infestans* has the potential to provide unpleasant surprises due to its high evolutionary potential (reproduction and genetic flexibility) (Cooke *et al*., 2011). The pathogen can reproduce by sexually as well as asexually and able to produce large amount of variation in their progeny. Mutation, mitotic recombination, and parasexual recombination are the most common mechanisms of genetic variability (Fry, 2008) in absence of sexual reproduction. Mutation is the primary source of new genetic variation. Analyses of US-1 isolates worldwide have detected a number of probable mutations both in nuclear and mitochondrial (mt) DNA (Goodwin, 1997). Recently, mutations in PiAvr4 have been detected in Nicaraguan and Swedish population of *P. infestans* (Sjöholm, 2012). In addition, new variants may also be generated asexually following hyphal fusion and nuclear fusion. Fusion of different nuclei can produce polyploidy in progeny (Howard *et al.,* 1998; Stukenbrock & McDonald, 2008). Polyploidy in *P. infestans* was reported by Catal *et al*. (2010). Oospores produced by sexual reproduction have higher influence in population dynamics (Sjöholm, 2012). The Nordic countries including Norway are one of the regions outside Mexico where sexual reproduction occurs (Brurberg *et al*., 1999; Brurberg *et al*., 2011; Widmark *et al*., 2011). Brurberg *et al*. (2011) and Cooke *et al*. (2011) reported the similar patterns of genotype presence all over Nordic country which suggest that, the Nordic *P. infestans* lineages belong to the same population.

1.5 Measurement of variation

The variation between populations of *P. infestans*, within population and between individual can be measured with several phenotypic and genotypic markers.

1.5.1 Genotypic characterization

Genetic markers characterize genetic variation between individual organisms mainly based on the laws of Mendelian inheritance. Amount and distribution of genetic variations can be measured at morphological, biochemical or DNA level. Different techniques have been developed to measure genotypic variation of *P. infestans*, such as, allozyme patterns, the moderately repetitive RFLP (Restriction Fragment Length Polymorphism) probe RG57, amplified fragment length polymorphism (AFLP), mitochondrial DNA haplotype determination,

simple sequence repeat (SSR) markers and single nucleotide polymorphism (SNP) (Cooke and Lees, 2004).

SSRs are tandemly repeated motifs of one to six bases found in the nuclear genomes and frequently used to characterize *P. infestans* population (Knapova and Gisi, 2002; Cooke and Lees, 2004; Lees *et al*., 2006; Brurberg *et al*., 2011; Li *et al*., 2013). The tandemly repeated motifs are used for fingerprinting, parentage analyses, genetic mapping or genetic structure analysis (Guichoux *et al.*, 2011). Monitoring of genetic diversity of *P. infestans* through SSR has recently proved effective for defining multilocus genotypes (Lees *et al*., 2006). The first SSRs for *P. infestans* were Pi4B, Pi4G, PiG11, Pi1D, Pi2D and Pi2H which were developed by Knapova *et al.,* (2001) (cited in Knapova and Gisi, 2002). Subsequently twelve other SSR markers Pi02, Pi04, Pi16, Pi26, Pi33, Pi56, Pi63, Pi65, Pi66, Pi70, Pi89 and D13 were identified by Lees *et al*., (2006). After evaluation of those SSR markers using number of European isolates, PiG11, D13 and Pi02 markers were found to be highly polymorphic. Recently, Brurberg *et al.*, (2011) used nine SSR markers (Pi02, Pi04, Pi16, Pi26, Pi33 4B, 4G, G11and D13) to determine variability in Norwegian *P. infestans* population.

1.5.2 Phenotypic characterization

Mating type, virulence, aggressiveness and fungicide resistance test have been performed to find out the phenotype of *P. infestans* in several studies (Brurberg *et al*., 1999; Hermansen *et al*., 2000; Lehtinen *et al*., 2008; Blando Diaz *et al*., 2012; Chmielarz *et al*., 2013; Runno-Paurson *et al.*, 2013)

1.5.2.1 Mating type testing

Mating type determination is the earliest marker used for *P. infestans* characterization. Mating type determination is used in almost every characterization study of this pathogen to understand the potentiality for sexual reproduction. Mating type is determined by pairing unknown isolate of *P. infestans* with known A1 and A2 tester isolate on artificial growth media. Presence of both mating types enables formation of oospores. An isolate that produces oospores when paired with a known A1 tester isolate mating type, is designated an A2 mating type, and *vice versa*. Testing isolates that produce oospores with both testers are termed self-fertile. These days, PCR based

techniques have been developed to determine mating types. PCR based PHYB-1 and PHYB-2 primers were developed by Kim & Lee (2002) to identify A2 mating type and primers INF-1, INF-2 (Kim *et al.*, 2005) for A1 mating type. Mating type determination is based on presence and absence of PCR product. However, this technique is still unpopular for the large population due to lack of consistency.

1.5.2.2 Fungicide resistance

Disease caused by *P. infestans* can be controlled through intensive use of fungicide. The first effective fungicide for control of potato late blight was the Bordeaux mixture, discovered by Millardet (Torgeson, 1967; Haverkort et al., 2008.). Later, phenylamide fungicide 'metalaxyl' became popular to control *P. infestans* and was first introduced during 1977 in Western Europe (Schwinn and Margot, 1991). Due to its high potential to inhibit mycelial growth and sporangia formation at low concentration, it was extensively used at the beginning. Soon after its introduction, resistant isolates were discovered in Ireland (Dowley and O'Sullivan, 1981) and in Netherland (Davidse *et al*, 1981) in 1980. The rapid development of resistance against metalaxyl may be due to presence of single dominant gene influenced by minor genes in the pathogen (Lee *et al*., 1999). In Norway, metalaxyl resistant isolates were detected first time in 1988 (Magnus & Hjønnevåg, 1989). Hermansen *et al*. (2000) and Lehtinen *et al* (2008) also found high frequency of metalaxyl resistance isolates in Norway. Due to frequent detection of metalaxyl resistant isolates, only one application of metalaxyl per season has been permitted in Norway (Cooke *et al*., 2011).

Propamocarb hydrochloride is a systemic carbamate fungicide developed in 1966 and released in 1974 for commercial purpose to control oomycetes (Cohen, 1986). This fungicide affects the synthesis of phospholipids and fatty acids necessary for cell wall formation. Resistance to this fungicide is not as common as metalaxyl in *P. infestans*. Metalaxyl resistance or sensitivity is not related to toxicity of propamocarb however, Samoucha and Cohen (1990) found that metalaxyl resistant isolates were less sensitive to propamocarb than metalaxyl sensitive isolates. In case of Norway, all previously tested isolates were found sensitive towards propamocarb even those isolates that are resistance to metalaxyl (Lehtinen *et al*., 2008).

1.6 Management of *P. infestans*

Effective management strategy of *P. infestans* should consider all those factors that can reduce the population growth rate and their size. Control of primary inoculum sources, use of *P. infestans* resistant cultivars, and use of judicious amount of fungicide can help to manage *P. infestans*. Pathogen can survive for a week as a free living zoospore or as saprophytic mycelium. Tubers containing mycelium and sexually reproduced oospores are the major over wintering inoculums sources in Norway (Cooke *et al*., 2011). Treatment of tuber with chemical fungicide can reduce the inoculums of tuber, but tuber treatment is not allowed in Norway. Crop rotation with non-Solanaceae crops can reduce the primary inoculum sources of *P. infestans* (Hannukkala *et al.*, 2007).Use of resistance varieties is another potential method to control late blight, but most of the commercial varieties have moderate to little resistance (Liu and Halterman, 2009; Mayton *et al*., 2009). Commercial parameters such as yield, quality and earliness are usually not possible to combine with resistant varieties thus most of the European growers are not so interested to grow resistance varieties. However, potato varieties having partial resistance could be complementary to the fungicide applications, which can help to reduce the frequency of fungicide application (Nærstad *et al.*, 2007). A large variety of commercial fungicides to control late blight are available in the markets. Metalaxyl, propamocarb, mancozeb, cyazofamid, mandipropamid, fenamidone, cymoxanil, zoxamide are some of the registered fungicide in the Nordic countries and most of them are applied in the mixture. To control late blight in Norway, three to eight applications are carried out per season (Cooke *et al*., 2011)

1.7 Objectives

General objective of the study was to find out the sources of primary inoculums of *P. infestans* in Norway and characterization of Norwegian *P. infestans* isolates.

Specific Objectives

- To find the primary inoculums sources for late blight epidemics in Norway.
- To find the proportion of mating type of *P. infestans*.
- To find out the metalaxyl resistance level on Norwegian *P. infestans* isolates.
- To find out the propamocarb resistance level on Norwegian *P. infestans* isolates.

- To find out genetic variation among Norwegian *P. infestans* isolates.

1.8 Justification of the study

P. infestans is a major constrain for potato cultivation in Norway. Due to presence of considerable diversity of *P. infestans* (Andersson *et al*., 1998; Brurberg *et al*., 1999; Hermansen *et al*., 2000; Flier *et al*., 2007; Widmark *et al*., 2007; Brurberg *et al*., 2011) and frequent late blight epidemic in Norway, it is necessary to find out the primary inoculums sources of pathogen for development of sustainable management strategy. In addition, frequent monitoring of over their population character (mating type, fungicide resistance) is also important. This study had been conducted with following hypothesis:

(i) Tubers as the source of primary infections result in infection foci individually caused by a single or very few genotype; since it is less likely that a single tuber carries several genotypes or that a number of infected tubers are planted together.

(ii) Infection foci caused by oospores in the soil consist of many genotypes, each coming infections from different oospores.

2. Materials

2.1 Laboratory equipments

Tubes		
1.5 ml micro centrifuge tube	20170-333	VWR International
96 well semi skirted PCR plate	Ab-0900	Thermo Scientific
PCR strip tubes	PCR-0208-CP-C	Axygen
Bio-spin column	732-6204	Bio-Rad
Tubes	227-261	Cellstar
Bottle and flask		
Flask		Pyrex
Sterilized bottle 1/2 l and 1 l		Pyrex
Electric devices		
Biological safety cabinet		Thermo Scientific (Hera Safe)
Autoclave		Matachaa
Incubator		Termaks
Magnetic stirrer		Thermo Lab
Microwave		Sanyo
Vortex (mini shaker)		IKA
Weighing scale		Sartorius, Kebolab, Metter Toledo
Electrophoresis electricity supply		Bio-Rad Power pack
Gel doc		Bio-Rad
Gel Plate		Bio-Rad
Gene scan		ABI 3730 DNA analyzer
PCR cabinet		Bioair instruments
PCR thermal cyclers		GeneAmp® PCR System 9700
Pipettes		
Pipette	4500, 4501 and 4510	Thermo labsystems
Pipette tips 100-1000µl	94300220	Thermo scientific

Pipette tips 5-200 µl	94300120	Thermo scientific
Pipette tips up to 10 µl	94300310	Thermo scientific
Pipette tips 10 µl	Ref: M-0011-9FC	Biotix
Pipette tips 20 µl	Ref: M-0020-9FC	Biotix
Pipette tips100 µl	Ref: M-0100-9FC	Biotix
Pipette tips 1250 µl	Cat: 8045	Thermo scientific
Microscope		
Stereo microscope		Wild Heerbrugg
Compound microscope		Leica
Inverted microscope		Leica
Centrifuge Machine		
Plate centrifuge		Eppendorf centrifuge 5810R
Mini centrifuge	Galaxy mini	VWR
Centrifuge fresco 21		Thermo scientific
Various tools		
Mortar and pestle		Morgan Technical Ceramics Haldenwanger
Plastic bags with lock		Grippie
Disposable spatula	80081-188	VWR International
Burker cell	-	-
Leaf disc cutter		Na
Measuring cylinder 1000ml		PMP Plasti Brand
Measuring cylinder 50-100 ml		Duran/Hirschmann

2.2. Chemicals

Chemicals	**Ref. number**	**Supplier**
10% Polyvinylpolypyrrolidone powder	P6755	Sigma
Agar	214010	Becton, Dickinson and Company
Agarose	A9539	Sigma
Chloroform	32211	Sigma Aldrich
Ethidium bromide 0.07%		VWR, BDH, PROLABO

10x PCR buffer	Lot:B01545	Perkin Elmer
Geneamp 10x PCR gold buffer and		
$MgCl_2$ solution	Lot:R06361	Applied Biosystem
$MgCl_2$ solution	Lot: E11264	Applied Biosystem
Isopropenol	200-661-7	Kemetyl Norge AS
Metalaxyl		APRON® XL
Neomycin		Sigma
Propomacarb		PREVICUR® N
Rectified spirit	600051	Kemetyl
Tris(hydroxymethyl)aminomethane	T1501	Duchefa
Sodium acetate	1.06268.0250	Merck
Ethylenediaminetetraacetic acid		Sigma
Hi-Di- formamide		Applied Biosystems

2.3 Marker and DNA

Marker	**Ref. number**	**Supplier**
100 bp ladder	N3231L	Biolabs
GeneScan 500 LIZ size standard		Applied Biosystems
dNTPs	10297-018	Invitrogen

2.4 Enzymes

Enzyme	**Ref. number**	**Supplier**
Ampli Taq polymerase 5u/µl	Lot: p15533	Applied Biosystem
Ampli Taq gold	Lot: R04816	Applied Biosystem
BSA	B9001S	Biolabs

2.5 Reference isolates

Mating type	**Isolate name**	**Supplier**
Standard isolate A1	90209	Cyanamid Forschung GmbH, Germany
Standard isolate A2	88055	Cyanamid Forschung GmbH, Germany

2.6 Primers

2.6.1 SSR primers

Marker	SSR primer sequence	Size range (bp)	Dye
Pi02	F:CAGCCTCCGTGCAAGA	142–166	NED
	R:AAGGTGCGCGAAGACC		
Pi04	F:AGCGGCTTACCGATGG	162–170	VIC
	R:CAGCGGCTGTTTCGAC		
Pi26	F:GCAGTAGCCGTAGTCCTCAG	171-187	6FAM
	R:GTTCCAAATCGTCAACCAAC		
Pi33	F:TGCCGACGACAAGGAA	203–209	PET
	R:CGGTCTGCTGCTGCTC		
4B	F:AAAATAAAGCCTTTGGTTCA	205–217	VIC
	R:GCAAGCGAGGTTTGTAGATT		
G11	F:TGCTATTTATCAAGCGTGGG	142–166	PET
	R:TACAATCTGCAGCCGTAAGA		

2.6.2 Internal transcribed spacer primers

Primer	Direction	Primer sequence
ITS1	Forward	TCCGTAGGTGAACCTGCGG
ITS3	Forward	GCATCGATGAAGAACGCAGC
ITS4	Reverse	TCCTCCGCTTATTGATATGC

2.7 Buffers

SPCB buffer (CTAB-PO_4 buffer)

For 1000 ml

Sodium phosphate Na_2HPO_4	- 21.4 gm
CTAB (Hexadecyltrimethylammonium bromide)	- 20.0 gm
Sodium chloride NaCl	- 87.7 gm

All the chemicals were dissolved in Luke-warm distilled water at volume up to 1 L and pH was adjusted to 8.

TE buffer

For 1000 ml

1M Tris(hydroxymethyl)aminomethane (pH 7.5)	-10ml
0.1M Ethylenediaminetetraacetic acid (pH 8)	-10ml
Distilled H2O	-980ml

TBE solution

10x TBE stock Solution for 1000 ml

Tris base- Tris(hydroxymethyl)aminomethane	-108g
Boric acid	- 55 g
0.5M Ethylenediaminetetraacetic acid	- 40ml

Tris and boric acid were dissolved to 600ml of distilled water. Then, EDTA was added to the solution and brought to the 1 liter volume.

2.8 Preparation of culture media

2.8.1 Pea agar

Pea agar was prepared as described by Le *et al.* (2008). 125 g of frozen peas were boiled in 1.2 liter of ion-exchanged water for 45 min. Then the peas were removed by filtering through tetra layered cheese cloth and the broth was autoclaved after adding 15 g/L agar. After autoclaving, 0.2 g ampicillin and 10 mg pimaricin L^{-1} were added to suppress the bacterial growth in the plate.

2.8.2 Rye B agar

Overnight soaked 60 gram rye grains (on 200 ml distilled water) were strained through tetra layered cheesecloth and liquid was transfer to a separate beaker. Soaked grains were boiled in 800 ml of distilled water for 45 minutes and strained through tetra layered cheesecloth. Liquid from soaked grain and the broth were mixed together and brought to 1000 ml volume with distilled water. The broth was autoclaved after adding 15 g/L agar for 121^{o}C for 20 minutes.

2.8.3 Mixed pea and rye B agar

Mixed pea and rye B agar was prepared by mixing pea agar (without ampicillin and pimaricin) and rye B agar into 1:1 ratio and poured in plate.

2.9 Preparation of gel agarose

To make 1% of agarose gel of 100 ml volume, 1 g of agarose was mixed with 100 ml of 1x TBE in a 250 ml conical flask. The mixture was boiled in a microwave oven for about 1-2 minutes to completely dissolve the agarose. The solution was brought down to about 60°C by keeping it into cold running water. Two drops of ethidium bromide (0.07%) was added to the solution and was mixed well. The gel was poured in to a gel tray with comb giving required number of wells. Bubbles were removed by using disposable pipette tip. The gel matrix was ready to use after 45 minutes.

3. Methods

3.1 Condition of sampling

To study the origin of the primary inoculums, samples from early in the epidemic is necessary to avoid interference from airborne-sporangial inoculum sources.

3.2 Collection of sample

A total of 237 infected potato plant parts were collected early in the epidemic from 10 different commercial potato growing farms from three districts in Norway (Table 1 and Figure 3). Three leaflets (each with one single lesion), one stem lesion and one mother tuber were collected from each plant in July 2012. However, some of the tubers were found rotted and were not possible to sample. Most of the sampling plants from Sande were found in very early epidemic and only single leaves from individual plant were possible to sample. The infected plants and plant parts used for testing were selected randomly, excluding those that had several or no lesions. Samples were placed in small plastic bags and kept in an icebox to prevent overheating.

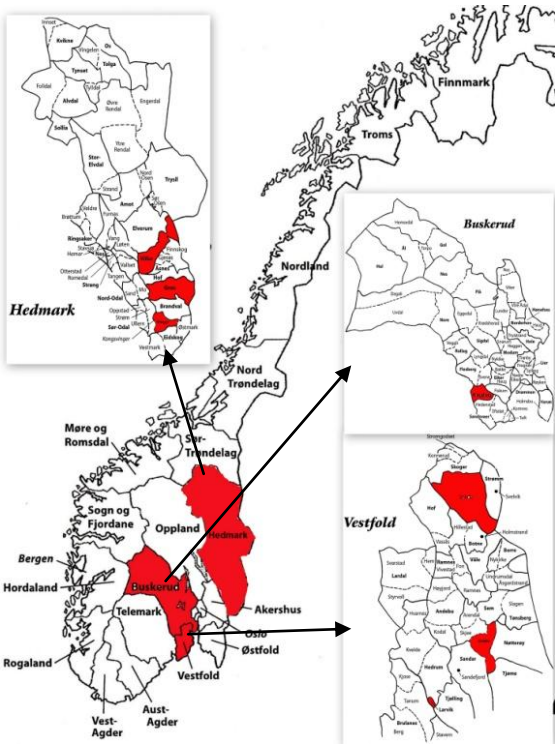


Figure 3: Map of Norway showing area of *P. infestans* infected potato sample were collected.

Map source: https://familysearch.org/learn/wiki/en/Norway_Maps

In Laboratory, stem samples and half of the leaf portion used for DNA extraction were stored in -20^{o}C. The tuber portion around (2 cm diameter) the infected shoots germinated were cut into 1mm thin slices and kept in -20^{o}C until DNA extraction.

Table 1: Number of fields, sample code, fungicide spray before sample collection, number of plants and plant parts sampled

S.N.	Sampling area	Sample code	Fungicide sprayed before collection	Number of plants	Sample size		
					Leaf	Stem	Tuber
1	Sande	S	Ridomil	12	14	2	1
2	Melsomvik	M	-	6	16	5	6
3	Passebekk-1	P	Ridomil	5	15	5	5
4	Pessebekk-2	G	-	5	15	5	5
5	Passebekk-3	O	-	5	15	5	5
6	Larvik	L	Ridomil	5	15	5	5
7	Kongsvinger	K	-	5	15	5	2
8	Namnå-1	N	-	5	15	5	1
9	Namnå-2	H	Ridomil	5	15	5	5
10	Våler	V	-	5	15	5	5
Total				**58**	**150**	**47**	**40**

3.3 Isolation of *P. infestans*

The infected leaf samples were blotted by clean blotting paper and divided into two equal parts with some healthy tissue around. One of the parts was transferred to a 1.5 ml sterilized micro centrifuge tubes tube for DNA extraction and stored at -20^{o}C after 24hrs of incubation at room temperature. The remaining half was cut into three equal parts and used for *P. infestans* trapping as described by Lehtinen *et al* (2008). The cut leaf parts were placed abaxial side up under a flame sterilized 1 cm thick tuber slice (cv. Bintje) in a Petri plate. Tubers used for *P. infestans* cultivation were flame sterilized. Tubers of cultivar Bintje were washed carefully with detergent under running cold water. Tubers then dried with blotting paper. Dried tubers were dipped in to 96% alcohol for 30-45 seconds and flamed immediately. Petri plates were incubated 4–7 days at 18° C. Small pieces of mycelium were collected from the upper side of the potato slice (Figure 4) and transferred to pea agar with the help of sterile needle. Plugs of agar with growing hyphal tips were cut from the colony margins and transferred to Petri plates having mixed pea and rye B agar and incubated at 18° C in darkness. The isolates were maintained on pea and rye B mixed agar by transferring to fresh media at 4 week intervals.

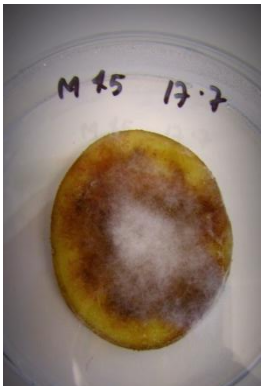


Figure 4: Trapping of *P. infestans* by potato slice. Mycelium growing on potato.

3.4 Mating type determination

The mating type of each isolate of *P. infestans* was determined by pairing them with Dutch reference isolates 90209 (A1) and 88055 (A2) previously used by Le *et al*. (2008). Referance isolates were obtained from Cyanamid Forschung GmbH, Germany (provided by Vinh Hong Le). The mating type was examined on pea and rye B mixed agar as described by Hermansen *et al.* (2000). An agar plug from the testing isolate containing actively growing mycelium was obtained and transferred to one side of new plate. A similar sized agar plug of a tester (Dutch reference) isolate A1 or A2 containing mycelium was placed on the other side. The plates were incubated in the dark for 14–21 days at 18^{o} C. Plates were scored for oospore formation at the hyphal interface between the developing colonies under the light microscope. The isolates that produce oospore in the presence of A1 tester was scored as A2 mating type, and the isolates that produce oospore in the presence of A2 tester was scored as A1 mating type. The isolates that can able to produce oospore only by them self or without any teaser isolate were categorized as self fertile.

3.5 Fungicide resistance tests

3.5.1 Production of potato leaves

Potato cultivar Bintje was cultivated in green house at approximately 18^{o}C under artificial light condition from September to December 2012. Three small seed tubers were planted in 5 L pot containing compost soil and grown for approximately 5-6 weeks.

3.5.2 Production of sporangia

The sporangia were multiplied on healthy leaflets obtained from green house grown plants. Petri plates of 14 cm diameter were lined with filter and sprinkled with sterile distilled water. Leaflets were placed with the abxial side up and inoculated with mycelial plugs from actively growing

Figure 5: *P. infestans* mycelium inoculated leaves producing whitish mass of sporangia.

margins (Figure 5). Inoculated leaves were incubated for 1 week at 15–18°C in artificial light. Sporangia were harvested in distilled water and stored in cold condition.

3.5. 3 Calculation of sporangia

Sporangia counts were performed by using a Bürker counting chamber (0.1 mm depth) under compound microscope with 400 times magnification. About 20 µl of spore suspension was used. 12 random squares (1 mm^2) were counted for each sample. All empty sporangia were discarded from the reading. Sporangia concentration was calculated by using the following formula

Number of sporangia in 1 ml= $n*10^4/z$

Where, n is the whole number of sporangia in all the counted squares (1 mm^2) and z is the number of counted squares (1 mm^2)

3.5.4 Fungicide resistance tests

Figure 6: Floating disk method for fungicide resistance test.

The fungicide sensitivity to metalaxyl and propamocarb- HCL were determined by floating leaf disc method (Figure 6) as previously described by Hermansen *et al*. (2000) and Lehtinen *et al.* (2008). Leaf disk of 15 mm diameter were obtained from leaflets of susceptible cultivar Bintje using cork borer. The leaflets were picked from five week old greenhouse grown plants. Six leaf discs were floated abaxial side up in Petri plates (5 cm diameter) each containing 7 ml metalaxyl-M (APRON® XL) solution at concentrations of 1.0 or 10.0 or100.0 mg L^{-1} or propamocarb- HCL (PREVICUR® N) solution at concentrations of 10.0 or 100.0 or 1000.0 mg L^{-1}. Leaf discs floated on distilled water was used as a control. Harvested sporangia from leaflets were adjusted to 10,000-20,000 sporangia ml^{-1}. Twenty micro liters of the spore suspension was placed in the centre of each leaf disc floating on water, metalaxyl or propamocarb solution. The inoculated leaflets were incubated for seven days in artificial daylight at 15–18°C on green house benches. Production of sporangia on leaf disc was examined under stereomicroscope. The test was repeated if the disc on control plate containing distilled water did not produce spores on four or

more than four disc. Isolates sporulating on the discs floating on water containing 100 mg L^{-1} metalaxyl or 1000 mg L^{-1} propamocarb were rated resistant; those on 1 or 10 mg L^{-1} metalaxyl or 10 or 100 mg L^{-1} propamocarb were rated intermediate; those that sporulated only on water were rated as sensitive.

3.6 Isolation of DNA

DNA was extracted following a protocol developed by Scottish Crop Research Institute (Refined version of Cullen *et al.* (2001) provided by supervisor 'May Bente Brurberg'). Tuber samples were crushed in mortar and pestle in presence of 2 ml SPCB (also called CTAB-PO4 or Hexadecyltrimethylammonium bromide) buffer. 1.5 ml of the tissue solution was transferred to a 2 ml micro centrifuge tube. In the case of stem and leaves, liquid nitrogen was used to make a fine power. About 0·5 g powder was transferred to a 1.5ml micro centrifuge tube containing 1 ml SPCB buffer. All crushed samples (tubers, stems and leaflets) were centrifuged at 6000 rpm for 5 minutes. About 0.9 ml of supernatant was transferred to another 2ml tube and equal volume of chloroform was added, mixed and centrifuged at 13000 rpm for 4 minutes. The aqueous phase (0.9ml) was removed to a fresh tube and mixed with 90 µl 3M NaOAc and 0.9 ml isopropanol. The mixture was then incubated for 1 hour at room temperature to precipitate the DNA followed by centrifugation 13000 rpm for 4 minutes. The pellet was washed in 70% ethanol and dissolved in TE buffer.

A spin column method was used to purify the DNA. 400 µl of distilled water was added to a spin column filled with polyvinylpolypyrrolidone powder and centrifuged to 4000 rpm for 4 minutes. The DNA suspension was added to same spin column insert in to a new 1.5 ml micro centrifuge tube and the tube was centrifuged to 4000 rpm for 4 minutes.

3.7 Evaluation of DNA

The DNA quality was evaluated by electrophoresis on 0.8% agarose gel. Electrophoresis separates DNA fragments with respect to size and charge. In this method, fragments of negatively charged DNA are forced to migrate towards the anode through a highly cross-linked agarose matrix in response to an electric current. This sieving process greatly depends on size of the DNA, conformation of the DNA, and ionic strength of the running buffer, gel concentration

and voltage. To evaluate quality of extracted DNA, 2 µl of DNA sample was mixed with 1.0 µl of loading buffer (6 x bromophenol blue dye) and 3 µl of dH_2O. The mixture was subjected to electrophoresis in 1x TAE buffer for 45 minutes at 90V on 0.8% agarose matrix. Gels were photographed with UV light under Bio-Rad gel documentation system.

3.8 PCR amplification

The polymerase chain reaction (PCR) is a powerful tool for repetitive duplication of a DNA template (Raeymaekers, 2000). It is an extremely sensitive method and trace amounts of nucleic acids can be amplified. The DNA to be amplified is completely dependent on the primer set (single-stranded, synthetic DNA molecules) used. The process comprises three thermo phases on each cycle: denaturation (breaking of H-bonds across double stranded DNA), annealing (binding of oligonucleotide according to primer) and elongation (extend the primers by addition of dNTPs to create a new strand DNA). At the end of each cycle, original double helix molecule has been replicated into 2 copies.

3.8.1 Amplification of ITs region

Quality and quantity of extracted DNA template were also evaluated by amplifying with Internal Transcribed Spacer (ITS) primer. Two sets of primer i.e ITS 1 and ITS 4 as well as ITS 3 and ITS 4 were used for this evaluation. Amplifications were carried out in 25 µl with 2.5µl of 10x PCR buffer (15mM $MgCl_2$), 2µl of 2.5 µM deoxynucleotide triphosphates (dNTPs), 2.5µl of 10x bovine serum albumin (BSA), 0.5 µl (50 pmol/µl) each of forward and reverse primer, 0.125µl (5u/µl) Taq polymerase and 3µl of template. Reactions were carried out on PCR strip tubes using following thermal cycling parameters: initial denaturation for 5.0 minutes followed by 35 cycles of: 30 sec at 94 ^oC, 30 sec at 55 ^oC, 1.0 minute at 72 ^oC and final extension for 7.0 minutes at 72 ^oC , before cooling to 4^oC. PCR reactions were performed with a GenAmp PCR System 9700 (PE Applied Biosystems). Two µl of PCR product were analyzed by gel electrophoresis on 1% agarose gel matrix.

3.8.2 Amplification of SSR region

Amplification reactions were carried out under identical reaction conditions, in volumes of 10 µl with 1 µl of 10x PCR buffer (15mM $MgCl_2$), 0.1µl of 25 µM $MgCl_2$, 0.8µl of 2.5 µM dNTPs, 0.25µl of 10 pmol each of forward and reverse primer, 0.1µl of Ampli Taq Gold 5ui and 6.5µl of sterilized distilled H_2O. A total of 1 µl from the DNA preparations was used as a template in each reaction. All SSR amplifications were conducted on in a Thermo Scientific 0.2ml semi-skirted 96-well PCR plate. The thermal cycling was carried out as follows: 95^oC for 10 min, followed by 35 cycles of 95 ^oC for 30 s, 59 ^oC for 30 s, 73 ^oC for 1 minute, and a final extension of 7 min at 73 ^oC, before cooling to 4^oC.

Six polymorphic SSR regions were amplified using PCR with primers develop previously: Pi02, Pi04, Pi26, Pi33 (Lees *et al*., 2006); 4B and G11 (Knapova & Gisi, 2002). The primers were obtained from Applied Biosystems. For automated fragment analysis, one primer of each locus was labeled 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-over lapping ranges of allele sizes.

3.9 SSR-fragment analysis

The fluorescently labeled PCR products were analyzed by using an automated ABI 3730 DNA analyzer as described by Brurberg *et al* (2011). This system, each array contains 48 36 cm long capillaries. Before analyzing all samples, optimal concentration of PCR product from each primer was determined. PCR products from twenty four randomly selected samples including three controls were used for optimal concentration test. All six set of PCR products (amplified with six primer set) were mixed together in a separate Thermo Scientific 0.2ml semi-skirted 96-well PCR plate with different dilutions. Two different dilution sets were used for that test (Table 2). After finding the optimal concentration, 1 µl of 26.4 to 43.3 fold (depending on the different markers according to reference of optimal concentration test) diluted PCR products were used for the test. 1 µl of diluted PCR mixture was added to 9 µl of loading buffer containing 0.25 µl of GeneScan 500 LIZ size standard (Applied Biosystems) and 8.75 µl Hi-Di- formamide (Applied Biosystems). The mixture was analyzed at 66^oC and at 15 kV, for 20 min using a 3730 DNA Analyzer (Applied Biosystems). The software called Data Collection v 2.0 (Applied Biosystems)

was used to collect data. The length of the labeled fragments (allele size) were determined by using GeneMapper v 4.0 (Applied Biosystems) and the known fragment lengths of the LIZ-labelled marker peaks. Allele size was determined by using the marker and by comparing with three reference isolates (C5, C6 and C10) previously used by Brurberg *et al* (2011).

Table 2: Dilution of PCR product with different primers to find optimal concentration

PCR product	Dilution A	Dilution B
Pi26	23.1 Times	45.8 Times
Pi4B	23.1 Times	45.8 Times
Pi04	23.1 Times	45.8 Times
Pi02	23.1 Times	45.8 Times
PiG11	18.1 Times	36.0 Times
Pi33	23.1 Times	45.8 Times

3.11 Data analysis

Normalized Shannon's diversity index (H_s): $H_s = - \Sigma P_i \ln P_i / \ln N$ was calculated to find out genotypic diversity of the population following the calculation done by Brurberg *et al.* (2011). N defines the sample size where as P_i defines the frequency of the i^{th} multilocus genotype. Values for H_s may range from 0 (single genotype present) to 1 (each isolate in the sample has a different genotype).This diversity index corrects for differences in sample size.

Nei's genetic distance $D = -\ln I$ (where $I = \Sigma x_i y_i / (\Sigma x_i^2 \Sigma y_i^2)^{0.5}$) was calculated using POPGENE software following the method used by Brurberg *et al* (2011). The diversity within farm and between farms was calculated as mean value of pairwise F_{ST} by using the Arlequin software package, version 2000 following the method used by Brurberg *et al* (2011).1023 permutations were used to test the significance of F_{ST} values.

The percent inhibitions of sporulation of *P. infestans* by test fungicides were calculated using the formula: Percent of sporulation inhibition = 100-(a* 100/b). Where, a = number of leaf disk that gives the sporulation on fungicide solution, b=number of leaf disc able to give sporulation on control.

Other statistical analyses were done by using MS Excel-2007.

4. Results

4.1 Recovery of isolates

Phytophthora infestans were isolated from early epidemic late blight. From 148 leaf samples, 73 isolates were recovered. Twenty two samples were found to be infected with three different *Fusarium* species during recovery of *P. infestans* from infected leaves (Figure 7). A total of 68, 69 and 70 isolates were analyzed for mating type, metalaxyl resistance and propamocarb resistance test respectively (Table 3).

Table 3: Number of isolates recovered from infected leaves from different farms and number of isolates tested for mating type, metalaxyl and propamocarb resistance test

S.N.	Farm	Recovered isolates	Mating type testing	Fungicide resistance test	
				Metalaxyl	Propamocarb
1	Kongsvinger	8	7	7	7
2	Larvik	13	13	13	13
3	Melsomvik	10	10	9	9
4	Namnå-1	2	1	2	2
5	Namnå-2	9	9	9	9
6	Passebekk-1	12	9	12	12
7	Passebekk-2	6	6	6	6
8	Passebekk-3	4	4	4	4
9	Sande	5	5	5	5
10	Våler	4	4	2	3
Total		**73**	**68**	**69**	**70**

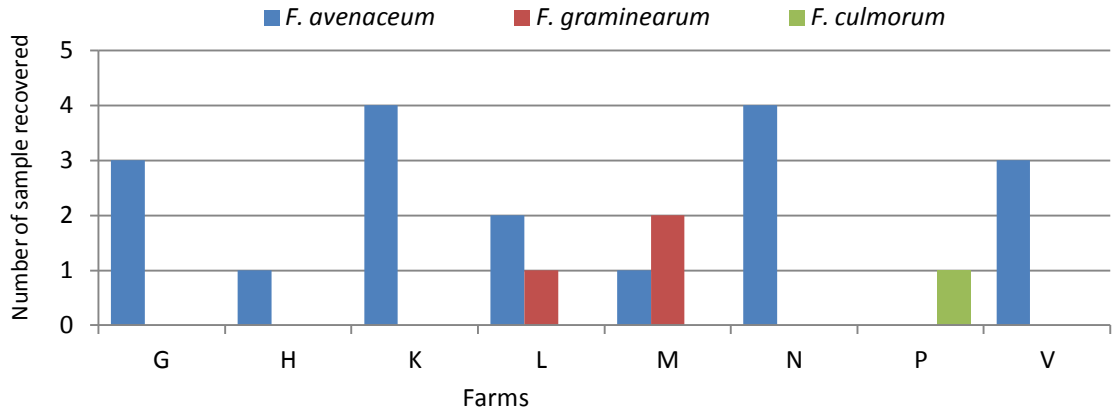


Figure 7: Different *Fusarium* species recovered from the samples of different farms: Passebekk-2 (G), Namnå-2 (H), Kongsvinger (K), Larvik (L), Melsomvik (M), Namnå-1 (N), Passebekk-1 (P) and Våler (V).

4.2 Mating type determination

A total of 68 isolates were analyzed for mating type. Majority (62) of the tested isolates were found mating type A1 (Figure 8). All isolates from Kongsvinger, Melsomvik, Namnå-1, Namnå-2, Passebekk-1, Passebekk-2 and Passebekk-3 were found as A1 mating types and able to produce oospores with A2 reference isolate (Figure 9). In addition, 1 isolate from Våler, 3 isolate from Larvik, and 2 isolates from Sande were detected as A2 mating type. All isolates used for testing were never able to produce oospores when grown in a pure culture and it indicates that, there was no isolates were self fertile.

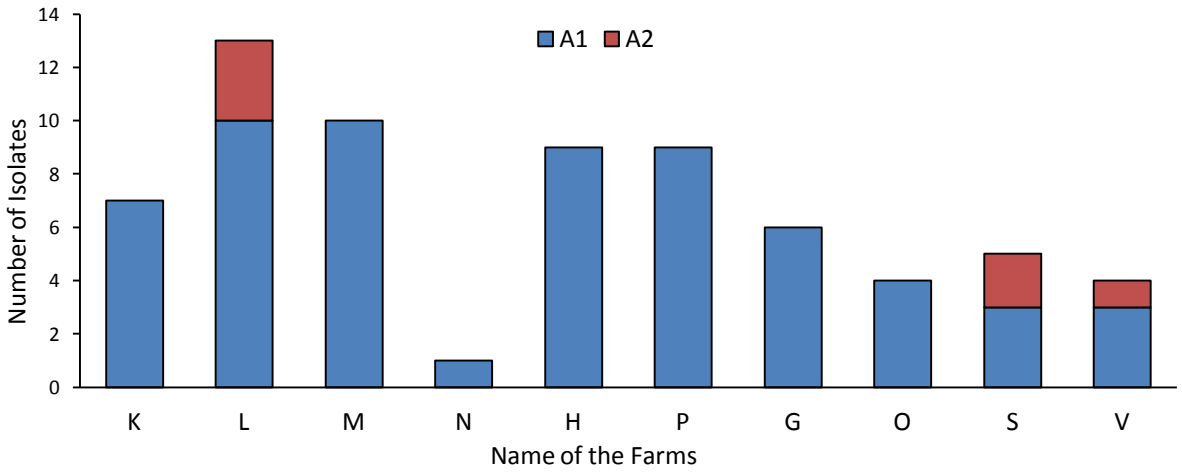


Figure 8: Number of P. infestans isolates and their mating types (A1 and A2) of from Kongsvinger (K), Larvik (L), Melsomvik (M), Namnå-1 (N), Namnå-2 (H), Passebekk-1 (P), Passebekk-2 (G), Passebekk-3 (O), Sande (S) and Våler (V).

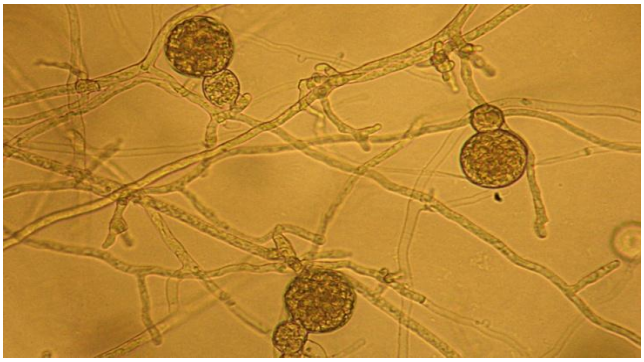


Figure 9: Oospores produced when compactable mating types (A1 and A2) were grown in a same plate containing mixed pea and rye B agar. Oospores observed at 200 times magnification under inverted microscope.

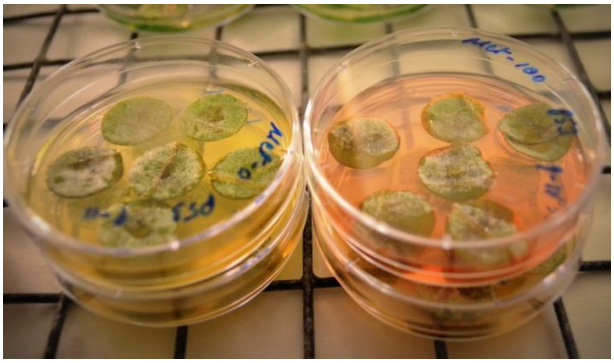
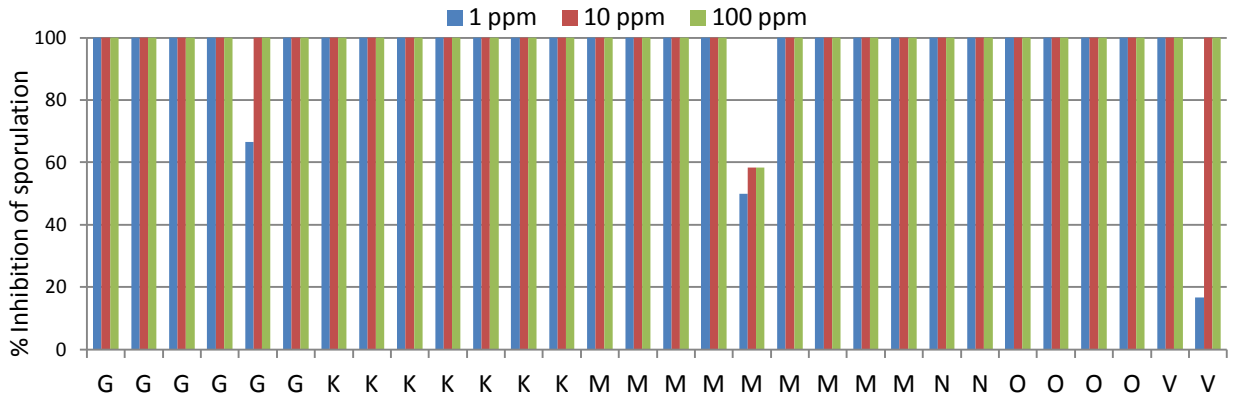


Figure 10: Leaf disk floating on control (met-0) and 100ppm of metalaxyl have no effect on sporulation.

4.3 Metalaxyl resistance test

In total, 69 isolates were screened for metalaxyl resistance. Nearly, all isolates obtained from, Kongavinger, Namnå-1, Passebekk-3, Melsomvik, Passebekk-2 and Våler were found sensitive to metalaxyl (Figure 11a). One third of the isolates (31.8%) were able to sporulate in all tested concentrations of metalaxyl (Annex 1). Metalaxyl resistant isolates were from Passebekk-1, Namnå-2, Larvik (Figure 11b). In addition, 69 %, 16% and 60% of isolates from Larvik, Passebekk-2 and Sande respectively were found intermediate resistance.

a)



b)

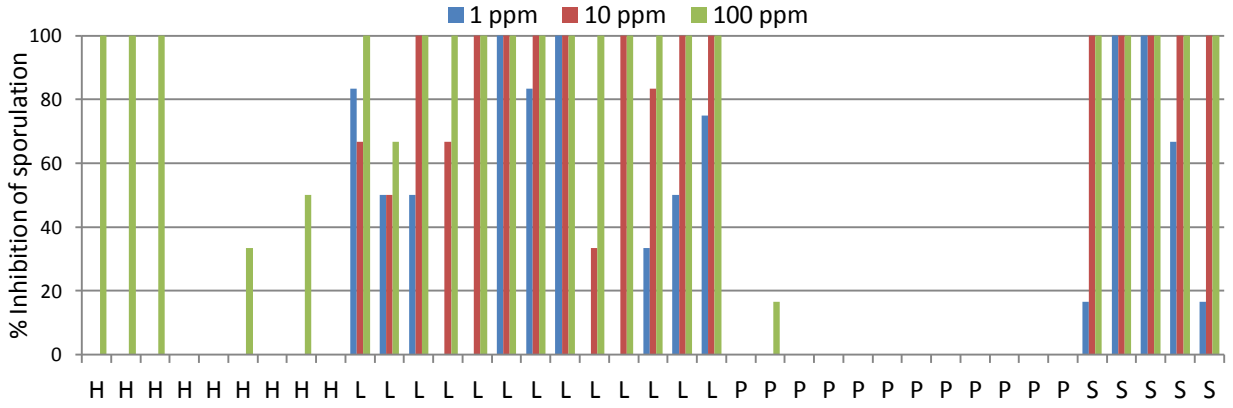
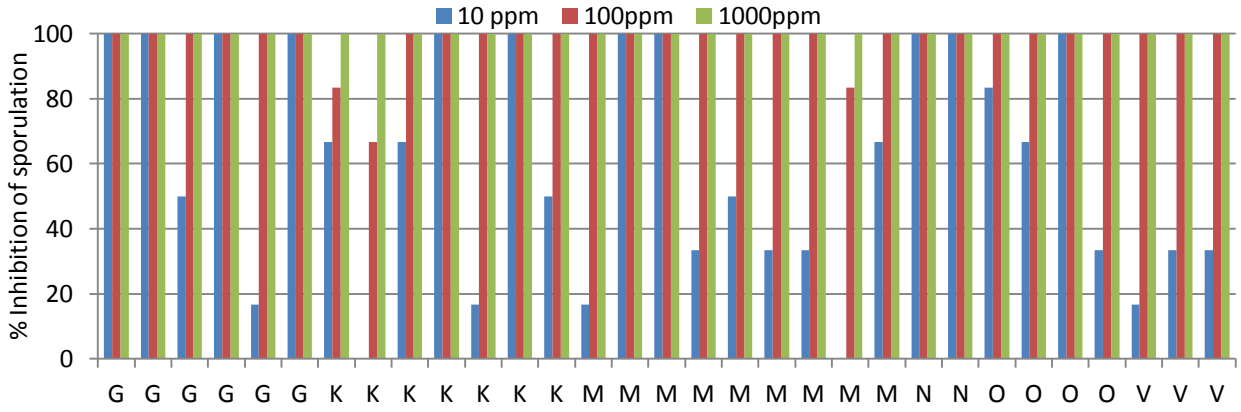


Figure 11: Effect on sporulation of isolates of *Phytophthora infestans* on leaf disc floating on 1, 10, and 100 ppm of metalaxyl as compared to control. **a)** isolates from the field sprayed without Ridomil before sample collection (Kongsvinger (K), Melsomvik (M), Namnå-1 (N), Passebekk-2 (G), Passebekk-3 (O) and Våler (V)) and **b)** isolates from the field sprayed with ridomil before sample collection (Larvik (L), Namnå-2 (H), Passebekk-1 (P) and Sande (S)).

4.4 Propamocarb resistance test

In total 70 isolates were screened for propamocarb resistance. Except two isolates (from Passebekk-1), 1000 ppm of propamocarb concentration sufficiently inhibited the sporulation of all isolates (Figure 12). All isolates from Namnå-1, 71 % from Kongsvinger, 50% from Passebekk-2 were sensitive to propamocarb (Annex-2). Fifty out of sixty-seven isolates were intermediate resistance.

a)



b)

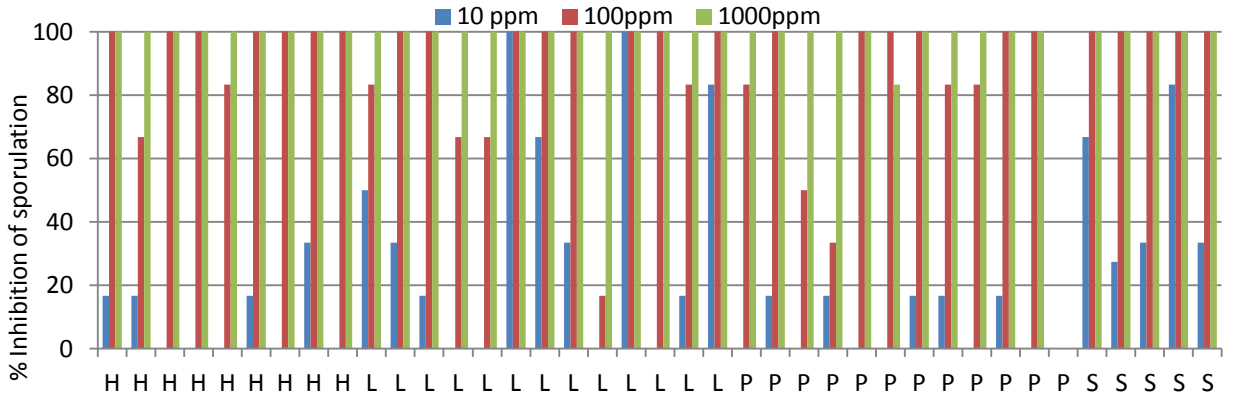


Figure 8: Effect on sporulation of isolates of *Phytophthora infestans* on leaf disc floating on 10, 100, and 1000 ppm of propamocarb as compared to control. **a)** isolates from the field sprayed without ridomil before sample collection (Kongsvinger (K), Melsomvik (M), Namnå-1 (N), Passebekk-2 (G), Passebekk-3 (O) and Våler (V)) and **b)** isolates from the field sprayed with ridomil before sample collection (Larvik (L), Namnå-2 (H), Passebekk-1 (P) and Sande (S)).

4.5 Genetic diversity

A total 237 *P. infestans* infected plant samples were analyzed with 6 pairs of SSR markers (Pi02, Pi04, Pi4B, PiG11, Pi26, and Pi33). Tuber samples from all the farms and 10 samples including leaves and stems failed to give result from all primer sets. These samples were omitted from the result analysis.

Genetic differentiation among all ten farms and farms within same location were measured by pairwise fixation index (F_{ST}) value. F_{ST} value for all ten farms was found moderately high i.e. 0.56. Almost similar result (0.59) was found when F_{ST} was calculated for the three farms at Passebekk. However, genetic variation between two farms of Namnå showed low F_{ST} value (0.27).

The genetic diversity of populations of *P. infestans* at each farm was calculated using the Shannon's diversity index (H_s) value in which 0 means no diversity in the population and 1 means maximum diversity present. Among the ten farms, highest diversity was found in Passebekk-2 and Sande with H_s value of 0.25 (Table 4). However, lowest diversity was observed in Passebekk-1 and Passebekk-3 (H_s: 0.06).

Table 4: Genetic diversity of *P. infestans* population from different farms

Farms	Genetic diversity
Kongsvinger	0.07
Larvik	0.09
Melsomvik	0.21
Namnå-1	0.15
Namnå-2	0.07
Passebekk-1	0.06
Passebekk-2	0.25
Passebekk-3	0.06
Sande	0.25
Våler	0.22

In total 30 alleles were detected at six SSR loci ranging from two at Pi33 to nine at PiG11. Lowest frequency (0.01) was found in allele 166 of Pi02 and allele 187 of Pi26 (Table 5). Highest frequency (0.84) was found in allele 203 of Pi33. 66 multilocous genotypes were

identified from 191 *P. infestans* infected samples. A new allele 146 of PiG11 was detected from 26 samples (allele frequency: 0.07).

Table 5: Allele frequencies of SSR markers and allele genotypes in the 191 tested *P. infestans* infected samples

SSR locus	Allele	Allele Frequency	Genotype Number	SSR locus	Allele	Allele Frequency	Genotype Number
PiG11	142	0.33	25	Pi04	160	0.03	9
	146	0.07			166	0.42	
	148	0.03			168	0.07	
	152	0.11			170	0.48	
	154	0.15		Pi26	173	0.07	12
	156	0.09			177	0.21	
	158	0.05			179	0.20	
	160	0.09			181	0.44	
	162	0.08			183	0.05	
Pi02	152	0.13	11		185	0.02	
	158	0.30			187	0.01	
	160	0.29		Pi4b	205	0.26	6
	162	0.27			213	0.36	
	166	0.01			217	0.38	
				Pi33	203	0.84	3
					206	0.16	

The genetic relationship among all isolates from all field were examined by UPGMA cluster analysis (Figure 13). The molecular cluster analysis revealed that 187 isolates were clustered into 5 major groups when the dendrogram was truncated at genetic similarity (GS) value of 0.58. A total of 52 isolates were in cluster 1 which contains all the isolates from Kongsvinger, all isolates except one from Melsomvik, 9, 4 and 1 isolate from Våler, Passebekk-2 and Sande respectively. The second cluster contains 69 isolates which were all isolates from Namnå-2, Passebekk-2, all isolates except one from Namnå-1 and most of the isolates from Passebekk-3. Cluster 3 contain 48 isolates including all isolates from Larvik and Passebekk-1, a few isolates from Sande, two isolates from Våler, and one isolate each from Namnå and Melsomvik. Cluster 4 and 5 contains only a few isolates. Three isolates from Sande and seven isolates from Våler belongs to cluster 4 where as cluster 5 constitute all 8 isolates from Sande. Most variability was found in the isolates from Passebekk-2, Sande and Våler.

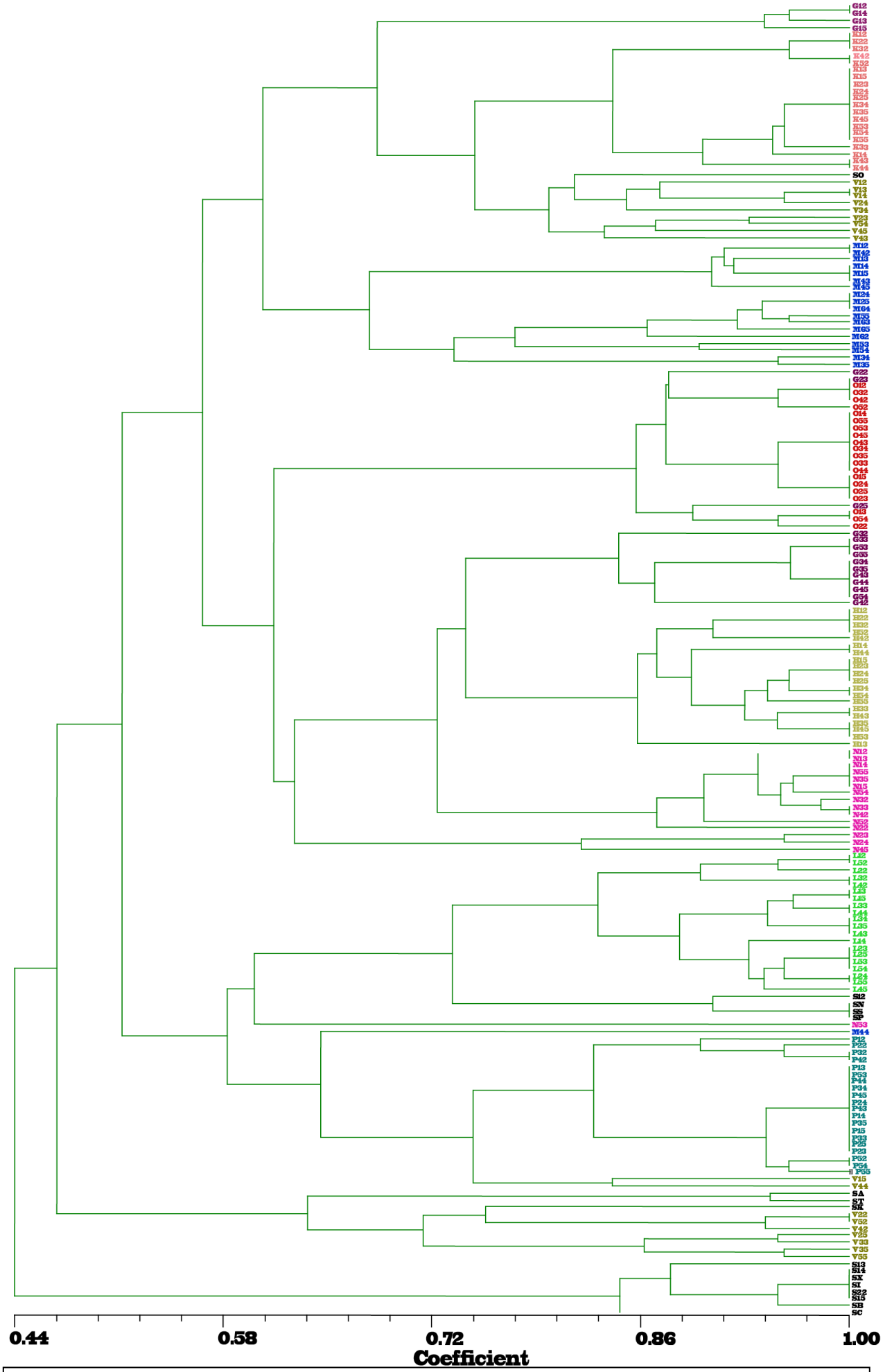


Figure 9: Dendrogram of 187 *P. infestans* infected samples from 10 Norwegian farms generated from matrices of similarity based on SSR cluster analysis.

5. Discussion

Reports published earlier suggest that the population structure of *P. infestans* in Norway has been changing dramatically after invasion of the A2 mating type in Europe. Presence of considerable variation over population increases the importance of frequent monitoring of their population structure. This study was conducted to characterize present population of *P. infestans* and to find out the inoculums sources for early epidemics.

Seventy-three isolates of *P. infestans* were recovered from leaf samples from 10 farm fields. *Fusarium* species were also recovered from 22 samples. Associated *Fusarium* spp. possibly came from contaminated leaf sample with *Fusarium* spores. Insufficiently sterilized tubers used to trap pathogen may carry *Fusarium* spp. (Peters and Lees, 2004) and (Estrada, *et al.*, 2010).

Both, A1 and A2 mating types were only detected on isolates from Larvik, Sande and Våler. This result indicates that the sexual cycle may play an important role to produce genetic variability and contribute to adaptation and epidemiology of the pathogen on those farms. The total percentage of A2 mating type discovered in this study was only 9%. Current proportion (10:1) of A1:A2 mating type is considerably higher than detected in previous studies in the Nordic countries including Norway (Brurberg *et al*., 1999; Hermansen *et al*., 2000; Flier *et al*., 2007 and Lehtinen *et al*., 2008). Lehtinen *et al*. (2008) found that the mating type ratio of Nordic *P. infestans* was close to 1:1, which is lower than the present result. Present study indicates that the population of A2 mating type in Norway has been declining. Similar results were also found in Ireland (Carlisle *et al*., 2001) and in China (Li *et al.*, 2009). In Estonia, the ratio of A1:A2 was recorded 3:2 by Runno-Paurson *et al.* (2013). In Poland, 71% tested isolates in 2006 to 2009 were A1 mating type (Chmielarz *et al*., 2013). In Nicaragua, *P. infestasns* population was composed of only A2 (Blando Diaz *et al*., 2012) however population of Ecuador comprises only A1 mating type (Delgado *et al*., 2013). In addition, both A1 and A2 mating types were detected in the same plant during this study. Coexistence of both mating types in same plant can increase the possibility of oospore formation in foliage (Turkensteen *et al.*, 2000). The presence of oospores may act as primary inoculums for next season as well as increase the diversity within the population (Widmark *et al*., 2011).

Metalaxyl resistance has previously been reported in Norway (Brurberg *et al*., 1999; Hermansen *et al*., 2000; Lehtinen *et al*., 2008). The proportion of metalaxyl resistant isolates in Norway has been fluctuating in recent years. During early and mid 90s, 60% of 491 isolates were resistance to metalaxyl (Hermansen *et al.*, 2000). In a new survey in 2003, the proportion of metalxyl restant isolates was significantly reduced (12%) (Lehtinen *et al.*, 2008). The present study shows that the resistant population is again increasing. Fluctuation in proportion of metalaxyl resistant in Estonian *P. infestans* was also reported by Runno-Paurson *et al.* (2010) and Runno-Paurson *et al*. (2013). Most of the isolates collected from ridomil (metalaxyl-M) sprayed field were resistant to metalaxyl and it is also logical that high resistance could be expected in isolates facing frequent fungicide application. In contrast, almost all tested isolates from rarely sprayed field were also found resistant in Nicaragua (Blando Diaz *et al*., 2012). High frequencies of metalaxyl resistance were also found in China (Li *et al*., 2009) where 86% metalaxyl resistance when tested on agar plate amended with 10mg/l of metalaxyl.

Runno and Koppel (2006) found the efficacy of metalaxyl to be decreased in extreme late blight pressure while efficiently protecting during moderate pressure. That finding also supports the farm condition of Passebekk-1 where extreme late blight was developed within a week. 100 % of the isolates from Passebekk-1 were resistance to metalaxyl. Though the metalaxyl resistance on *P. infestans* has been frequently encountered since 1980, this fungicide is still effective and popular among farmers. If the metalaxyl is applied according to the instruction, it is possible to reduce the generation of resistance isolates. In Norway, metalaxyl is allowed for one spray per season (Cooke *et al*., 2011) however metalaxyl resistant isolates seems to persist in this region.

Propamocarb-HCL is highly specific for organisms belonging to the order Peronosporales. However, 2 out of 70 isolates were found resistance and 52 out of 70 were intermediate resistant to propamocarb. Previously, propamocarb resistant isolates (at 1000 ppm) have not been found in Norway. Though, some isolates from Sweden and Finland (sampled in 2003) were found resistant (Lehtinen et al., 2008). These results indicate that sensitivity of pathogen population of Norway has changing towards resistance.

In total, 30 alleles and 66 multilocous genotypes were detected from 191 *P. infestans* infected samples obtained from 10 farms in Norway. Six SSR markers that were previously used by

Brurberg *et al.* (2011) to study *P. infestans* populations in Norway were also used on this study. In the previous study a large number of multilocus genotypes (169 of 191 isolates) with 49 different alleles were detected (Brurberg *et al*., 2011) when tested with nine SSR markers. That large number of genotypes could be the result of increased number of markers used (Lees et al., 2006). Different numbers of genotypes were found in different country. A study conducted in China by Li *et al*. (2009) identified 72 alleles in 288 isolates using 14 SSR markers. In Poland, 57 alleles and 66 genotypes were detected in 96 isolates using 12 SSR markers (Chmielarz *et al*., 2013).

Most of the alleles found in the current study, except 187 of Pi26 and 146 of PiG11 were previously reported in isolates from Norway (Brurberg *et al.* 2011). To the best of my knowledge, the allele 146 of PiG11 was never detected previously. For some of the markers, three and four alleles were detected in the same locus. *P. infestans* is a diploid organism and maximum of two alleles per locus can be expected. The presence of more than two alleles in the same locus may indicate polyploidity or presence of two or more isolates in same sample. Presence of more than two alleles at a specific locus has already been reported by Knapova & Gisi (2002), Lees *et al.* (2006), Brurberg *et al.* (2011) and Delgado *et al*. (2013)

The current study also discovered that, both the mating type A1 and A2 have similar genetic structure and share identical alleles for all the tested loci. This result indicates that both the mating types might have a common ancestor (oospores). Oospores can segregate in A1 and A2 mating type in F_1 Progeny (Knapova, *et al.* 2002).

Moderately high F_{ST} values (0.56 and 0.59) indicate that, there is variability between farms. However, in the case of Namnå-1 and Namnå-2, The F_{ST} value was lower (0.27) and the farms are probably sharing the same population of the pathogens because of the closeness of the farms. This low diversity within individual farms (0.06- 0.25) shows that the populations within farms is genetically alike and probably share a common primary inoculum source. Low F_{ST} value (0.04) was observed by Brurberg *et al*. (2011) between Denmark, Finland, Norway and Sweden.

The clustering patterns of 187 samples show that the population from same field may have the same ancestor (except some farms). Variability between isolates within single farms in this study

was very low. However, population from Passebekk-2, Sande, Melsomvik and Våler in dendrogram indicates the more variation. From the results, we could suspect that the populations from those farms were the result of sexual reproduction, meaning oospores from soil or seed tubers. Remaining populations were clustered together according to their origin farms. The lack of genetic variability among isolates from the same farm indicates the primary inoculums sources must be asexual propagules which may come from seed tubers.

6. Conclusion

The present data demonstrate that the A2 mating type of *P. infestans* has declined in Norway. Low level of A2 mating type will in principle reduce genetic variability in the pathogen populations and consequently oospores as overwintering primary inoculums. However, this is still threat for potato growers having farms with both mating type. The genetically diverse inoculums from those farms posses the potential risk for the country as well. The increased number of metalaxyl resistant *P. infestans* isolates suggests that the widespread usage of metalaxyl strategy should be reduced. Appearance of propamocarb resistant isolate suggests that this chemical could be used for chemical protection against late blight, but need caution. Low diversity in *P. infestans* populations within farms, clustering pattern of isolates, and presence of low level of A2 mating type supports hypothesis, that most of the plants became infected from inoculums carried by tuber. However, further studies should be performed on association of *P. infestans* inoculums in seed tuber to conform the exact sources of primary inoculums in Norway.

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Annex-1: Frequency of metalaxyl resistance *P. infestans* isolates from different farms.

		Percentage of isolates		
S.N.	**Farm**	Sensitive	Intermediate resistance	Resistance
1	Kongsvinger	100	0	0
2	Larvik	23.1	69.2	7.7
3	Melsomvik	89	11	0
4	Namnå-1	100	0	0
5	Namnå-2	0	0	100
6	Passebekk-1	0	0	100
7	Passebekk-2	83.3	16.7	0
8	Passebekk-3	100	0	0
9	Sande	40	60	0
10	Vålar	100	0	0
	Total	**47.8**	**20.2**	**31.8**

Annex-2: Frequency of propamocarb resistance *P. infestans* isolates from different farms.

		Percentage of Isolates		
S.N.	**Farm**	Sensitive	Intermediate resistance	Resistance
1	Kongsvinger	71.4	28.6	0
2	Larvik	15.4	84.6	0
3	Melsomvik	33.3	66.7	0
4	Namnå-1	100	0	0
5	Namnå-2	0	100	0
6	Passebekk-1	0	83.3	16.7
7	Passebekk-2	50	50	0
8	Passebekk-3	25	75	0
9	Sande	0	100	0
10	Vålar	0	100	0
	Total	**24.3**	**72.9**	**2.9**