170 x 240 mm

Multiplex SSR analysis of *Phytophthora infestans* in different countries and the importance for potato breeding

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170 x 240 mm

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Multiplex SSR analysis of

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countries and the importance for

potato breeding

Ying Li

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General introduction

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GENERAL INTRODUCTION

Potato

Potato (*Solanum tuberosum* L.) was currently, declared as food hero at the 7th World Potato Congress in 2009. Almost 320 million tons of potato is consumed every year, making it the third most important food crop in the world after rice and wheat. The International Year of the Potato has highlighted the contribution that potato is making to economic development and food security in Africa, Asia and Latin America, where potatoes have become an important staple food and cash crop. Potato can play a vital role in feeding the world's population which is expected to reach 9 billion by 2050. Potato tubers are used in a wide variety as fresh table potato, processed food potato, starch potato, livestock feed, and source of non-food industrial use (Feustel, 1987, Talburt, 1987).In ancient time, potato was already an important food crop for people inhabiting the South American Andes region. Potato was brought from South America to Europe in the late 16th century, in Spain (1570) and England (between 1588 and 1593) (Hawkes, 1990). Potato was introduced in the Philippines during the late 16th century and to China during the 17th century, firstly recorded in Chinese in 1700.

Phytophthora infestans

The destructive disease on potato is known as late blight, caused by oomycete *Phytophthora infestans*. It created a major disaster in the 1845 Irish and 1846 Highland potato famines, leading to mass emigration and millions death in Ireland (Woodham-Smith, 1962). The pathogen causes a destructive foliar blight and also infects potato tubers and tomato fruit (Fig. 1). Consumption of potato in blight years was associated with birth defects (Anencephaly and spina bifida) (Renwick, 1973). In Europe and USA late blight is mainly controlled with chemicals and harvested potatoes are stored in controlled conditions during the winter. This solution is not valid in poverty areas and environment protection results in other solutions like natural resistance. The pathogen can be transported overlong distances by infected plant materials like seed potatoes. The disease has reached epidemic proportions in North America, Russia, China and Europe due to the development of resistance to phenylamide fungicides in populations of the pathogen and the widespread occurrence of new genotypes (Zhang *et al.*, 1996, Judelson, 1997, Wangsomboondee *et al.*, 2002b, Grunwald & Flier, 2005, Varshney *et al.*, 2005, Gotoh *et al.*, 2007, Lees *et al.*, 2009, Kawchuk *et al.*, 2011).

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Fig. 1. A and B: *P. infestans* infection in the field; C: Asexual sporangia are produced on infected tissue and germinate directly or release; D: The sexual oospores are produced if both mating types are present in the infected tissue.

P. infestans is an oomycete pathogen and, unlike fungi, contains the vegetative mycelia (without cross-walls), which contain diploid nuclei. The organism is more closely related to brown algae than to fungi (Goodwin *et al.*, 1992a). The life cycle of *P. infestans* can be separated into an asexual cycle and a sexual cycle (Fig. 2). The asexual cycle is the driving force behind rapid polycyclic epidemics that can be observed in potato crops during the growing season. Numerous sporangia (Fig. 1C) are produced on infected leaflets and stems. Sporangia are released into the atmosphere under dry conditions or they can be splashed and dispersed by rain. When released into the atmosphere sporangia may cause new foliar infections in the same crop or neighboring crops. When washed into the soil, sporangia may cause tuber infections. In both cases, the ambient temperature determines whether the sporangium germinates directly (optimum at ±23°C) or indirectly (optimum at ±12°C). Direct germination results in formation of a germ tube. Indirect germination results in formation of motile zoospores. When zoospores lose their flagellae, they become cystospores which germinate and infect through a germ tube. The asexual stage of the pathogen was thought to

be the primary mode of reproduction occurring in most fields before 1980 (Varshney *et al.*, 2005). The pathogen typically survives from season to season as mycelium (Fig. 2) in infected potato tubers and debris when the asexual cycle is predominant. Infected tubers are an important source of inoculum and contribute to epidemic development on subsequent potato crops (Turkensteen *et al.*, 2000, Cooke *et al.*, 2011).
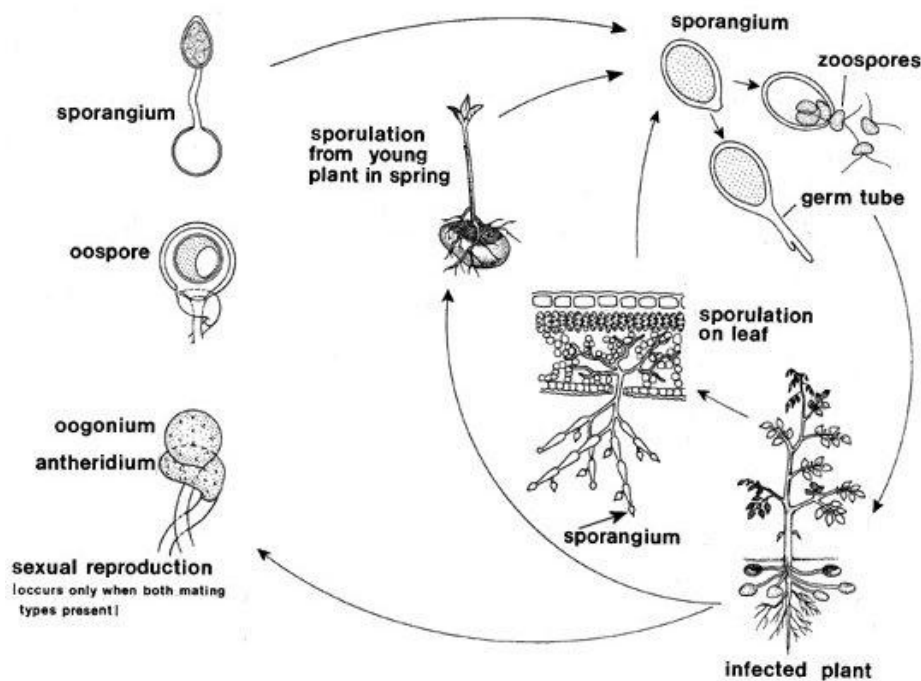
Fig. 2. Life cycle of late blight (*Phytophthora infestans*)

P. infestans is a heterothallic oomycete and reproduces sexually by outcrossing of two mating types termed A1 and A2 (Judelson, 1997, Goodwin *et al.*, 1999). These mating types are actually compatibility types and do not correspond to dimorphic forms of the organism (Goodwin *et al.*, 1992b, Goodwin *et al.*, 1992a, Goodwin, 1997). Mating types are distinguished by the production of specific hormones that induce the formation of gametangia in the opposite mating type. Fusion of the gametangia results in the formation of diploid oospores (Fig. 1D) that can survive for long periods in soil. During sexual reproduction, genetic recombination occurs as the oospore is formed and leads to genetic diversity in subsequent generations of the pathogen (Judelson & Blanco, 2005, Haas *et al.*, 2009). The sexual cycle is completed only once per growing season. In Mexico, the presumed center of origin of *P. infestans*, both mating types are present in approximately equal frequencies, and

oospores are commonly found in infected potato crops.

Population changes of *Phytophthora infestans*

To learn the population change of *P. infestans*, it is better firstly to explore the origin of the late blight pathogen. It is for sure, the origin is in South America, while there are three views about where is the center of the origin is as reviewed by Ristaino (2002). The first view is that the center of origin and diversity of the late blight pathogen is in Mexico, which is also the source of inoculum for the 19th century epidemics that led to the Irish potato famine. The second one is that Peru represents the center of origin and the source of inoculum of *P. infestans*. The third possibility is that Mexico is the center of origin, but Peru is the source inoculum for the 19th century epidemics in Europe and the US. However, all these theories are based on hypotheses with limited evidence. Later with studies of host resistance of *Solanum* species, it appeared that Mexican *Solanum* species, coevolved with *P. infestans* providing a source of resistance (*R*) genes (Grunwald & Flier, 2005). It is now apparent that Mexico is the center of origin not only of *P. infestans*, but also of several related *Phytophthora* species (Grunwald & Flier, 2005). But for migration, human activities undoubtedly were an important tool in the dispersal of this pathogen over long distances. A satisfactory answer to the center of origin and source inoculum questions is yet to be resolved.

Both mating types have been found before 1980's in the Toluca Valley, in the highland of central Mexico (Niederhauser & Mills, 1953). Outside central Mexico, only the Al mating type was known. In the early 1980's, A2 mating type isolates appeared in Europe and currently, the A2 mating type is present all over world (Hohl & Iselin, 1984, Tantius *et al.*, 1986, Frinking *et al.*, 1987, Andrivon, 1996). Sexual reproduction of results in large genetic variation might lead to increased and more rapid evolution of this pathogen. Various studies reported that the population structure of *P. infestans* has undergone major changes in the past 20 years (Fry *et al.*, 1993, Drenth *et al.*, 1994, Fry & Goodwin, 1997b, Fry & Goodwin, 1997a, Schiermeier, 2001, Shattock, 2002). During the 1980's, following a renewed global migration of both (A1 and A2) mating types, new *P. infestans* populations rapidly displaced the old clonal lineages (Spielman *et al.*, 1991, Drenth *et al.*, 1993, Zhang *et al.*, 2001, Garelik, 2002, Ryu *et al.*, 2003). One of the driving forces behind this displacement may have been the higher levels of aggressiveness and fitness in the new population as compared to the old population (Flier & Turkensteen, 1999). The newly introduced *P. infestans* genotypes in

combination with the occurrence of sexual reproduction considerably raised the level of genetic diversity in the global *P. infestans* population leading to a more variable population (Drenth *et al.*, 1994) with a presumed higher level of adaptability as compared to the previously, purely asexually, reproducing population.

After two decades since 1980s, investigators from the UK reported that a single *P. infestans* genotype with A2 mating type, EU13_A2 or "Blue_13" is dominant in the UK (Lees *et al.*, 2009), which is much more aggressive to most (resistant) potato varieties than earlier strains (White & Shaw, 2009). "Blue_13's" dominant position was hypothesized to have emerged from superior levels of fitness in combination with resistance to the frequently used metalaxyl and a "Blue_13" favorable choice of commonly grown cultivars (White & Shaw, 2009).

Molecular tools used to identify the population structure and migration pattern

A range of genotypic and phenotypic marker systems has been used to study diversity in populations of *P. infestans,* including phenotypic assays such as virulence, mating type or allozymes, and genetic assays such as RFLP fingerprints and AFLPs, mitochondrial haplotyping, microsatellites (SSR) (Fry *et al.*, 1991, Fry *et al.*, 1992, Drenth *et al.*, 1993, Drenth *et al.*, 1994, Van der Lee *et al.*, 1997, Van der Lee *et al.*, 2004). The first population studies relied on determination of virulence phenotypes using the potato differential set for *R1- R11*, mating type, and allozyme specific genotype. The population of *P. infestans* in central Mexico differed from other populations by other frequencies of specific virulence factors. The moderately repetitive RFLP fingerprinting probe RG57 allowed for the resolution of up to 27 distinct genetic loci showing that every isolate, originating from Toluca, had a unique banding pattern (Goodwin *et al.*, 1992a). After 1990's, AFLP replaced RFLP as a new useful fingerprinting technique for *P. infestans*. AFLP fingerprinting of 170 isolates collected in 1997 yielded 158 distinct loci of which 135 were polymorphic (Flier *et al.*, 2003). However, the main problem of AFLP in the population analysis is the information on distinct loci is unknown.

Co-dominant markers such as microsatellites, also known as simple sequence repeats (SSRs), have improved the ability to detect cryptic outcrossing in fungi (Vandenkoornhuyse *et al.*, 2001), and also have been used to investigate the genetic structure and reproductive biology of numerous plant pathogens . Among different classes of molecular tools, SSR markers are useful for a variety of applications in genetics and breeding of crop plants

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because of their reproducibility, multi-allelic nature, co-dominant inheritance, relative abundance and good genome coverage (Varshney *et al.*, 2005). SSR technology has become popular for studies on population genetics and genetic identity due to its favors like co-dominant inheritance, high abundance, enormous extent of allelic diversity, ease of assessing SSR size variation through PCR with pairs of flanking primers and high reproducibility (Varshney *et al.*, 2005). However, the development of microsatellites requires extensive knowledge of DNA sequences, and sometimes they underestimate genetic structure measurements. With the establishment of expressed sequence tag (EST) sequencing projects and the whole genome sequencing project of *P. infestans*, a wealth of DNA sequence information has been generated and deposited in online databases, but also it allows the selection of SSR markers in *silico* (Haas *et al.*, 2009).With the advantage of sequence information, SSR markers may provide a better understanding of the overall genetic structure of pathogenic species like *P. infestans*.

Virulence on the traditional differential set

Sexual reproduction of *P. infestans*, associated with genetic recombination during meiosis in the antheridium or the oogonium, is a major mechanism of genetic variation in this predominantly diploid organism. However, other mechanisms of genetic variability may have a significant role in creating new variants of this pathogen. Mutation, chromosomal instability, mitotic recombination, and parasexual recombination are proposed options of genetic variability in the absence of sexual reproduction (Goodwin, 1997). The most important aspect of genetic variability in plant pathogens is the variability in pathogenicity and virulence toward the host. Virulence variability in *P. infestans* populations is recognized as a major reason for failure of race-specific major genes for resistance in cultivated potato as a disease management strategy.

The race concept as applied to *P. infestans* refers to possession of certain virulence factors. The factors are actually the effectors that trigger effector-triggered immunity (ETI) in plants (Chisholm *et al.*, 2006). Characterization of isolates to different races is based on their interaction with major genes for resistance in potato. Major *R*-genes in *Solanum spp.* and their corresponding avirulence genes in *P. infestans* were first characterized by Black *et al.* (Black *et al.*, 1953), who based their analysis on the gene-for-gene concept of Flor (Flor, 1971). They identified 11 major genes for resistance which have been identified in *Solanum spp*. This is the basis for a differential set of potato cultivars used worldwide to identify the

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virulent races occurring in the *P. infestans* population. Determining virulence towards race-specific *R* genes is a prerequisite to understanding the response of pathogen populations to resistant cultivars and, therefore, to assess the durability of these *R*-genes and the performance of resistance management strategies. However, the characteristics of *R*-gene expression, the sensitivity of the phenotype to environmental and physiological parameters, and the diversity of experimental protocols make the comparison of data from different studies problematic. Only recently a more or less standardized protocol for the virulence test on *P. infestans* diversity has been developed (Andrivon *et al.*, 2011). A high level of consensus in the determination of virulence/avirulence to *R1*, *R3*, *R4*, *R7*, *R8*, *R10* and *R11* was achieved among the collaborators. However, *R2*, *R5* or *R9* genes are known to be highly sensitive to host and environmental conditions, the consensus determination was often markedly different suggesting virulence instability. Therefore, this existing differential set is not suitable for accurately diagnosing isolates is not adapted to the *R*-genes which have or will become available. It may suggest to develop a complementary differential set inserted with new cloned *R*-genes which are used in resistance breeding. The combination of both sets can be used for fine tuning of the spectrum of isolates collected in the region where the cloned *R*-genes can be applied.

Resistance breeding of potato to late blight

The strategy for breeding late blight resistant potatoes during the first half of the 20th century was utilization of the major dominant *R*-genes which had been discovered in the Mexican wild species *S. demissum*. However, they failed to provide durable resistance, either singly or in combination, owing to the evolution of new races of *P. infestans*. This pathogen evolves by mutation of effectors frequently and rapidly. Consequently, individual *R*-genes can be overcome very fast after their introduction into the potato crop (Turkensteen, 1993).

A promising strategy for breeding more durable resistance is to stack multiple broad spectrum *R*-genes in one genotype. This type of resistance is expected to be more effective and durable in plants (Halpin, 2005, Douglas & Halpin, 2010), especially with *R*-genes originating from different *R*-gene clusters, representing different HR interactions between *R-Avr* effector combinations. Recently it has been observed over the years that the cultivar Sarpo Mira shows more durable resistance (White & Shaw, 2009). More detailed investigations showed that in this case multiple *R*-genes are present (Rietman, 2011; PhD thesis) and probably responsible for this broad spectrum and more durable resistance. Even in

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the differential set, it has been shown that several differentials like *R8* and *R9*are showing more durable resistance, which is combined with *R*-gene stacking (Kim *et al.*, 2011). The fact that cv Sarpo Mira is only one of the very few varieties with broad spectrum resistance shows the complexity of classical introgression breeding, which is directly connected with the problem of linkage drag (Jacobsen & Schouten, 2007). For example, stacking of 3 or more broad spectrum *R*-genes by classical introgression breeding is only possible, 1) by using marker assisted breeding in order to recognize, indirectly, the presences of all these stacked *R*-genes and 2) by solving multiple linkage drag problems because of the use of more *R*-gene sources. Another more fast and efficient way to introduce and domesticate multiple *R*-genes into an existing cultivar is genetic modification. It has to be investigated if stacking of multiple *R*-genes in one plant by transformation can be connected with undesired inhibitors of these *R*-genes, called epistatic effects.

Population dynamics and the need for resistance

It is clear that farmers, the potato industry, consumers and the environment could greatly benefit from more efficient and environmentally friendly ways to control late blight through e.g. the introduction and durable exploitation of host plant resistance. *P. infestans*, however, is renowned with a high evolutionary potential that can rapidly evolve virulence on resistant plants (McDonald & Linde, 2002). Since the A2 mating type was introduced to outside of South America, both ways of sexual and asexual reproduction are found throughout the world. The review of Goodwin (1997) describes the global migration of *P. infestans*. Striking parallels of sexual and asexual reproduction reveals that *P. infestans* is to limit sexual reproduction and thereby generate clonal populations with rare bursts of sexual reproduction. However, reproductive isolation is not limited to heterothallics passing through genetic bottlenecks. A similar process could occur with homothallics as mutation followed by inbreeding would cause clonal populations to diverge. While sexual reproduction challenges efforts to control the late blight, better knowledge of local *P. infestans* characteristics and high level understanding of population dynamics in indispensable in order to avoid unnecessary erosion of resistance and development of fungicide resistance. With advanced knowledge of *R-Avr* interaction, monitoring of population diversity in specific region or country, could help to define the breeding and the management strategies of potato resistance.

Scope of this thesis

*P. infestans*is well renowned for its capacity of fast adaptation, of its variability and adaptation towards virulence on specific *R*-genes. Therefore, as described in this introduction (**Chapter 1**), one of the prerequisites for durable management of late blight is a thorough up to date knowledge of local *P. infestans* characteristics and high level understanding of population dynamics in order to avoid unnecessary erosion of plant resistance and development of fungicide resistance.

As stated, SSRs offer the greatest combination of required attributes for population analysis and their potential should be explored more fully. However, SSRs have not, to date, been exploited widely in *P. infestans*, except a few studies (Knapova & Gisi, 2002, Knapova *et al.*, 2002, Lees *et al.*, 2006). With the present availability of a large amount of *P. infestans* expressed sequence tags (EST) and noncoding sequence data, a new set of highly informative SSRs for *P. infestans* population analysis is explored and assembled into an efficient multiplex (**Chapter 2**). Once the exploration and optimization phase are complete, throughput may be increased by amplifying more than one locus per PCR, termed multiplexing. Such a system would be a tremendous resource for *P. infestans* research. This thesis describes the results of the development of efficient one-step genotyping analysis by SSRs (**Chapter 3**) allowing faster, more accurate and cost-effective acquisition of data, intending to be an international practice tool, allowing high-throughput screening of large numbers of samples of isolates representing the *P. infestans* population.

By the use of the multiplex SSRs, it is possible to perform a standard genotypic analysis of the *P. infestans* population at a global level. This thesis will apply this molecular tool on four potato producing countries with different host situations (China, the Netherlands, Ecuador and Tunisia). Investigation of the population structure of *P. infestans* in China shows four geographically bound clusters and reveals the dominant presence of EU genotype "Blue_13" (**Chapter 4**); **Chapter 5** assesses the population dynamics of *P. infestans* in the Netherlands during the first decade of the 21st century, analyzing the occurrence, dynamics and displacement of the ten most important genotypes in the Netherlands. Of Ecuadorian landraces, the newly *P. infestans* isolates are investigated by comparing with previously reported isolates collected on commercial potatoes to assess the impact of small scale farming on the *P. infestans* population and to study the genetic diversity within the population (**Chapter 6**). In Tunisia, *P. infestans* population structure is not influenced by the factor of importing seed potatoes from Europe, but reserves its own clonal lineage; whilst the clonal lineage is being replaced by a more complex, genetically diverse and sexually propagating population (**Chapter 7**).

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In the general discussion (**Chapter 8**), we recapitulate the results and discuss their further implications in population study of *P. infestans* and its potential application for late blight resistance breeding in potato.

CHAPTER 2

A new set of highly informative SSR markers for *Phytophthora infestans* population analysis assembled into an efficient multiplex

CHAPTER 2

SUMMARY

A database of ESTs and genomic sequences from *Phytophthora infestans*, the late blight pathogen of potato and tomato, was scanned with an automated software pipeline for di- and tri-nucleotide units repeated tandemly six or more times. From a screening of 300 non-redundant SSR loci that were identified in ESTs and genomic sequences from *P. infestans* we selected eight SSR markers based on their positions on the genetic linkage map and the information content in populations. These eight highly informative SSR markers were assembled into two multiplex PCR sets using fluorescent labeled primers that allow all markers to be scored after a single capillary electrophoresis run.

INTRODUCTION

Phytophthora infestans is an oomycete that causes a serious disease on potato known as late blight. The organism can also infect tomatoes and some other members of the *Solanaceae*. To date, the pathogen is still a global problem for all potato and tomato areas. The use of numerous applications of both protective and curative fungicides is common practice to control potato late blight. *P. infestans* is heterothallic, both A1 and A2 mating types are required for sexual recombination. Sexual reproduction results in genetic variation in the pathogen and might lead to an increased and more rapid evolution of the pathogen. Both mating types have been found before 1980's in the Toluca Valley, highland of central Mexico the presumed center of origin of the late blight pathogen (Fry *et al.*, 1992). In the early 1980's, in addition to the A1 mating type, isolates with the A2 mating type appeared in Europe (Goodwin *et al.*, 1992a, Ristaino *et al.*, 2001). Currently, this A2 mating type is present all over Europe, South and North American, Asia, and part of Africa.

It has been repeatedly reported that the population structure of *P. infestans* has undergone major changes in many parts of the world over the past 20 years. The predominant "old" populations of *P. infestans* in Europe, North-America and Asia were apparently displaced by "new" populations (Ryu *et al.*, 2003, Zhang *et al.*, 2001). Molecular markers provide a great opportunity to monitor the individual isolate genotypes and population diversity. In the past, genotypic characterization of *P. infestans* isolates included allozyme pattern, mitochondrial DNA haplotype and RFLP fingerprints with RG57 (Cooke & Lees, 2004) and AFLP. Microsatellites (SSR) technology has become recently popular for studies on population genetics and genetic identity due to its favors like high information value, multiallele detection, and integration of datasets (Varshney *et al.*, 2005).SSR markers have been used to investigate the genetic structure and reproductive biology of numerous plant pathogens (Tenzer *et al.*, 1999).

Genomic and EST sequences of *P. infestans* (Tyler *et al.*, 2006, Randall *et al.*, 2005) provided the opportunity to identify and evaluate potential SSR markers for investigating the population structure. SSR markers will also be instrumental for efficient analyses of *P. infestans* populations and will facilitate integration of the various genetic datasets available for this important plant pathogen. The objective of this study is to generate SSR markers that would allow efficient monitoring of worldwide populations of *P. infestans*.

RESULTS

CHAPTER 2

Identification and development of SSR markers

Genomic and EST sequences were scanned for the presence of di- and tri-nucleotide units that are repeated six or more times. We identified 333 unique SSR loci using an automated software pipeline. Di-nucleotide repeats were the most common, making up 60% of all repeats. Sequences flanking these SSR markers were used to develop primer pairs using Primer 3 software. We produced 300 primer pairs for 90% of the SSR markers, and they were tested on a set of 12 previously characterized *P. infestans* field isolates (Table 1). Of the 300 primers pairs tested, 210 pairs (70%) generated a clear amplicon of the expected size, of whom 110 showed multiple alleles. Some SSR markers seemed to be very variable in the *P. infestans* isolates with up to 9 different alleles detected within the ten genotypes.

Linkage group mapping

In total 23 SSR markers that showed polymorphism between the parents of cross 71 (80029 and 88133) were tested on this sexual progeny. Twenty-three of these SSR markers could be positioned on the genetic linkage map using a LOD score of 6 or higher (Table 2). These markers were scattered over chromosomes except for a cluster of markers on LG A1-b. Three markers were present on the LG that contains the mating type locus. The eight most informative markers were selected based on their high level of polymorphism and their position on the genetic linkage map (Table 3).

Table 1. Isolates used in this study

Isolate	Year	Country	Mating type
T30-4	1992	-[1]	A1
IPO428-2	1992	Netherlands	A2
80029	1980	Netherlands	A1
88133	1988	Netherlands	A2
88069	1988	Netherlands	A1
VK1.4	1958	Netherlands	A1
Mex580	-	Mexico	A1
90128	1990	Netherlands	A2
IPO-0	before 1980	USA	A1
USA 618	-	Mexico	A2
3417	2001	Ecuador	A1
B/VK98014	1998	Netherlands	A1
Dutch field isolates[2]	2004, 2005, 2006	Netherlands	A1, A2

[1] This isolate is not a field isolate but derived from a cross between isolates 80029x88133.

[2] The Dutch field isolates are a clone corrected set of 38 isolates collected between 2004 and 2006 and consists of the isolates NL04304, NL04291, NL04265, NL04274, NL05686, NL05394, NL05399, NL06033, NL06110, NL06249, NL04222, NL04232, NL04238, NL06227, NL05642, NL05638, NL06162, NL04215, NL6091, NL06138, NL04297, NL04300, NL04294, NL04282, NL04323, NL05397, NL05476, NL05466, NL05667, NL06029, NL06047, NL06140, NL06081, NL05627, NL06231, NL06100, NL06166, NL06215.

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Table 2. Most informative SSRs mapped in the genetic linkage groups

Locus name	Repeat	Repeat number	Size (bp)	Marker code	Allele number	Position	Forward primer	Reverse primer
micro_gca_0012.seq	gca	6	103		3	LGIII	CATGACGAGCGTGGCGAG	AGGAATCATCGTGAAGGCAG
micro_gaa_0015.seq	gaa	6	212		3	LGXI	ACGTGATCAATGGACGGAAG	TCCAGAGTCGAGGGTTTGTC
micro_at_0111	at	7	262		8	LGII	GTCCTCTTGCCGCAACTACG	CGCAAGGCTTGTGGCATGG
micro_ag_0009.seq	ag	6	138		3	NP	CGCGTCCCTGCTCAACTAC	TTTTTCGGTGAGTAACAAGCG
micro_at_0255	at	8	226	1	4	LGIIIA	GGCGCCTACCCACCGTC	GCGCCTCTTCGCGGACGC
micro_gt_0269.seq	gt	8	284		3	LGA1-b	TTATCGCTAGGTGTCCTCGG	AACCAGGAAGCGTACACACC
micro_at_0612.seq	at	6	324	2	4	LGVI	CATCATGGAGCGTAGGATGG	TCGCCACAAGATTTATTCCG
micro_ct_0003.seq	ct	6	168	3	3	LGIX??	CGACTTCTACATCAACCGGC	GCTTGGACTGCGTCTTTAGC
micro_ac_0041.seq	ac	6	252	7	4	LGXI	AATCTGATCGCAACTGAGGG	ACAAGATACACACGTCGCTCC
micro_at_0982.seq	at	10	285	4	6	LGII	TCTTGTTCGAGTATGCGACG	CACTTCGGGAGAAAGGCTTC
micro_ctt_0006.seq	ctt	6	329		?	NP	AAGAAAGGTGTGTTCCACGC	GCGAGCTATGCTTTGAGTGAC
micro_gt_0118	gt	8	171	8	3	LGA1-b	GTCCTCGGCGTTCTATGAC	CCGAGTACCGAATGAGGC
micro_gt_0023.seq	gt	10	260	5	3	LGV	ACTTGCAGAACTACCGCCC	TTGACCACTTTCCTCCGGTTC
micro_gt_0268.seq	gt	8	303		3	LGA1-b	TGCAAGACGTAGCGTGAGTC	GAAGAGATTCGAGTGGAGCG
micro_gaa_0008.seq	gaa	7	333	12	8	NS	CCACGCGTCCGTTTATCTC	ACAAGTTTCAGGCGACAAGC
micro_gcc_0004.seq	gcc	6	187		3	LGIII-b	TCTTGTCGGTGACAGAGTCG	TCTGAGGATGATACGGAGGC
Pinf_matSSR_3	aag	6	260	9	2	LGIII	CGGTCGATGCCCATGGCAG	GTAGGAGTCGCCTCAGCG
micro_ac_0096.seq	ac	6	303	6	5	LGIII	AGGAAAGCGGAAGGAGATTG	GCACCGAAGTAGGGAGAAAG
micro_gt_0300.seq	gt	10	338		3	NS	CAGCCACATTTCACATTTC	CAGCGGATAGCGTTTTGTTC
micro_ag_0129.seq	ag	6	223		3	NP	TACACCACACATGCACAACG	TCGTCCACTTTGACCATTCC
micro_gt_0107.seq	gt	8	283		3	LGA1-b	TCGCTAAGTATGTCCTCGGC	AACCAGGAAGCGTACACACC
micro_at_1068.seq	at	6	304	10	4	LGXIII	CATGCAAGCTTGTGCTGAAC	GCTCGCATGACTGGTGTATG
micro_ag_0175.seq	ag	7	338	11	3	LGA2-a	TTAAGCCACGACATGAGCTG	AGACAATTGTTTTGTGGTCGC

Table 3. Selected eight SSR markers for the multiplex amplification set

ID	Primer sequence (5' to 3')	Label	Primer conc. (µM)	Motif	Size range	Genome location[1]	Contig[2] (based on BLASTn)	Number of amplified isolates[3]	Number of alleles[4]	PIC value[5]	HWE Exact Tests[5,6] P value
PinfSSR1	F: GGCGCCCTACCCACCGTC R: GTTTGCGCCTCTTCGCGGACGC	HEX	0.05	AT	231-245	LGIIIA	1334	46	4 (3)	0.66	0.8498
PinfSSR2	F: CGACTTCTACATCAACCGGC R: GTTT GCTTGGACTGCGTCTTTAGC	FAM	0.03	CT	165-177	LGA2-a	836	46	3 (2)	0.45	0.9615
PinfSSR3	F: ACTTGCAGAACTACCGCCC R: GTTT GACCACTTTCCTCGGTTC	HEX	0.0625	GT	254-274	LGV	5502	46	3 (6)	0.55	0.0153[7]
PinfSSR4	F: TCTTGTTCGAGTATGCGACG R: GTTTCACTTCGGGAGAAAGGCTTC	FAM	0.06	CT	280-302	LGII	75	46	6 (6)	0.68	0.9860
PinfSSR6	F: TCGCCACAAGATTTATTCCG R: GTTT-CATCATGGAGCGTAGGATGG	FAM	0.04	AT	211-225	LGIII	3839	46	5 (4)	0.59	0.9970
PinfSSR7	F: GTCCTCGGCGTTCTATGAC R: GTTTCCGAGTACCGAATGAGGC	HEX	0.05	GT	191-203	LGA1-b	5224	46	3 (4)	0.41	0.0509
PinfSSR8	F: AATCTGATCGCAACTGAGGG R: GTTT ACAAGATACACACGTCGCTCC	FAM	0.05	AC	256-274	LGXI	2232	46	4 (6)	0.67	0.9964
PinfSSR11	F: TTAAGCCACGACATGAGCTG R: GTTTAGACAATTGTTTTGTTGGTCGC	HEX	0.075	AG	325-357	LGIX[8]	4845	46	3 (3)	0.51	0.5253

[1] named according to Van der lee et al., 2004
[2] BLAST analyses performed at http://www.broad.mit.edu/annotation/genome/phytophthora_infestans/Blast.html
[3] Amplified isolates consist 8 reference isolates T30-4, IPO428-2, 80029, 88133, VK1.4, 90128, IPO-0, B/VK98014, and 38 Dutch field isolates
[4] Number of alleles identified in the reference set and the Dutch population between brackets
[5] Polymorphism Information Content (PIC) based on 38 Dutch field isolates
[6] Estimation of exact P-Values by the Markov chain method from Hardy–Weinberg equilibrium
[7] Statistically significant after Bonferroni correction ($P > 0.05/8$)
[8] Mapped with lower confidence

CHAPTER 2

Development of SSR combination set

Locus alleles were determined for 12 reference isolates at eight selected SSR loci and all amplicons were sequenced. Sequence data were generated for all eight SSR loci, including non-repetitive flanking regions. Sequence alignments indicated allelic variation almost exclusively due to variation in SSR repeat numbers for some SSR's small InDels outside the repeat were found and in these cases new primers were generated to eliminate these size polymorphisms outside the repeat units. Except for SSR11 the size differences were due to larger insertions. SSR allele sizes were scored. The average number of alleles observed at each locus was three. Sequence analysis of cloned PCR products amplified from the eight microsatellite loci revealed a range of fragment lengths between 170 - 352 bp among all loci.

The eight SSR loci were integrated to two PCR combinations. The primer sequences were redesigned based on the sequencing data using one primer labeled with FAM or HEX, so that no two markers with the same dye label had overlapping allele size ranges. The reverse primers were redesigned with extra tail "GTTT" at the 5' end, which reduced stuttering amplification and improved the scoring quality. To optimize the multiplex PCR conditions, several variables were considered and balanced. Different combination of the 4 primers pairs were examined at different primer concentrations on eight of twelve reference isolates (428-2, 80029, 88133, 90128, 98014, IPO-complex, T30-4, and VK1.4). Four different primer pairs in each combination were balanced and optimized. The PCR's had a volume of 20 μl, 10 ng template DNA, 200 μM of each dNTPs, 0.08 U *Taq* DNA polymerase (Roche Diagnostics, Leiden, The Netherlands), 50 mM KCl, 1.5 mM MgCl2, the final primer concentration ranged from 0.03-0.075 μM (Table 3). This resulted in two multiplex PCR mixtures that could be pooled after PCR and separated in a single run with ABI capillary electrophoresis.

To test for reliability of the combination quality, the optimized PCR and capillary electrophoresis processes were separately repeated on a minimum of three different reactions with the reference set. All repeats were consistent on allele numbers and allele sizes. No stutter patterns or null alleles were found among any attempts and all alleles were scored correctly. To ensure the genotyping analysis is consistent throughout the duration of a study, eight reference isolates were used in every genotyping set.

Evaluation of SSR markers

The mapping of the SSR markers on different chromosomes and different contigs in the genome assembly (Table 2) demonstrates that the eight selected markers are independent. The SSR markers were performed on the 38 Dutch field isolates (Table 1 and Table 3) and then calculated for their HWE. Since some isolates can be triploid or trisomic (Van der Lee *et al.*, 2004), which complicates calculating the deviation from the HWE. After Bonferroni correction, all 8 SSR markers did not significantly deviate from the HWE (Table 3).

DISCUSSION

Simple sequence repeats were identified in genomic and EST sequences of *P. infestans*, over 300 non-redundant SSR markers were identified *in silico*. Primers were developed and tested on a reference set of *P. infestans* isolates. 27% of total SSR markers were non-redundant and polymorphic. Finally, only eight (2%) SSR markers with the most polymorphic, known location on the genetic maps and robust scoring were selected. It was reported that in oomycetes transcribed sequences the number of SSR markers is very low and long SSR markers are rare; compared with other oomycete pathogens, *P. infestans* had the highest number of sequences analyzed but showed the lowest total SSR count and percentage of SSR-containing sequences (Garnica *et al.*, 2006). The eight markers were assembled into two multiplex PCR sets using fluorescent labeled primers. Addition of 5′ PIGtail "GTTT" (Brownstein *et al.*, 1996) to reverse primers allowed the easy scoring of length polymorphisms. The two primer combination sets made SSR analysis easily and cost efficient. All population analyses could be done with two multiplex PCR reactions and one run with ABI capillary electrophoresis. It is the first attempt of multiplex SSR assay in *P. infestans*.

This paper recommends some key applications of our eight SSR markers in approach and reports on the status and potential of monitoring *P. infestans* genetic diversity. In oomycetes, SSR markers have had important applications (Dobrowolski *et al.*, 2002, Ivors *et al.*, 2006, Prospero *et al.*, 2004). The first report on the use of SSR markers in the study of *P. infestans* (Knapova & Gisi, 2002, Knapova *et al.*, 2002) demonstrates both the difficulties and the great potential of SSR markers. Two of these three loci were tested against 176 isolates from Switzerland and France and revealed four and six allele sizes, respectively, in 21 different combinations,

indicating that they have a resolution appropriate for population analysis. Null alleles were recorded with one SSR locus (Knapova & Gisi, 2002) and some of the loci recently developed at SCRI (Lees *et al.*, 2006) show limited allele diversity. So far, however, only a limited number of informative microsatellite loci have been identified in *P. infestans* and none have been mapped. Our eight SSR markers show few null alleles and limited allele diversity, but has been mapped on the genetic linkage groups and been successfully used in several applications of population analysis.

A disadvantage of SSR markers is the relatively long lead-in time. Once the discovery and optimization phase is complete, throughput may be increased by amplifying more than one locus per PCR. Our eight SSR markers can be done with two amplification reactions and put in one run of ABI sequencer. Such a system would be a tremendous resource for *P. infestans* research, as the examples showed in this paper. Our further aim will be to integrate our eight markers with other available SSR markers in *P. infestans* and develop a robust one-step multiplex PCR assay for *in planta* fingerprinting of *P. infestans* isolates. As a practical method, population researchers of *P. infestans* could use this standard SSR set to rapidly *in planta* detect the fingerprinting of the whole target isolate population.

MATERIALS AND METHODS

Isolates and DNA extraction

Isolates used in this study (Table 1) were subjected to SSR analysis. All isolates were grown in pea broth on a rotary shaker at room temperature for three to four days. Genomic DNA was isolated from 20 mg of lyophilized mycelium using the PUREGENE DNA isolation kit (Gentra, Minneapolis, MN) following the manufacturer's instructions and eluted with 50 l ultra-pure water. DNA extracts were stored at -20 $^{\circ}$C.

Identification and development of SSR markers

Genomic sequences with a 7x coverage of *P. infestans* isolate T30-4 and 75,757 high quality EST sequences of isolate 88069 (Randall *et al.*, 2005) were submitted to analysis in a bioinformatics pipeline for SSR detection, which consists of a primer development module and a selection for non-redundant sequences (Goodwin *et al.*, 2007). It was estimated at 65 Mbp in size with over 1 million reads assembled into 2576 scaffolds. At the time during our SSR study in October 2003, the sequence consisted of 445,030 FASTA reads at 4X. The computer software used for SSR mining and primer design was developed at the Plant Research International (PRI). This software comprised a compilation of 5 different modules, generating a dataset of unique sets of PCR primers for the amplification of microsatellite sequences. Primers were designed using the following criteria: min. T_m= 60°C, GC content $\geq$ 50%, primer size 18-21 bp, PCR product size between 100 and 350 bp, and GC

clamp=1. For each SSR containing sequence, 5 different forward and reverse primer combinations were developed.

In silico detection of polymorphic SSR loci

Since the EST sequence data and the genomic data were derived from different isolates and *P. infestans* is diploid in its vegetative cycle, polymorphic SSR could be present in the data set. To identify these polymorphic SSR markers we developed a set of PERL scripts that would detect highly homologous sequences based on the BLAST analysis but still had different numbers of repeat units. In the standard pipeline only one of these sequences would end up in the non-redundant set of SSR markers. But these additional scripts allowed identifying potentially more informative SSR loci.

PCR amplification

300 SSR's primer pairs (Illumina Inc., San Diego, CA) were selected and screened for polymorphism within a subset of 12 samples, the isolates T30-4, 80029, 88133, 88069, VK1.4, Mex580, 90128, IPO-0, 3364, 3417, its potato host cultivar Bintje and Bintje infected with VK1.4. Amplification reactions consisted of 10 ng template DNA, 200 µM dNTPs, 0.8 U *Taq* DNA polymerase (Roche, Indianapolis, IL), and 10 ng of the forward primer and 10 ng of the reverse primer in a 10 µl reaction volume in PCR buffer 1.5 mM MgCl$_2$. The forward primers were labeled by phosphorylating the 5'end with [γ-^{33}P]ATP. Amplifications were run in a 9600 thermocycler (Perkin Elmer, US), with initial denaturation at 94°C for 2 min, followed by 13 touch-down cycles of 94°C for 30 sec, from 66 to 53°C for 30 sec, and 72°C for 30 sec, then by 28 cycles of 94°C for 30 sec, 53°C for 30 sec, and 72°C for 30 sec, and a final extension at 72°C for 7 min. 10 µl of de-ionized formamide loading buffer (50% formamide with bromophenol blue and xylene cyanol as tracking dyes) was added to the PCR reactions and the samples were denatured at 92°C for 3 min and separated on 6% denaturing polyacrylamide gels electrophoresis, dried and the radioactive labeled fragments were visualized as described by van der Lee *et al.* (1997).

SSR loci mapped on the genetic linkage groups

SSR markers that were polymorphic between the parents of the *P. infestans* mapping population were tested on 77 progeny of cross 71 (Van der Lee *et al.*, 1997). Those showing the expected Mendelian segregation by χ^2 analysis were added to the existing genetic linkage map (Van der Lee *et al.*, 2004) using JoinMap® version 3 (van Ooijen & Voorrips, 2001).

Development of highly informative multiplex SSR set

For high through-put and efficient use, eight SSR markers were optimized for two multiplex combinations depending on amplicon size difference and PCR conditions. Each combination was labeled with the same dye, either FAM or HEM (Table 3). Amplification program used the touch-down PCR profile described above. The PCR products were diluted (5-20 times) based on the intensity on the agarose gel, pooled, denatured using formamide and separated on an ABI3100 or ABI3700 (Applied Biosystems, US). The fragments were sized using the molecular standard GeneScan-500 ROX and scored using GeneMapper 3.7 (Applied Biosystems, US).

Hardy-Weinberg equilibrium of SSR markers

SSR markers were evaluated by the Markov chain method with the web version of GenePop 4.0 (Raymond & Rousset, 1995). In case of theheterozygote excess (Goodwin, 1997), the U test was

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used (Rousset & Raymond, 1995). Then Bonferroni correction (Rice, 1989) was applied to correct the deviation.

CHAPTER 3

Efficient one-step genotyping of the oomycete plant pathogen *Phytophthora infestans*

Ying Li, David E. L. Cooke, Theo van der Lee, Evert Jacobsen

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SUMMARY

Genotyping is fundamental to population analysis. To accommodate fast, accurate and cost-effective genotyping, a one-step multiplex PCR method employing simple sequence repeat (SSR) markers is developed for high-throughput screening of *Phytophthora infestans* populations worldwide. The SSR markers reported for this specieswere evaluated and the twelve most informative and easily scored were selected. To accomplish a single step genotyping procedure, we optimized primers, fluorescent labels and PCR conditions to genotype using a capillary electrophoresis system with four fluorescent labels (FAM, NED, PET and VIC) and a labeled LIZ standard for sizing of the SSR fragments. The results obtained using commercially available multiplex PCR kits on a set of reference isolates were in agreement with that obtained using primer pairs in simplex reactions. In testing on many thousands of isolates, we have found the markers appropriate for resolving distinct multilocus genotypes (MLGs) of isolates of European and wider populations. Here we demonstrate the utility of the assay on a set of 19 reference isolates plus 77 others sampled from the Netherlands and Great Britain. In most isolates one to two alleles were observed at each locus but the presence of three alleles at a single locus in some isolates was consistent with aneuploidy. Methods are presented that are appropriate for the analysis of datasets comprising isolates of mixed ploidy levels. We also report on the direct *P. infestans* genotyping from infected field material using FTA cards to collect, store and extract pathogen DNA. A critical step in this method was the standardization of the protocol between two laboratories in the Netherlands and Scotland. Reference isolates were exchanged and an allele nomenclature and scoring system agreed. Such co-operation is facilitating the genotyping of international collections of *P. infestans* isolates in wider networks of laboratories and providing the data required to expand an existing international database of pathogen diversity.

INTRODUCTION

Genotyping pathogen populations poses several challenges, i) in general large numbers of isolates should be examined the analysis thus requires a fast, affordable and robust genotyping method; ii) most analysis is performed at a national level but the pathogens move across national boundaries and standardized methods between laboratories are thus required to integrate data sets; iii) in addition the marker resolution should be sufficiently high to discriminate MLGs and also identify sub-clonal variation; iv) finally, isolation of the pathogen from infected tissue is time consuming and often a rate-limiting step that could be aided by *in planta* fingerprinting. Co-dominant microsatellites (SSRs) have been used increasingly since the late eighties for applications such as fingerprinting, parentage analyses, genetic mapping or genetic structure analyses (Tenzer *et al.*, 1999, Guichoux *et al.*, 2011). Despite growing competition from new genotyping and sequencing techniques, the use of these versatile and cost-effective markers continues to increase, boosted by successive technical advances. First, next-generation sequencing technologies allow the identification of large numbers of SSR loci at reduced cost in non-model species. Second, methods for multiplexing PCR have considerably improved over the last years, thereby decreasing genotyping costs and increasing throughput. A technical advantage of fluorescence-based SSR genotyping is that several SSRs can be simultaneously separated in a single column by separating the loci by allele size and fluorescent dye labels. In order to reduce the costs samples may be combined in different ways. In post-PCR multiplexing, also called multi-pooling, whereby individual PCR assays are pooled and run through the capillary together. However PCR multiplexing is a more efficient approach in which several SSR are amplified in a single PCR reaction (Hayden *et al.*, 2008). As a consequence, SSRs are currently the preferred type of marker as they are highly polymorphic, reproducible, neutral, co-dominant, relatively easy to automate and score. Mutational relationships between alleles and thus MLGs may be inferred using the stepwise mutation model. However few methods are available that can effectively accommodate variation in ploidy within the population.

Phytophthora infestans is an oomycete plant pathogen that causes the serious late blight disease of potatoes. It was responsible for the crop losses that lead to the nineteenth century Irish famine (Woodham-Smith, 1962). This pathogen also infects

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tomatoes and many other cultivated and non-cultivated members of the *Solanaceae*. Potato late blight remains a significant threat to global potato production. *P. infestans* is heterothallic, with A1 and A2 mating types required for the sexual oospores to be formed. Sexual recombination generates new genotypes that may prove more difficulties to manage resistance, for example, they evolve to overcome host resistance, to resist fungicides and to increase aggressiveness. Prior to the 1980s both mating types were only found together in the Toluca Valley, in Mexico (Ristaino, 2002), which may be its center of origin. In the early 1980s, the A2 mating type was first found in Europe (Ristaino *et al.*, 2001, Goodwin & Drenth, 1997) and later, the A2 mating type has subsequently been found in other countries (Fry *et al.*, 1993, Fry *et al.*, 1992). Since this migration the pathogen population in Europe has undergone a major transition with the pre-1980 population being replaced by new clonal lineages and a move to sexual recombination in some regions such as the Netherlands (Drenth *et al.*, 1994, Zwankhuizen & Zadoks, 2002).Tracking such changes in the populations has involved a range of different methods as reviewed by Cooke and Lees (Cooke & Lees, 2004). However, SSR technology is currently the favored approach and has great value in, for example, discriminating MLGs, tracking pathogen spread on local and international scales and examining the presence and significance of sexual recombination. Such studies are proving valuable information on contemporary populations benefits the growers, advisors as well as the agrochemical industry and potato breeders.

Several SSR markers have been used for *P. infestans* population studies (Cooke & Lees, 2004, Knapova & Gisi, 2002, Lees *et al.*, 2006). Li, *et.al* in 2010 (Li *et al.*, 2010c) first reported a multiplex SSR set of eight markers available for *P. infestans*. In this study our aim is to combine the most informative and easily scored markers from the previous studies into a one-step multiplex PCR assay. The assay must allow faster, more accurate and cost-effective data acquisition for examining the pathogen on an international scale and ideally be applicable to both pure *P. infestans* DNA as well as being suitable for use on DNA extracted directly from infected plant tissue. We developed the assay in two different laboratories (Wageningen University, The Netherlands and the James Hutton Institute, UK) and standardize the allele binning and nomenclature across all 12 multiplexed loci. This harmonized assay will serve as a single means of scoring *P. infestans* diversity. Importantly we also explored means of analyzing such data using a sub-sample of European isolates representing clonal

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Table 1. Reference *P. infestans* isolates used in the study

Isolate	Source	Origin	Mating type	Year isolated	MLG
T30-4	WUR	-*	A1	1992	
IPO428-2	WUR	Netherlands	A2	1992	
80029	WUR	Netherlands	A1	1980	
88133	WUR	Netherlands	A2	1988	
VK1.4	WUR	Netherlands	A1	1958	US-1
90128	WUR	Netherlands	A2	1990	
IPO-0	WUR	USA	A1	Before 1980	
VK98014	WUR	Netherlands	A1	1998	
US467 (C6)	W. E. Fry, Cornell Univ.	USA	A2	1998	US-8
EC1 (C7)	G. Forbes, CIP	Ecuador	A1	1998	EC-1
AR4 (C8)	unknown	Argentina	A2	1997	n/a
96.17.5.3 (C1)	JHI	Scotland	A1	1996	4_A1
95.16.3.1 (C9)	JHI	Scotland	A1	1995	8_A1
95.17.3.2 (C2)	JHI	Scotland	A1	1995	8_A1
97.38.2.2 (C3)	JHI	Scotland	A1	1997	8_A1
96.9.5.1 (C4)	JHI	Scotland	A1	1996	5_A1
96.13.1.3 (C5)	JHI	Scotland	A1	1996	5_A1
97.28.1.2 (C10)	JHI	Scotland	A2	1997	'misc'
06_3928A	JHI	England	A2	2006	13_A2

*This isolate is not field isolate but derived from a cross between isolates 80029x88133.

Table 2. The SSR primers used in the study and the final concentration of each primer in multiplex PCR reaction

SSR locus	Dye	Product size range (bp)	Primer sequence	Final con. (uM)	PIC	Rare allele[#]
PiG11[a]	NED	130-180	FwdNED-TGCTATTTATCAAGCGTGGG	0.05	0.78	8/13
			Rev-GTTTCAATCTGCAGCCGTAAGA	0.05		
Pi02[b,d]	NED	255-275	FwdNED-ACTTGCAGAACTACCGCCC	0.05	0.55	2/4
			Rev-GTTTGACCACTTTCCTCGGTTC	0.05		
PinfSSR11[c]	NED	325-360	FwdNED-TTAAGCCACGACACATGAGCTG	0.05	0.33	1/4
			Rev-GTTTAGACAATTGTTTGTGGTCGC	0.05		
D13[b]	FAM	100-185	FwdFAM-TGCCCCCTGCTCACTC	0.16	0.66	13/16
			Rev-GCTCGAATTCATTTTACAGACTTG	0.05		
PinfSSR8[c]	FAM	250-275	FwdFAM-AATCTGATCGCAACTGAGGG	0.3	0.50	2/4
			Rev-GTTTACAAGATACACACGTCGCTCC	0.3		
PinfSSR4[c]	FAM	280-305	FwdFAM-TCTTGTTCGAGTATGCGACG	0.05	0.61	3/7
			Rev-GTTTCACTTCGGGAGAAAGGCTTC	0.05		
Pi04[b]	VIC	160-175	FwdVIC -AGCGGCTTACCGATGG	0.05	0.57	1/4
			Rev-GTTTCAGCGGCTGTTTCGAC	0.05		
Pi70[b]	VIC	185-205	FwdVIC - ATGAAAATACGTCAATGCTCG	0.05	0.19	1/3

Locus	Dye	Size	Primer	Conc.	Freq	Ratio
			Rev-CGTTGGATATTTCTATTTCTTCG	0.05		
PinfSSR6[c]	VIC	230-250	Fwd-GTTTTGGTGGGGCTGAAGTTTT	0.05	0.44	0/3
			RevVIC - TCGCCACAAGATTTATTCCG	0.05		
Pi63[b]	VIC	265-280	FwdVIC - ATGACGAAGATGAAAGTGAGG	0.05	0.58	0/3
			Rev-CGTATTTTCCTGTTTATCTAACACC	0.05		
PinfSSR2[c]	PET	165-180	FwdPET-CGACTTCTACATCAACCGGC	0.05	0.35	1/3
			Rev-GTTTGCTTGGACTGCGTCTTTAGC	0.05		
Pi4B[a]	PET	200-295	FwdPET - AAAATAAAGCCTTTGGTTCA	0.3	0.62	2/5
			Rev-GCAAGCGAGGTTTGTAGATT	0.3		

[#]The ratio of the rare alleles and observed alleles and the frequency of the rare alleles is less than 10%.

Primers previously reported by [a]Knapova and Gisi 2002 [b]Lees et al 2006 [c]Li et al 2010. [d]Primers for Pi02 locus modified to amplify a larger fragment than previously published(Lees et al 2006). The equivalent allele sizes are reported in www.euroblight.org. Note Pi02 (Lees et al 2006) and PinfSSR3 (Li et al 2010) are the same locus. The primer concentrations are for guidance only and may need to be adjusted for other PCR and electrophoresis conditions in other laboratories.

and diverse sexual populations that accommodate the challenges of the variable ploidy levels observed.

RESULTS

Primer optimization

To optimize scoring of the length polymorphism the addition of a 5' PIG tail "GTTT" was made to the unlabeled primer sequences. For PinfSSR2, PinfSSR3, PinfSSR4, PinfSSR6, PinfSSR8, PinfSSR11, it has been previously confirmed that this can reduce stutter peaks (Li *et al.*, 2010, Lees *et al.*, 2006, Cooke & Lees, 2004). In this study, PIG tails were tested on primers for markers G11, D13, Pi04, Pi70, Pi63 and Pi4B. In the case of G11 and Pi04 the 5' PIG tail improved the quality of scoring as the "stutter" of the main SSR peaks is reduced. For the other SSR loci, no improvement was noted and the primers were not modified.

The development of multiplex PCR with 12 SSRs

The reaction conditions for the PCR assay were optimized to ensure that all the target gene sequences were satisfactorily amplified. We determined the optimal concentration of locus-specific primer that was required to achieve strong amplification of a PCR fragment(s) of the expected size. Initially, a standard concentration (0.1 ìM) of each set of primers was used but this resulted in uneven peak intensities or absence of amplification of some markers. To overcome this, the proportions of the various primers in the reaction mixture were adjusted. The final concentration of the primers varied from 0.05 μM to 0.3 μM (Table 2). All PCR primers were prepared as 10 mM stock solutions which were frozen at -20°C in 20 μl aliquots to minimize thawing and freezing of the reagents. All SSRs present in the multiplexed PCR were successfully amplified as assessed by ABI3730 fluorescence intensities that fell within a range optimal for semi-automated allele sizing, i.e. 200-5000 relative fluorescence units.

Robustness of multiplex PCR (genotyping error and null alleles)

To assess the robustness of multiplex PCR to variation in the concentration and quality of DNA samples, two experiments were performed. A range of different concentrations of DNA and the effect of DNA quality were determined by comparing the different approaches of DNA extraction, kit extraction from isolates mycelia, FTA

tyity170 x 240 mm

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cards and robot DNA extraction from infected leaves. The method shown in Methods and Materials was found robust for the multiplex PCR.

The multiplex PCR has been applied to over 60,000 isolates of global origin in the WUR (NL) and JHI (UK) laboratories. The error rates were negligible amongst reference isolates that were run in every batch of samples (WUR, NL). Furthermore, many hundreds of isolates of some dominant clonal lineages, have been genotyped with this system and alleles recorded were identical at a wide range of DNA concentrations in extractions using a range of methods. The standard reference allele names and peak sizes reported to date are presented (Supplementary Table S1) and will be continually updated on the Euroblight website (see protocols section of www.euroblight.net). Null alleles were called in cases where amplification of only a single locus in the 12-plex assay failed repeatedly. To date null alleles have only been recorded at a low frequency in the markers D13, G11 and PinfSSR8. Reference samples have been run using both the QIAGEN multiplex kits mentioned above with identical results. Similarly, we have found that the same isolates run on several different PCR machines generated identical fingerprints.

Case study: the analysis of SSR data from 96 isolates

The reference isolates and those selected from NL and UK populations were fingerprinted using the 12-plex assay and the findings reported as a case study to illustrate the potential of the method. It was not the intention of this study to explore the specific details of the populations in question. The samples were chosen as representative of some representative clonal lineages (Cooke *et al.*, 2007, Fry *et al.*, 2009), some of which are known to be present in both the Netherlands and UK and a mixed group of diverse isolates whose fingerprints (Supplementary Table S2) are consistent their originating from sexual recombination (Brurberg *et al.*, 2011). The latter set were defined as 'miscellaneous' (hereafter 'misc'). The PIC levels of the markers ranged from 0.19 (Pi70) to 0.78 (PiG11) and 80 distinct fingerprint patterns (MLGs) were discriminated amongst the 96 isolates. Three peaks were recorded at one or more loci of 26 of 96 isolates which suggests different ploidy levels were present in the sampled population. This is consistent with previous studies of altered levels of ploidy or trisomy of *P. infestans* (Tooley & Therrien, 1991, van der Lee *et al.*, 2004). Such a mixture of isolates of different levels of ploidy makes the analysis of the resultant SSR data complex (Bruvo *et al.*, 2004, Clark & Jasieniuk, 2011). The

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R package POLYSAT infers the ploidy from the number of alleles at a locus and calculates a genetic distance matrix and principal co-ordinate analysis of populations of mixed ploidy levels. A plot of the first two principal components exported from POLYSAT (Fig. 1) provides an overview of the variance of the dataset and indicates a broad scatter of data points amongst isolates defined as 'misc' with clusters of isolates representing the clonal types. In particular, minor variation was observed amongst a cluster of 15 isolates defined as the EU_13 MLG (equivalent to 13_A2 in Cooke *et al* 2007; Fry *et al* 2009). The variation in such cases was predominantly at highly polymorphic loci such as D13, G11 and SSR4. The dendrogram indicating the genetic relatedness of the isolates (Fig. 2) similarly indicates considerable diversity amongst the collection with an overall 'star' or 'bushy-like' shape indicating there is no clear sub-structuring amongst this sample of 96 isolates. As in the case of the principal co-ordinate analysis (PCA) clusters of closely related isolates representing clonal types were evident. An additional analysis using minimum spanning trees indicated a broadly similar pattern (data not shown).
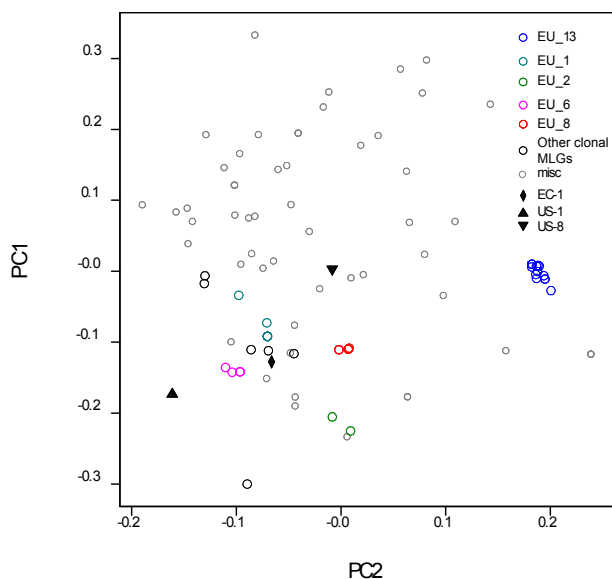

Fig. 1. A plot of the first two principal components representing 96 selected isolates which were characterised by using 12 SSR markers and examined in POLYSAT.
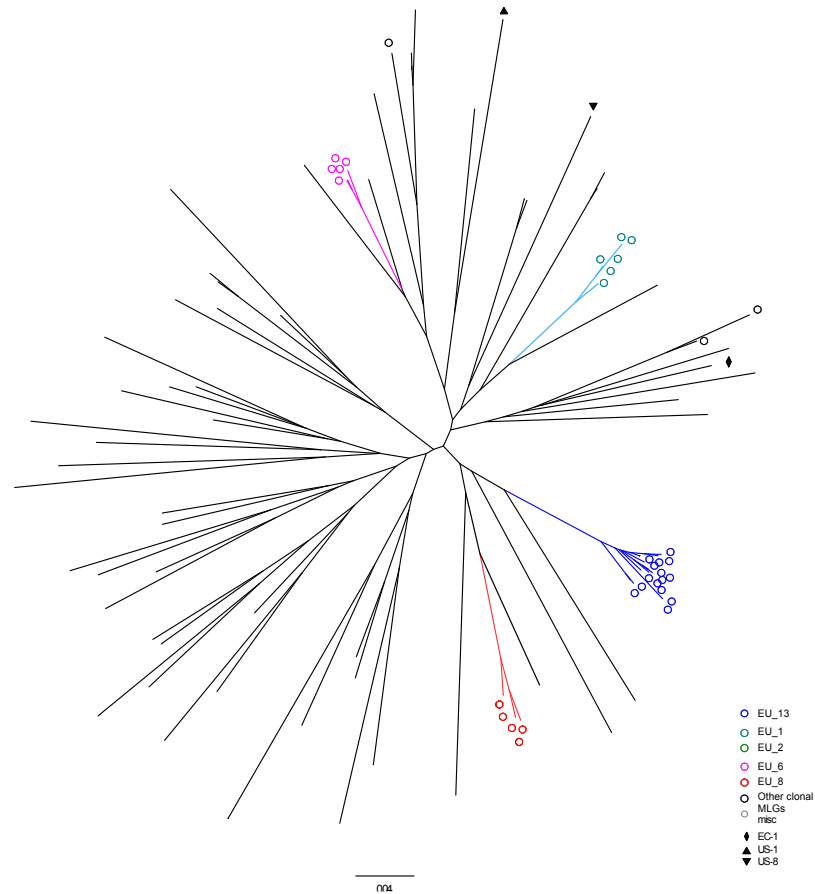
Fig. 2. The NJ dendrogram of the genetic diversity of 96 isolates. The isolates of some clonal lineages are marked with symbols and the branches with no labels represent 'misc' isolates.

Determination and naming of clonal lineages

P. infestans isolates examined to date comprise several major distinct clonal lineages based on a range of marker systems including SSR, mitochondrial haplotype combined with mating type analysis. The distinct evolutionary lineages of *P. infestans* were determined in dendrograms with significant bootstrap support based on multilocus SSR data supplemented with that of mitochondrial markers and mating types (data not shown). In accordance with other proposals (Grünwal *et al.*, 2008), we suggest that lineages are named with a two letter identifier for the region in which they were first found (e.g., EU = Europe, CN = China) followed by a number indicating the order in which they were described. In such a system lineage EU_13

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would be the name for what was called 13_A2, a lineage of mating type A2 and mtDNA haplotype Ia that was first reported in Europe. We do not propose standardizing nomenclature for different genotypes in this study but the standard nomenclature and alleles of genotypes will be presented in detail on the Euroblight website.

Fast and high-throughput *in planta* fingerprinting in the season

With the multiplex SSR analysis, the whole genotyping procedure from field sampling to the final genotyping results can be completed in seven days. The first step of mycelium preparation requires the majority of this time and cannot be shortened. Automation of the DNA extraction procedure can shorten the process to provide a rapid feedback to growers within a season. However, an alternative is to examine the pathogen directly from lesions either pressed onto FTA cards or subject to direct DNA extraction. In this study we have comprehensively tested over 1,000 lesions pressed onto FTA cards and find the method is very effective with as many as 90% of pressed lesions successfully fingerprinted. To account for the lower levels of DNA released from the 2 mm diameter disk, additional cycles of PCR are added according to the QIAGEN kit instructions. This provides great potential for a rapid, easy, high-throughput and inexpensive detection pipeline for the field potato in the blight season. FTA cards also allow the convenient typing of lesions from remote sites without access to laboratories and materials for pathogen isolation.

DISCUSSION

Fluorescence-based SSR detection and allele sizing on an automated DNA fragment analyzer is one of the fastest and most accurate methods for SSR genotyping. The assay described in this study is a one-step multiplex PCR amplification to facilitate highly parallel, fluorescence-based SSR genotyping. This multiplex PCR for *P. infestans* typing is (i) simple, as only one PCR is needed to perform multilocus typing with twelve markers; (ii) rapid, as the genotyping results can be available in one day; (iii) reproducible and suited to large numbers of isolates.   The reproducibility of the assay has been tested on isolates collected from different countries by the different laboratories (see Acknowledgments) and has proven to have a suitable level of resolving power. The genotyping data from different geographic populations is to be submitted to the Euroblight database offering a powerful approach to studying

population change on broad geographic and time scales. The standardized bin set and reference allele calls are also available from Euroblight website.

Alleles may be scored by different means; true allele size calling, i.e. using decimal numbers, and binning, i.e. the conversion of alleles from actual DNA fragment sizes into discrete units to which an integer label is assigned. The allelic binning is very critical. In one comparative study, 83% of discrepancies between laboratories in scoring dinucleotide alleles were caused by arbitrary decisions in binning (Weeks *et al.*, 2002). It confirms the necessity of well-established reading rules. In our study, a consistent rule for scoring was used in case of naming a new allele. Users should be aware that the bin label is not necessarily the true allele size. In our study, the difference between the bin label and true size is from 0 to 2 bp. Accurate binning of alleles is critical. For dinucleotide markers, where alleles normally differ by multiples of 2 bp, ideal bins should have small ranges (<0.8 bp) and large interbin distances (>1 bp) (Ghosh *et al.*, 1997). Such consistent allele naming and scoring is particularly important in establishing a database of SSR alleles. For a new allele to be authenticated partners should ideally distribute an image of the new allelic peaks, ideally to a reference laboratory, so that this allele may be added to those in the database. Within the Euroblight database, data entry is limited to pre-set allele sizes to minimize inconsistencies in allele naming. All submitted datasets are also subject to rigorous quality control to ensure data is of the required standard.

Without a standard marker set the clonal lineages in different countries cannot be resolved and compared easily by different laboratories. The SSR loci described in this study exhibit fixed lineage-specific alleles that easily distinguish the lineages at the molecular level. However, as seen in this study isolates within a given lineage have diverged considerably at the faster-evolving SSR loci. Such differences amongst clonal lineages of *P. ramorum* lineages have also been reported (Goss *et al.*, 2009). The markers in this 12-plex assay provide a useful mix of those with a lower mutation rate (those in open reading frames) with more variable ones (those in noncoding regions and with longer repeat units).

The analysis methods presented here provide a means of accounting for differences in ploidy and will be appropriate for the analysis of large data-sets. PCA is useful for an overview of the variance in the data. The Neighbor Joining tree is a powerful means of displaying clusters of isolates that are variants of clonal lineages. However, the deeper roots of the tree will not be truely representative of the

CHAPTER 3

evolutionary history as it is known that SSRs are less suited than sequence analysis for resolving such evolutionary histories. In populations with large numbers of clones and variants of those clones minimum spanning trees (or networks) that present nodes may be more appropriate. In such a system nodes of different sizes (in proportion to the number of isolates of MLGs) are displayed and the links between these nodes represent the step-wise mutation of the clonal lineage. Such studies have proved suitable for *P. ramorum* analysis (Goss *et al.*, 2009, Vercauteren *et al.*, 2010) and will be appropriate for largely clonal *P. infestans* populations.

MATERIALS AND METHODS

Samples

For thisstudy 96 *P. infestans* isolates were selected to demonstrate the method. Diverse reference isolates are from the previous studies (Li *et al.*, 2010, Lees *et al.*, 2006) and were combined with other samples selected from pathogen surveys as representatives of known clonal lineages and novel and diverse MLGs in both the Netherlands and Great Britain sampled over the period 2001 to 2011.

DNA extraction

Agar plugs of each individual *P. infestans* isolate were taken from the edge of a seven day old actively growing colony in pea agar medium (120 g of frozen peas, 15 g of agar, 1 liter of distilled water) and transferred to liquid pea broth (120 g of frozen peas, 1 liter of distilled water). After 3-4 days of incubation at 20ºC in dark, sufficient mycelium was available for DNA extraction. Genomic DNA was isolated from 20mg of lyophilized mycelium using the DNeasy 96 Plant Kit (QIAGEN Catalogue Number 69181). The procedure followed the detailed manufacturer's instructions and elution was made with 200 μl ultra-pure water. DNA extracts were stored at -20ºC until further use.

In planta genotyping using FTA cards

Sap from the margins of actively spreading foliar blight lesions was pressed onto FTA Classic cards (Whatman, WB120205) using a pair of pliers. After air-drying, the cards can be used immediately or stored for several years. Small disks were cut from the card using a 2mm Harris Micro Punch (Whatman, WB100007) and processed according to the manufacturer's protocols in a FTA purification reagent (Whatman, WB120204) before being placed directly into the PCR mix for multiplex SSR analysis (see below).

SSR primer design

The twelve SSRs used in this study were selected from the 20 previously published sets according to their map position, ease of scoring and allelic diversity (Knapova & Gisi, 2002, Lees *et al.*, 2006, Li *et al.*, 2010, Cooke & Lees, 2004). If required the primers were redesigned to adjust the PCR product size for the multiplex assay (Table 2) and each locus was assigned one of the four different fluorescent labels (blue, 6-carboxyfluorescein (6FAM); NED; VIC, PET (AppliedBiosystems)) in such a manner that no two markers with the same fluorescent dye had overlapping in allele size ranges. Addition of 5′ PIGtail "**GTTT**"to **reverse** primers has been reported to reduce the stutter peaks and assist the scoring

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of length polymorphisms (Binladen *et al.*, 2007). In the previous study (Li *et al.*, 2010), it was confirmed that the reverse primer of markers PinfSSR2, PinfSSR3, PinfSSR4, PinfSSR6, PinfSSR8, and PinfSSR11 offered a benefit. In this study, the benefit of adding a PIGtail to reverse primers of another six SSRs (G11, D13, Pi04, Pi70, Pi63, Pi4B) was tested.

Primer optimization

The optimal concentration of locus-specific primer required to amplify the target sequence was determined empirically. Initially 0.4, 0.2, 0.1 and 0.05 µM of locus-specific primer was tested. PCR products were separated on 3% agarose gels. The optimal primer concentration was determined by visual inspection as the strong amplification of a PCR fragment of the expected size. In instances where it was desirable to improve PCR specificity and yield, additional locus-specific primer concentrations were tested.

Multiplex PCR reaction

The amplification of twelve SSRs by uniplex and multiplex PCR was performed under identical reaction conditions. Uniplex PCR was performed in a volume of 20 µl, 10 ng template DNA, 200 µM of each dNTPs, 0.08 U Taq DNA polymerase (Roche Diagnostics, Leiden, The Netherlands), 50 mM KCl, 1.5 mM $MgCl_2$, and a range ofconcentrations (Table 2) ofthe locus-specific primers.For multiplex PCR, locus-specific primers for several markers were added to each reaction at the optimal concentration determined in uniplex assays.The QIAGEN multiplex PCR kit (QIAGEN Catalogue Number 206145) was used at WUR and the QIAGEN Type-it Microsatellite PCR Kit (QIAGEN Catalogue Number 206243) was used at JHI. Amplification reactions were as described by the manufacturer. Amplifications using the QIAGEN multiplex PCR kit were run in a PTC200 thermocycler (MJ Research, Waltham, Massachusetts, USA), with an initial denaturation at 95°C for 15 min, followed by 30 cycles of 95°C for 20 sec, 58°C for 90 sec, and 72°C for 60 sec, and a final extension at 72°C for 20 min. At JHI using the QIAGEN Type-it Microsatellite PCR Kit the manufacturer's protocols were followed; PCR conditions 95°C for 5 minutes followed by 28 cycles (33 cycles for FTA cards) of 95°C for 30 seconds, 58°C for 90 seconds, and 72°C for 20 seconds, and a final extension at 60°C for 30 min. Several different PCR machines have been tested. At JHI the manufacturer's recommended reactions sizes were reduced to 12.5 µl.

Error protection

To avoid discrepancies among studies and across time in different laboratories, reference (or control) isolates should be included in each study. In this study we included a reference set of eight Dutch isolates into each batch of 96 samples to calibrate and account for differences such as polymer batch, buffer concentration, array quality, ambient laboratory temperature, fluorescent label and variation in internal size standards that may affect the reproducibility of denaturing capillary electrophoresis. In this study, eight Dutch reference samples are loaded along with the test samples (Table 1). The reference samples of known SSR marker genotypes (i.e., reference alleles) consist of a set of unrelated template DNAs amplified alongside the test samples in the same PCR conditions. Reference isolate DNA templatesare maintained in the laboratory and should be shared with other groups to standardize allele calling between and within laboratories.

SSR scoring and quality control

CHAPTER 3

Electrophoresis and visualization of SSRs was performed on a Geldoc system of Herolab, type RH-5 and ABI3730 DNA analyser (Applied Biosystems). Five μl of PCR products was mixed with 1/6 volume of gel loading buffer (Orange G loading buffer) and separated on 3% agarose gel. For ABI3730 analysis at WUR, the PCR products were diluted 1,000 times and 1 μl of diluted SSR product was added to 9μl of deionised formamide (Hi-Di Formamide Part no. 4311320, Applied Biosystems) containing 0.045μl of GeneScan-500LIZ standard (Part no. 4322682, Applied Biosystems). The sample was run on an automated ABI 3730 capillary sequencer according to the manufacturer's instructions. SSR allele sizing was performed and scored using GeneMapper v3.7 software (Applied Biosystems). At JHI the PCR product was diluted 100 times and 0.6 of the diluted product added to a HiDi and GeneScan-500LIZ mix as described above.

Within the GeneMapper software kits, panels and binsets were generated defining the markers and their known allele bins. The required text files for GeneMapper used in this study as well as images of scored peaks and the MLGs of reference isolates are available on the Euroblight website (www.euroblight.net). Once loaded into GeneMapper, the panels and binsets are applied and peaks sizes calculated according to the size standard before the manual curation of the reference samples' according to their previously assigned allele (i.e., bin) name. If possible the primer and dye combinations should remain fixed as differences between expected and observed reference alleles due to dye shift have been reported (Sutton *et al.*, 2011). Any slight variation in the observed reference allele sizes compared to the expected sizes (within original binset file) from one laboratory to another was accounted for by adjustment of the bin centers, keeping the allele names the same. The resultant allele call table was exported from GeneMapper for further genetic analysis.

Data analysis

Evaluation of polymorphism information content (PIC) of the SSR markers was calculated using the formula below (D. Botstein *et al.*, 1980, Anderson *et al.*, 1993):

$$PIC = 1 - \sum_{i=1}^{k} Pi^2 ,$$

In which k is the total number of alleles detected for one SSR marker and the *P. infestans* is the frequency of the ith allele.

Genetic distances and principal co-ordinate analysis (PCA) were calculated from Bruvo distances (Bruvo *et al.*, 2004) using POLYSAT (Clark & Jasieniuk, 2011) implemented in R. The distance matrix was exported from POLYSAT for further analysis to either generate a minimum spanning tree in MINSPNET (Excoffier & Smouse, 1994)or a neighbor joining tree in NEIGHBOR in PHYLIP (Anderson *et al.*, 1993). Minimum spanning trees were drawn as part of GraphViz (http://www.graphviz.org) and neighbor joining trees viewed and exported using FigTree 3.1 (http://tree.bio.ed.ac.uk/). The first two principal co-ordinates were exported from R and plotted in GenStat for Windows (14th Edition).

ACKNOWLEDGEMENTS

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170 x 240 mm

SUPPLEMENTARY MATERIAL

Table S1. SSR allele names and sizes

Marker	Measurement																	
G11	**Allele name**	130	132	138	140	142	146	148	150	152	154	156	158	160	162	164	166	168	170
	Allele size	130.30	132.30	138.45	139.99	142.01	147.08	149.40	151.40	153.07	155.16	157.02	159.41	161.12	163.05	165.05	167.01	169.17	171.43
	Allele name	172	176	198	200	202	204	206	208	210	214	218	220	222					
	Allele size	173.21	176.72	198.27	200.21	201.96	204.07	205.82	207.68	209.93	213.62	217.45	219.45	221.40					
Pi04	**Allele name**	160	166	168	170	172													
	Allele size	166.06	171.15	173.45	175.41	178.52													
Pi4B	**Allele name**	205	209	211	213	215	217	221	225	#									
	Allele size	206.60	210.45	212.50	214.70	216.40	218.64	222.62	226.74										
Pi63	**Allele name**	270	273	279															
	Allele size	270.23	273.24	279.18															
Pi70	**Allele name**	189	192	195	198														
	Allele size	187.95	190.77	193.65	195.99														
	Allele name	108	110	112	114	116	118	120	122	124	126	128	130	132	134	136	138	140	142
	Allele size	106.22	108.17	110.04	112.07	114.24	116.33	119.01	120.89	123.06	125.09	127.06	128.78	130.55	132.62	134.78	136.62	138.91	141.13
D13	**Allele name**	144	146	148	150	152	154	156	158	160	162	164	166						
	Allele size	143.32	145.56	147.82	150.17	152.29	154.33	156.43	158.64	160.59	162.37	164.41	166.57						
	Allele name	188	190	208	210	212	214	216											
	Allele size	188.85	190.80	208.69	210.78	212.71	214.73	217.00											
SSR2	**Allele name**	163	167	173	175	177													
	Allele size	163.54	167.27	173.30	175.36	177.42													
	Allele name	258	264	266	268	270	272	276											
	Allele size	257.50	263.90	265.90	268.20	270.30	272.10	277											
SSR3*	Original allele name	152	158	160	162	164	166	168	170										
SSR4	**Allele name**	283	285	287	289	291	293	295	297	299	301	303	305	307	309	311	313	315	317

appears in the Pi4B row near the "152" column position.

	Allele size	281.41	283.67	285.65	287.78	289.72	291.86	293.61	295.70	297.62	299.73	301.85	303.98	305.71	307.90	309.86	312.07	314.02	316.27
SSR6	Allele name	232	236	240	242	244	246		254	256	258	260	262						
	Allele size	231.77	236.11	239.63	241.76	243.82	245.75		253.73	255.76	257.62	259.79	262.72						
SSR8	Allele name					260				264		266							
	Allele size					259.72				263.94		265.80							
SSR11	Allele name					331				341		356							
	Allele size					330.68				340.76		356.01							

* PRI SSR3=SCRI Pi02. Longer primers of SSR03 clearly match with peaks originally scored with Pi02 primers. New names suggested and comment in paper that 152=258 etc etc. Some rare alleles not yet confirmed with SSR03 primers.

Comment on larger alleles observed in isolates from Ecuador.

Table S2. SSR MLGs of *P. infestans* isolates tested in this study

Sample Name	D13 1	D13 2	D13 3	Pi4B 1	Pi4B 2	Pi4B 3	G11 1	G11 2	G11 3	Pi04 1	Pi04 2	Pi04 3	Pi63 1	Pi63 2	Pi63 3	Pi70 1	Pi70 2	Pi70 3	SSR2 1	SSR2 2	SSR2 3	SSR3 1	SSR3 2	SSR3 3	SSR4 1	SSR4 2	SSR4 3	SSR6 1	SSR6 2	SSR6 3	SSR8 1	SSR8 2	SSR8 3	SSR11 1	SSR11 2	SSR11 3
ipo_complex	148	164	0	217	217	0	142	156	0	160	168	0	157	157	0	192	195	0	173	173	0	268	268	0	288	294	0	244	244	0	260	266	0	341	341	0
NL03127	154	154	0	213	213	0	142	162	0	168	168	0	151	157	0	192	192	0	173	175	0	266	268	0	284	294	0	244	244	0	260	266	0	341	356	0
NL02054	136	136	0	213	217	0	142	162	0	166	170	0	148	157	0	192	192	0	173	175	0	266	268	0	284	294	0	244	244	0	260	266	0	341	356	0
NL03090	132	136	0	213	217	0	142	162	0	166	170	0	148	157	0	192	192	0	173	175	0	266	268	0	284	294	0	244	244	0	260	266	0	341	356	0
NL05034	132	136	0	213	217	0	142	162	0	160	170	0	148	157	0	192	192	0	173	175	0	266	268	0	284	294	0	244	244	0	260	266	0	341	356	0
NL05100	136	136	0	213	217	0	142	162	0	166	170	0	148	157	0	192	192	0	173	175	0	266	268	0	284	294	0	244	244	0	260	266	0	341	356	0
NL08078	136	136	0	213	217	0	142	162	0	166	170	0	148	157	0	192	192	0	173	175	0	266	268	0	284	294	0	244	244	0	260	266	0	341	356	0
NL05350	154	154	0	213	213	0	148	148	0	168	168	0	157	157	0	192	192	0	173	173	0	268	268	0	284	294	0	244	244	0	null	null	0	331	341	0
NL07564	118	118	0	213	217	0	148	148	0	168	168	0	151	157	0	192	192	0	175	175	0	266	268	0	284	284	0	244	244	0	266	266	0	null	null	0
NL01164	136	136	0	205	217	0	148	150	0	168	168	0	148	157	0	192	192	0	173	173	0	266	268	0	288	294	0	244	244	0	266	266	0	341	341	0
NL02059	136	136	0	213	215	0	148	150	0	168	168	0	148	148	0	192	195	0	173	175	0	268	268	0	288	290	0	240	242	0	260	266	0	341	341	0
NL00357	null	null	0	213	217	0	148	154	0	168	168	0	148	151	0	192	192	0	173	173	0	268	268	0	284	294	0	242	244	0	266	266	0	341	341	0

170 x 240 mm

NL01069
NL04178
NL05196
NL04055
NL01028
NL06010
VK1_4
NL01108
98014
NL05015
NL05246
NL05353
NL05557
NL05592
NL05869
NL07063
NL07548
NL08419
NL05204
MDJ08-12
NL03047
NL03081
T30_4
428_2
88133
NL02110
90128
NL01085
NL06025
NL06026

170 x 240 mm

NL03042
NL08510
NL06015
NL04001
NL04005
80029
FloDale_3_3
b
FloDale_3_3
d
09_7770B
09_7110A
PCL_10_793
0C
09_7186A
09_7610A
09_7458A
09_7410A
PCL_10_805
8A
2007_5622A
PCL_10_781
4A
09_7126A
PCL_10_807
8C
PCL_11_898
6A
PCL_10_805
0D

170 x 240 mm

170 x 240 mm

PCL_11_884
2B
PCL_10_788
6A
PCL_10_783
8C
PCL_10_805
4C
09_7722B
PCL_10_795
8A
C5
SC96.13.1.3
PIG_C7
P12201(SCR
1_C1)
P12210
(C10)
P12204
(C4-SC96.9.
5.1)
PIG_C2
PIG_C3
PIG_C6

170 x 240 mm

CHAPTER 4

Population structure of *Phytophthora infestans* in China shows four geographically bound clusters and reveals the dominant presence of EU genotype "Blue_13"

Ying Li, Theo van der Lee, Jiehua Zhu, Guanghui Jin, Chengzhong Lan, Suxian Zhu, Ruofang Zhang, Bowei Liu, Zhijian Zhao, Geert Kessel, Sanwen Huang, Evert Jacobsen

CHAPTER 4

SUMMARY

Phytophthora infestans in China has been the subject of several studies, resulting in conflicting reports on the population diversity in China. This study aims to perform a genetic analysis of the current population of *P. infestans* in China. A total of 229 *P. infestans* isolates, 188 newly collected in seven main potato growing regions in China between 2002 and 2009 and 41 from Guo *et al*., were genotypically characterized and identified the three mitochondrial haplotypes (Ia, IIa, IIb) and the three dominant clonal lineages (CN01, CN02 and CN03), with ten highly informative microsatellite markers. One of these three dominant clonal lineages is genetically similar to "Blue_13" a dominant genotype found in Europe since 2004. It is the first report of "Blue_13" outside Europe. Within the Chinese *P. infestans* population, the genotypes strongly clustered according to their sampling provinces. The mating type ratio and the SSR allele frequencies indicate that in China the contribution of the sexual cycle of *P. infestans* on population dynamics is minimal. The migration through asexual propagules and the generation of sub-clonal variation are the dominant driving factors behind the Chinese *P. infestans* population structure. The virulence spectrum with 10 potato differentials showed seven different virulence spectra varying from 3 to 10 factors and Chinese "Blue_13" isolates are more aggressive, one of whom was identified with virulence to all differentials tested. The results emphasize the differences that exist within China and are a clear warning against the transport of (seed) potatoes between the different regions in China, as this may quickly spread more aggressive and more virulent genotypes, and facilitate the reconvening of the two mating types which may result in the occurrence of the sexual cycle.

INTRODUCTION

Late blight, caused by the oomycete *Phytophthora infestans*, is one of the most devastating diseases of potato and tomato (Haverkort *et al.*, 2008). *P. infestans* has a heterothallic mating system and the two mating types (A1 and A2) are required for sexual reproduction (Judelson, 1997). The climate in the Chinese potato production regions is mostly favorable for *P. infestans* and potato late blight currently is the most serious threat for potato production in China (Huang *et al.*, 1981). Similar to observations in Europe and the USA, potato late blight epidemics have recently become more difficult to control, a feat attributed to population structure change in the USA and Europe.

Prior to the 1980's, only the A1 mating type was found outside Mexico. After this period, the presence of the A2 mating type was reported for several parts of the world over the past three decades. The A2 mating type was first found in northern China in 1996 (Zhang *et al.*, 1996) and later detected in other regions, including Yunnan in Southern China close to the Vietnamese border (Zhao & Zhang, 1999). Although both, the A1 and A2 mating types are found in China, to this date, no evidence of an active sexual cycle based on allele frequency was found (Yang *et al.*, 2008).

A new A2 clonal lineage, "Blue_13", was found in the Netherlands in 2004 and in the United Kingdom in 2005. Isolates with this genotype are highly aggressive, spread rapidly and now dominate *P. infestans* populations in large parts of Europe, including the U.K. (Lees *et al.*, 2009) and the Netherlands (Kildea *et al.*, 2010). It is considered a new threat to potato production and its spread is intensively monitored in Europe. So far, the "Blue_13" genotype was not reported outside Europe.

China's potato agribusiness has grown rapidly in recent years (Chen & Qu, 2008, Qu *et al.*, 2005). Nation-wide transportation of commercial (seed) tubers and potato commodities occur more frequently and will boom in the coming years. This increases the risk of introduction of new genotypes from abroad, the spread of more aggressive isolates and the reconvening of the two mating types.

Strategies for durable control of potato late blight (PLB) building on integrated pest management (IPM) are counteracted by population changes as described above for Europe and the USA. Monitoring of aggressiveness, virulence and fungicide resistance in the *P. infestans* population can thus be seen as a cornerstone for durable management of the *P. infestans* population and PLB control. Unfortunately, the current knowledge on the Chinese *P. infestans* population is limited and the conclusions from the different

studies were conflicting and data are likely out-dated despite the fact that several studies were undertaken (Guo *et al.*, 2007, Zhao *et al.*, 2007, Yang *et al.*, 2008, Yao *et al.*, 2008, Zhao *et al.*, 2008, Guo *et al.*, 2009b, Yao *et al.*, 2009) in the (recent) past. *P. infestans* isolates collected between 1997 and 2003 from Northern China all belonged to the same clonal lineage as determined by two SSR markers with some additional sub-clonal variation as determined by AFLP markers (Guo *et al.*, 2009). However, determination of the virulence spectrum towards *Solanum demissum R*-genes *R1-R11* revealed a high diversity within this clonal group (Guo *et al.*, 2009). In contrast, other studies showed more genetic variation among isolates but these studies included only isolates derived from parts of the country, were collected largely in the previous century and/or the isolates were only partly characterized (Yang *et al.*, 2008, Yao *et al.*, 2009, Zhao *et al.*, 2008, Yao *et al.*, 2008, Zhao *et al.*, 2007), resulting in fragmented information.

The objective of this study was to perform a comprehensive survey on *P. infestans* isolates from seven major potato regions for a better understanding of (i) the population structure, the contribution of (ii) the sexual cycle and (iii) the role of clonal variation within the population diversity, (iv) the national migration, and (v) virulence spectrum of the isolates.

RESULTS

Sampling of the isolates

In this study, 188 isolates were obtained from local scientists collected from seven regions, including 12 provinces during the years 2002 to 2009 (Table S and Fig. 1). In addition, a set of 41 isolates from the previous study was used for SSR genotyping analysis (Guo *et al.*, 2009). The latter set could only be analyzed genotypically, as only DNA and no viable cultures of these isolates were available. The isolates were divided into seven groups based on their regions of origin for further analysis. The seven regions were; HLJ (including Heilongjiang and Jilin Provinces), IM (Inner Mongolia Province), HB (Hebei Province), SC (including Sichuan and Guizhou Provinces), YN (Yunnan Province), FJ (Fujian Province), and NW (Northwestern China, including Gansu, Shanxi and Ningxia provinces).

Mitochondrial haplotype analysis

Three mitochondrial haplotypes (Ia, IIa, IIb, Fig. 1, 2) were found among the Chinese isolates in this study. Nearly all the isolates (59 of 60) derived from the Northern part of

China, i.e. Heilongjiang, Inner Mongolia, and Hebei had the IIa haplotype, and only one isolate (HLJ05-NL1 from Heilongjiang) had the mt haplotype Ia. All isolates derived from Sichuan and Yunnan had the Ia haplotype. All isolates collected in Fujian Province had the IIb haplotype. Mt haplotype Ib (US-1 clonal lineage) was not found in this study. The occurrence of three mt haplotypes, strongly correlated with the sampling origin of the isolates (P <0.0001, Fig.1, 2).
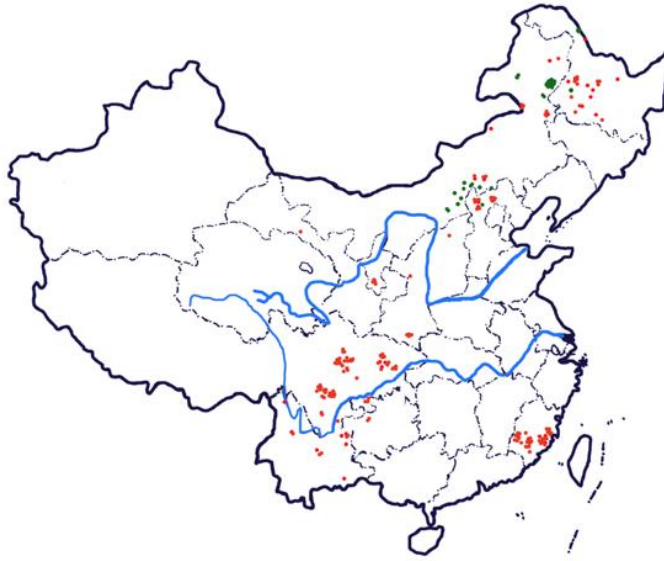
Fig. 1. Sampling location map of *P. infestans* isolates. Dots present the sampling locations by GPS coordinates; red dots indicate the samples collected in this study; green dots indicate the samples from the previous study of Guo *et.al*, 2010.

Table 1. Profile of SSR markers

ID	Size range	Label	Primer sequence (5' to 3')	Allele nr	PIC	He	Ho	P val (HWE)
G11	125-220	NED	F: TGCTATTTATCAAGCGTGGG R: TACAATCTGCAGCCGTAAGA	11	0.76	0.50	0.52	0.14
P4B	200-300	PET	F: AAAATAAAGCCTTTGGTTCA R: GCAAGCGAGGTTTGTAGATT	4	0.65	0.50	0.82	0.00
P63	265-285	VIC	F: ATGACGAAGATGAAAGTGAGG R: CGTATTTTCCTGTTTATCTAACACC	3	0.50	0.44	0.85	0.00
P70	185-200	VIC	F: ATGAAAATACGTCAATGCTCG R: CGTTGGATATTTCTATTTCTTCG	3	0	/	/	/
SSR 2	165-180	PET	F: CGACTTCTACATCAACCGGC R: GTTT GCTTGGACTGCGTCTTTAGC	3	0.44	0.36	0.59	0.00
SSR 3	254-274	NED	F: ACTTGCAGAACTACCGCCC R: GTTT GACCACTTTCCTCGGTTC	5	0.59	0.39	0.56	0.00
SSR 4	280-305	6-FAM	F: TCTTGTTCGAGTATGCGACG R: GTTTCACTTCGGGAGAAAGGCTTC	8	0.76	0.64	0.96	0.00
SSR 6	230-260	VIC	F: GTTTTGGGTGGGGCTGAAGTTTT R: TCGCCACAAGATTTATTCCG	3	0.43	0.26	0.34	0.04
SSR 8	256-274	6-FAM	F: AATCTGATCGCAACTGAGGG R: GTTTACAAGATACACACGTCGCTCC	3	0.53	0.33	0.50	0.00
SSR 11	325-360	NED	F: TTAAGCCACGACATGAGCTG R: GTTTAGACAATTGTTTTGTGGTCGC	3	0.55	0.46	0.69	0.00

Profile of SSR markers

The ten SSR markers yielded 44 alleles on the 229 isolates with an average of 4.4 alleles per locus. For Pi70 no polymorphism was found among Chinese isolates. For the polymorphic markers, an average of 4.9 alleles was detected for each locus. The mean PIC value was 0.58. G11 and PinfSSR4 were the most informative SSRs, with the highest PIC value (0.76). The mean expected (He) and observed (Ho) heterozygosity were 0.43 and 0.65, respectively. A complete list of loci and their variability is shown in Table 1. Except for G11, all loci deviated significantly from HWE expectations (P<0.05). Linkages between pairs of loci were detected, but were not consistent across geographic populations.

Genetic variation by province

SSR genotyping of the 229 *P. infestans* isolates resulted in 69 distinct genotypes (Fig.2), 49 of which show variations within the three dominant clonal lineages and are consistent with their sampling province. Careful inspection of genotypes revealed that these differences comprise losses of alleles for a particular locus or the presence of rare alleles in the hyper-variable loci PinfSSR4 and G11 and should therefore be considered as sub-clones. The dominant genotype named CN01_01 (Fig 2A) was found in four out of seven regions (HLJ, IM, HB and NW), located in the North and Northwest of China, (Fig.3). Its sub-clones CN01_07 and CN01_08 were also identified at multiple sites in the Northern part of China. The other two genotypes, CN03 and CN19 in Fig. 2 were found in two of Northern provinces, but are clearly distinct from the dominant clonal lineage CN01. A second dominant genotype CN02_01 was found in three regions located in Western China (NW, YN and SC). The other 65 genotypes including 25 genotypes belonging to clonal lineage CN03, were unique to the province from which they were collected. The genotypic variation within the seven regions varied from 4 (NW) to 25 (FJ) genotypes (Fig. 2).

To examine a potential substructure within the Chinese population, the isolates were divided into seven sub-populations based on the sampling regions. Based on the AMOVA analysis, 26.1% of the variation is found within sub-populations, while 73.9% of the variation is found between sub-populations. The variation between sub-populations is significantly higher than the variation within sub-populations (P<0.001). To visualize the genotypic relations, a similarity tree was constructed using only the SSR data by Neighbour-Joining analysis based on Nei's genetic distances. The

phylogram, rooted with the US-1 genotype from the Netherlands (VK1.4), is shown in Fig. 3. The genotypes group into four distinct clusters. When the sample position on the phylogeny tree was linked with the geographic regions, a clear pattern emerged. Cluster I contains all 100 isolates derived from Northern China with the addition of 7 isolates from NW and SC. Most isolates (86 of the 100) in Cluster I belong to the clonal lineage CN01 but 10 additional genotypes were identified. Isolates in cluster I have the A1 mating type and the mtDNA haplotype IIa with the exception of isolate HLJ05-NL1 which has the Ia haplotype. Cluster II consists of isolates from Yunnan and a few isolates from Sichuan and represents 8 distinct genotypes, none of them were found in large numbers. Isolates of Cluster II all have the A1 mating type and the Ia haplotype. Two isolates from Yunnan (YN07-501805 and YN07-401204) have a distinct genotype with different alleles at five different loci. One genotype representing four Sichuan isolates is also clearly distinct. The two isolates from Sichuan in this cluster (SC07-M0102 and SC07-M0103) were sampled in the same county.

Cluster III consists of isolates from Sichuan and a few isolates from NW with A2 and Ia. Nearly all isolates (67 of the 69) belong to the same clonal lineage CN02. They were collected from different years and fields in the mountain rich areas of Sichuan Province. Three Dutch isolates belonging to the "Blue_13" clonal lineage (NL05246, NL05238 and NL05147) that were included in the analysis grouped among the sub-clones of CN02 in Cluster III and one isolate even had the exact same genotype as one isolate (SC09-405) from Sichuan demonstrating that CN02_06 is in fact part of the sub-clonal lineage that includes "Blue_13". Also the mating type (A2) and the haplotype (Ia) fit with this genotypic profile.

Cluster IV groups all isolates from Fujian derived from both, potato and tomato. All isolates from cluster IV have the IIb haplotype and the A1 mating type. In this cluster all the isolates show a nearly identical genotype with the exception of the absence of alleles in specific loci and some variation in the alleles for G11 and PinfSSR4 and should therefore be considered to be sub-clones. The isolates collected in Fujian show a high level of subclonal variation. Only five SSRs (Pi4B, Pi63, Pi70, PinfSSR2 and PinfSSR6) show the same profile in all isolates, the other five polymorphic SSRs show 15 different alleles, 11 of whom have less than 11% variation. Eight isolates were obtained from potato plants whereas the rest of the Fujian isolates originated from tomato. In Fujian, the *P. infestans* isolates collected from potato and tomato are genetically similar (Fig. 3). The isolates collected from potato

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did not cluster in a particular clade, but were found to be dispersed among the majority of isolates collected from tomato.

Population structure

The genetic structure was analyzed with the model-based clustering algorithm implemented in the STRUCTURE 2.2 software. To avoid redundancy in the collection, we ordinated the data with the genotype correction, keeping only one isolate when more than one isolate were identified with the same SSR genotype at all loci. Thus, we ran the analysis of the genetic structure with 69 isolates. The Evanno *et al.* (2005) correction of the STRUCTURE 2.2 output was used (Fig. 4). For all K, memberships were consistent between all runs. The first peak of ΔK, for K=3, corresponded to the presence of three main groups. When individual isolates with a membership lower than 70% were not taken into account, two Sichuan isolates were misclassified SC09-424 and SC09-425. Consistent with this, these two isolates are also clearly separated from the main group in cluster III with bootstrap values of 90% in the phylogram (Fig. 3).

One STRUCTURE group contains the isolates from Fujian (shown in blue in Fig. 4), while the rest of the isolates are divided in two other groups colored in red (containing the isolates of phylogenetic Clusters I and II) and green (containing the isolates of the phylogenetic Cluster III). The results indicated that almost all isolates (>90%) had a high membership to their own group (>90%), indicating that there has been little or no gene flow between these three groups.

To validate the genetic structure identified by phylogeny and STRUCTURE, PCA was conducted. Plotted on the first two dimensions of PCA, the four clusters previously detected by the phylogeny analysis (Fig. 3) were adequately separated from each other (Fig. 5). In Fig. 5, Cluster I is close to Cluster II. In the phylogeny tree, the isolates from Yunnan grouped with isolates from the Sichuan cluster (IV) and Sichuan isolates with identified genotype were within the Yunnan cluster (II), while falling into the corresponding cluster in the PCA plot.

Fig.2. Genotype frequency of Chinese isolates. A, the 69 genotypes are grouped into 7 regions; B, each column presents a sampling regions.

HLJ00-B15
HLJ01-220
HLJ04-DA04-3
HLJ05-W22
HLJ05-W33
HLJ06-HH13
HLJ06-HL110
HLJ06-NH71
HLJ06-SH50
HLJ08-244
JL03-277
JL03-278
IM02-253
IM02-262
IM02-265
IM02-270
IM03-274
IM09-336
IM97-214
IM9.1
IM8.1
IM98-213
IM98-212
IM98-223
IM98-222
IM98-225
IM98-224
IM98-240
IM98-226
HLJ05-W13
IM99-217
IM99-216
HB01-W2
IM99-238
HB07-W0501
HB06-W0302
HB08-170
HB08-169
HB07-W0704
HB07-W0702
HB08-173
HB08-171
HB08-277
HB08-176
NX09-367
HB08-278
IM5.1
IM3.4
IM3.2
IM09-337
IM6.3
66 IM6.1
IM6.5
59 HLJ08-230
HLJ08-250
75 IM1.3
HLJ06-QG72
HB07-W0602
HLJ04-H1
IM2.4
IM2.2
HB07-Ch0301
66 HB07-ch0101
HB07-W0301
HB07-W0101
HB07-ch1001
HB07-ch0604
HLJ06-HH26
IM02-268
IM02-267
IM03-287
IM02-272
IM98-201
IM7.3
IM7.2
53 IM7.1
IM99-234
IM99-233
IM99-237
IM99-236
IM99-231
IM98-210
IM98-207
IM98-204
78 IM98-202
HLJ00-W1
IM03-281
IM03-279   *
82 SX02-256
IM03-283
IM03-282
HLJ08-222
HLJ08-219   *
HB06-W0201
HB06-W0102
66 SC09-401   *
SC09-399
SC09-398
HLJ08-223   *
97 HLJ06-QG51
HLJ04-HL043
GS09-357
89 HLJ06-KS632   *
HLJ06-KS622
HLJ05-NL1
63 ShX09-377
IM09-335

Cluster I
A1
IIA

SC09-432
100 SC09-431
SC09-434
SC09-433
80
73 YN07-501805
YN07-401204
YN07-4032072
YN05-XH05-5-4
YN07-400407
YN07-5032052
53
89 SC07-W0103
SC07-W0102
YN07-500504
YN07-404906
YN05-LSX18

Cluster II
A1
IA

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SC07-S0401
SC07-Pt0203
SC07-X0103
SC07-S0902
SC07-X0315
SC07-X0123
SC07-X0123
SC07-X0121
SC09-323
SC09-321
SC09-328
SC09-324
SC09-320
SC09-309
SC09-306
SC09-297
SC09-410
SC09-409
SC09-415
SC09-412
SC09-421
SC09-420
SC09-419
SC09-416
SC09-402
SC09-400
SC09-408
SC09-407
SC09-397
SC09-395
SC09-394
SC09-393
SC09-426
SC09-422
SC09-428
SC09-427
YN09-285
SC09-CQ390
SC09-CQ389
SC09-430
ShX09-382
ShX09-379
NX09-371
NX09-370
NX09-365
SC07-Pt0104
SC07-Pt0103
SC07-Pt0109
SC07-Pt0108
SC07-Pb0104
SC07-P20225
SC07-P20216
SC07-P20128
SC09-417
YN09-288
GZ09-346
GZ09-345
ShX09-381
ShX09-378
NL05246
SC09-392
NL05238
SC09-405
YN05-d5-2-2
SC09-303
SC09-CQ388
SC09-429
NL05147
SC09-404
SC09-403
SC09-391
SC09-424
SC09-425

**Cluster III
A2
IA**

100

82
65
79
57
67
97
90

FJ08-200
FJ06-t80
FJ07-t89
FJ08-197
FJ07-209
FJ07-203
FJ08-182
FJ08-182
FJ08-205
FJ08-207
FJ09-208
FJ02-p86
FJ02-p76
YN09-291
FJ05-t82
FJ05-t79
FJ06-t85
FJ06-201
FJ05-t88
FJ04-211
FJ02-t81
FJ02-p83
FJ07-t84
FJ07-t78
FJ07-t87
FJ06-t77
FJ06-t75
FJ07-198
FJ07-187
FJ08-178
FJ08-178
FJ07-183
FJ06-199
FJ09-193
FJ07-183
FJ08-186
FJ08-186
FJ09-195
FJ09-194
FJ08-181
FJ08-181
FJ07-190
FJ07-184

**Cluster IV
A1
IIB**

95
52
81
77

VK1.4

66

Fig. 3.1 and 3.2. The clustering tree of Chinese isolates based on the SSR data. The genetic distance estimation was performed according to Nei and Li (1979) and clustering was performed using Neighbor Joining. Bootstrap values in percentage (>50%) from 1000 replicate trees are shown at the nodes. The dark lines on the left side indicate the main clonal lineages. Asterisks indicate genotypes that do not belong to the main clonal group of the region and should be regarded as recent migrants or less successful lineages. The mating type and haplotype are shown on the right. The tree was rooted with VK1.4 a US-1 genotype originating from the Netherlands.
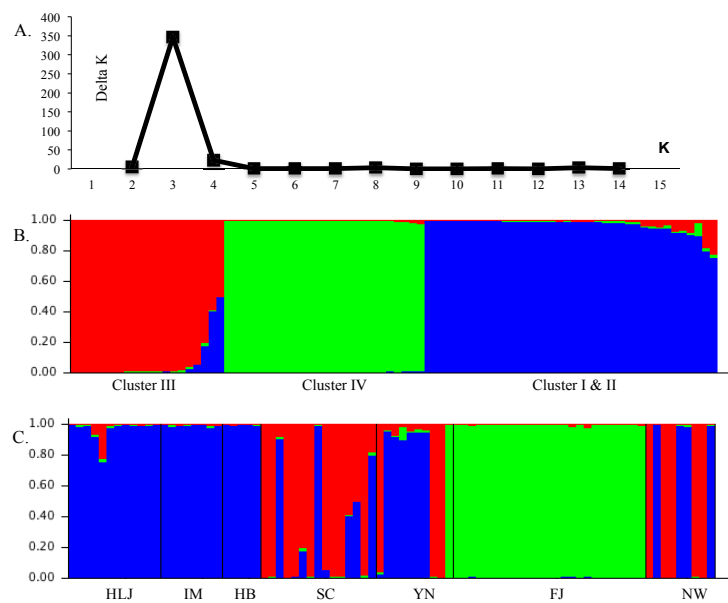
Fig. 4. STRUCTURE analysis of 9 microsatellite loci after clone correction. (A) Result of the ΔK calculation, the second order rate of change of LnP(D) with respect to K (1 to 15); ΔK for K=2-14 revealed a single distinct peak at K=3; (B) Plot example of the raw STRUCTURE output for one run (K=3) organized by the Q value.
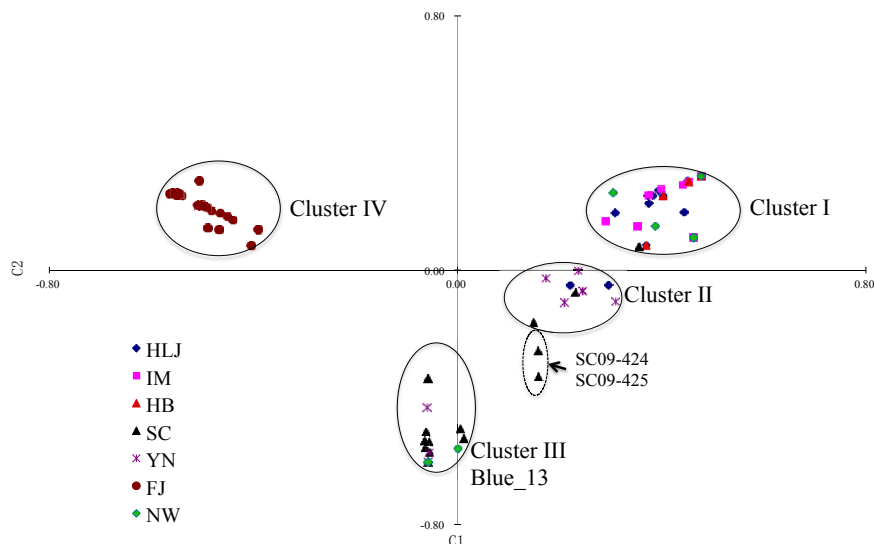
Fig. 5. Two-dimensional plot (X=PC1, Y=PC2) of a principal components analysis. Symbols represent the different sampling regions.

Mating analysis

For 114 viable isolates the mating type could be determined (Fig. 1). Allisolates produced oospores either with the A1 or A2 tester and no selfing isolates were found. Both mating types were found among the sampled isolates. The A1 mating type predominates and was found in all sampled provinces. Isolates from Heilongjiang, Inner Mongolia, Hebei and Fujian only had the A1 mating type. In Yunnan, Sichuan, Ningxia and Shanxi, both mating types were found. However, the frequency of the A2 mating type contrasted strongly between Yunnan and Sichuan. In Yunnan the frequency of A2 isolates was 10% (1 of 10, Table S), whereas the frequency of A2 isolates in Sichuan was 91% (21 of the 23) (Table S).

To confirm the mating potential of isolates with opposite mating types in China, nine cross combinations were performed. These crosses included both isolates originating from the same province as well as isolates from the Northern and the Southern part of China (Table 3). High densities of oospores were observed in all mating combinations tested (Fig. 6, Table 3).

Virulence analysis

The virulence spectrum was determined for a selection of 21 isolates representing the different SSR clonal lineages found. With the exception of the *R2* differential clone, that showed high variation between replicate virulence assays, all virulence scorings were consistent between the eight leaflet observations. Because of the variation in the results on the *R2* differential, these data were excluded from further analysis. Isolates selected from Cluster I and Cluster II, including representatives from Inner Mongolia, Heilongjiang, and Hebei, Yunnan and one isolate from Sichuan (SC07-M0102), all had the same virulence pattern (1, 3b, 4, 7, 10,11) with no virulence to *R5*, *R6*, *R8* and *R9*. Isolates from Cluster III largely comprised of sub-clones of CN02, showed complex virulence patterns (1, 3b, 4, 6, 7, 10, 11) with occasional virulence on potato lines that carry R5, R8 and R9. Isolate SC07-S0902 (CN02_01) had the most complex virulence pattern (1, 3b, 4, 5, 6, 7, 8, 9, 10, 11). Isolate YN-d5-2-2 from Yunnan, but grouped in Cluster III (CN02_08), had a complex virulence pattern (1, 3b, 4, 6, 7, 10, 11) as well as other Cluster III isolates. Genotype Cluster IV with isolates from Fujian showed a less complex virulence pattern. IsolatesFJ06-t77 and FJ07-t78 with an identical genotype also had the same virulence pattern (1, 3b, 4, 7), but FJ07-t82showed no virulence to potato lines carrying *R3b*. No virulence on *R10* and *R11* was detected among the isolates tested from Fujian. In general the Fujian isolates showed a lower virulence complexity than isolates from other regions and also appeared less aggressive on the susceptible cv Bintje.

All known virulence factors (*R1-R11*) were found among tested isolates (Table 2). The virulence between the different isolates varied between 3 and 10 factors, disregarding the results of differential *R2*. The virulence spectrum differed strongly per region. In isolates derived from Inner Mongolia, Heilongjiang, Hebei and Fujian Provinces no virulence was found for the R-gene differentials *R5, R6, R8* and *R9*. The virulence pattern correlates strongly with the genotype and shows high complexity with regional differences. The virulence patterns of the reference isolates 89148-9, IPO428-2 and H30P04 were as reported previously (Drenth *et al.*, 1995, Huang *et al.*, 2004).

The reference Chinese isolates for three main clonal lineages

To name the clonal lineages consistently following a standard nomenclature in the future studies, we recommend a specified reference isolate for each main clonal

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lineage. Our reference isolates were selected based on the total amount of information available (Table S).
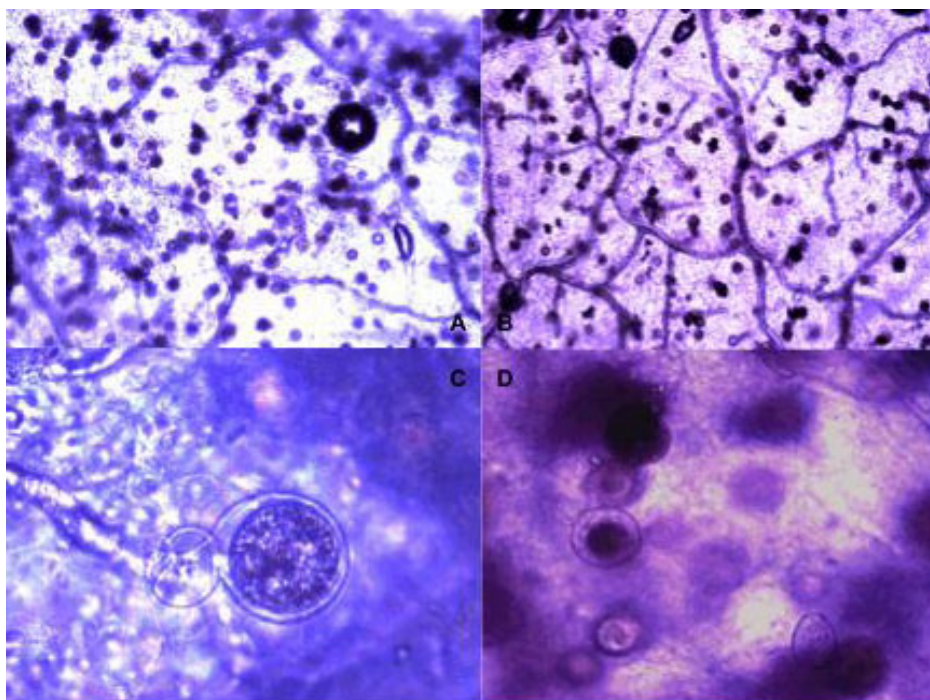
Fig. 6. Oospore formation *in planta* after a cross between selected Chinese *P. infestans* isolates. Fig. 6A, Cross YN05-d5-2-2 x YN07-501805; Fig.6B, Cross YN05-d5-2-2 x SC07-M0102; Fig. 6C, Cross YN05-d5-2-2 x YN07-500504; Fig. 6D, Cross SC07-M0102 x SC07-Pt0109.

Table 2. Virulence assay on a selection of 21 Chinese and three reference isolates using a differential set of 11 potato clones and the susceptible cv Bintje. The clusters I, II, III and IV are based othe phylogenetic tree of Chinese isolates shown in Fig. 4.

	Host	Location	Isolate	Bintje	Desiree	R0	R1	R3b	R4	R5	R6	R7	R8	R9	R10	R11
ClusterI	potato	IM	IM 07-6.1	+	+	+	+	+	+	-	-	+	-	-	+	+
	potato	IM	IM 07-3.4	+	+	+	+	+	+	-	-	+	-	-	+	+
	potato	IM	IM 07-6.5	+	+	+	+	+	+	-	-	+	-	-	+	+
	potato	IM	IM 07-7.3	+	+	+	+	+	+	-	-	+	-	-	+	+
	potato	HLJ	HLJ06-KS632	+	+	+	+	+	+	-	-	+	-	-	+	+
	potato	HLJ	HLJ 06-HH13	+	+	+	+	+	+	-	-	+	-	-	+	+
	potato	HLJ	HLJ 06-KS622	+	+	+	+	+	+	-	-	+	-	-	+	+
	potato	HB	HB07-W0501	+	+	+	+	+	+	-	-	+	-	-	+	+
	potato	HB	HB07-W0602	+	+	+	+	+	+	-	-	+	-	-	+	+
	potato	HB	HB07-ch0301	+	+	+	+	+	+	-	-	+	-	-	+	+
ClusterII	potato	YN	YN07-500504	+	+	+	+	+	+	-	-	+	-	-	+	+
	potato	YN	YN05-LSX18	+	+	+	+	+	+	-	-	+	-	-	+	+
	potato	SC	SC07-M0102	+	+	+	+	+	+	-	-	+	-	-	+	+
ClusterIII	potato	YN	YN05-d5-2-2	+	+	+	+	+	+	+	+	+	-	-	+	+
	potato	SC	SC07-S0401	+	+	+	+	+	+	+	+	+	-	+	+	+
	potato	SC	SC07-X0103	+	+	+	+	+	+	+	+	+	-	+	+	+
	potato	SC	SC07-Pt0109	+	+	+	+	+	+	-	+	+	-	+	+	+
	potato	SC	SC07-S0902	+	+	+	+	+	+	+	+	+	+	+	+	+
ClusterIV	tomato	FJ	FJ06-t77	+	+	+	-	-	+	-	-	+	-	-	-	-
	tomato	FJ	FJ07-t78	+	+	+	+	-	+	-	-	+	-	-	-	-
	tomato	FJ	FJ07-t82	+	+	+	-	-	+	-	-	-	-	-	-	-
			89148-9	+	+	+	-	-	-	-	-	-	-	-	-	-
			IPO428-2	+	+	+	+	+	+	-	-	+	(+)	-	+	+
			H30P04	+	+	+	-	+	-	-	-	+	-	-	+	+

Table 3. Artificial crossing experiments between Chinese isolates

Combination	A1 isolate	A2 isolate	Oospores
1	YN07-501805	YN05-d5-2-2	V
2	YN07-500504	YN05-d5-2-2	V
3	HLJ06-HH13	YN05-d5-2-2	V
4	IM07- 6.1	YN05-d5-2-2	V
5	YN05-LSX18	YN05-d5-2-2	V
6	SC07-M0102	YN05-d5-2-2	V
7	SC07-M0102	SC07-S0902	V
8	SC07-M0102	SC07-X0103	V
9	SC07-M0102	SC07-Pt0109	V

DISCUSSION

The Chinese *P. infestans* population shows multiple genotypes and clear genetic substructure correlated with the region of sampling

The genetic diversity reported here is not consistent with thenationwide admixed population reported previously (Montarry *et al.*, 2010). Nevertheless, the number of genotypes identified in this study exceeds those previously observed (Guo *et al.*, 2009) and it is clearly shown that different genotypes can be found in all regions, including Northern China which is different from what was reported previously (Guo *et al.*, 2009). The reasons for these differences most likely are the use of robust genetic markers and a larger number of isolates. Our analysis presents the first population study for *P. infestans* in China, using a high-resolution set of ten SSR markers and a wide sampling strategy including isolates from North, South, East and West China. The phylogenetic hypothesis of a nationwide admixed population was falsified by the model-based clustering method and PCA analysis.

Isolates from Fujian clustered as a clearly distinct group in the PCA with a wide range of sub-clonal variation, indicating that *P. infestans* in Fujian Province was isolated from *P. infestans* populations from other regions for a long time. The Fujian *P. infestans* population did not show sub-populations with respect to the different hosts, potato and tomato. Our observation contrasts results obtained by Lebreton and Andrivon (Lebreton & Andrivon, 1998) and others who reported that tomato and potato isolate collections showed clear differences in the frequency of genotypes

(Oyarzun *et al.*, 1998, Knapova & Gisi, 2002, Jyan *et al.*, 2004). Interestingly, we noted that isolates from Fujian were less aggressive on potato compared with the isolates from other provinces in our differential tests on potato, which could indicate a specialization towards tomato of these isolates. A recent *P. infestans* study reported the same rare IIb haplotype, found in Fujian, was also observed in Taiwan (Jyan *et al.*, 2004). As Fujian and Taiwan frequently exchanged agricultural products, it is hypothesized that these isolates from Fujian and Taiwan could have a common origin. However, more research on the genotypes found in Taiwan is required to confirm this hypothesis. Potato transport from other Chinese provinces to Fujian could result in the migration of more aggressive potato isolates and aggravate late blight problems for potato growers in Fujian. We hypothesize that the Fujian population is an older, less aggressive clonal lineage. This population survived in isolation possibly on tomato as its main host.

The European dominant strain "Blue_13" in China

A new A2 strain, called "Blue_13" with metalaxyl resistance was recently confirmed to take over a dominant position in the *Phytophthora population* in the UK, the Netherlands, and other European countries (Cooke *et al.*, 2009). This new *Phytophthora* strain is more aggressive. Our study is the first report of this aggressive strain outside Europe. Also, we found that the Chinese "Blue_13" clonal lineage (CN02) included several novel genetic variants. CN02/"Blue_13" firmly established itself in China in recent years. In Sichuan Province it was found from 2007 and we also identified one isolate from Yunnan that was collected in 2005. With the current data it was not possible to trace back when CN02/"Blue_13" established itself in China and where it came from. Sichuan and Yunnan Province are mountainous areas, farms are mostly small. Farmer-saved potato seed is common in Sichuan, which may provide a special niche for *P. infestans*. Official reports do not show any import of seed potato from abroad since 2000. It raises a question about the origin and spread of CN02/"Blue_13" in China. Was it present in the earlier isolates in Sichuan or other Chinese regions and perhaps migrated to Europe? Or does it originate from neighboring countries and migrate from there both to Europe and China. Fortunately, adequate data sets allowing these comparisons could be available in the near future by an internationally shared database. Now an European scale-based database developed

by the European Concerted Action on Blight with 37 European partners (http://www.euroblight.net), is heading to a broader international scale.

The identification of sub-clonal variation in China

Using ten highly informative SSR markers, it was possible for the first time to obtain identification with a high resolution of the sub-clonal variation among the larger number of Chinese isolates. The clonal lineage identified by Guo *et. al.,* 2009 (Guo *et al.*, 2009) is still dominantly present in Cluster I of the phylogenetic tree. Within Cluster I, some sub-clones were identified with genetic variation mostly characterized by the lack of alleles of specific SSR loci. The genotyping result did show some evidence for long-distance migration of *P. infestans* between the Northern, Xibei region and Sichuan province, with very few migrants. The major genotypes in Cluster I showed rare alleles in at least three different SSR loci. This can be the result of sub-clonal variation (Lees *et al.*, 2009). Sub-clonal variation, by occasional rare alleles and the loss of alleles in specific loci, was found in all regions in China. Consistent with the phylogeny analysis, in the STRUCTURE analysis, some evidence was found for gene flow or admixture between Yunnan isolates and Sichuan isolates. However, this may also indicate recent divergence between Cluster II and III assuming a common ancestry.

The sexual cycle is rare and sexual recombination seems low

In the previous study, the frequency of the A2 mating type in Northern China (Inner Mongolia and Hebei) was much higher than in Southern China (Sichuan and Yunnan) (Zhang *et al.*, 2001). However, in this study, we did not find any isolate with the A2 mating type in Northern China, corroborating previous studies reporting the decline of the A2 mating type in China (Li *et al.*, 2009) and the absence of the A2 mating type in Northern China (Guo *et al.*, 2009). Although, the presence of both mating types was observed (Zhang *et al.*, 1996), only Zhao *et. al.,* 2001 (Zhao *et al.*, 2001) reported the presence of oospores under field conditions in one single sample in 2000. While from the same field, self-fertile isolates were obtained. Crossings performed *in planta* in the present study yielded high densities of oospores in potato leaves for all combinations tested, indicating that Chinese isolates could initiate a sexual cycle, but a further progeny germination test is required to indicate that no post-zygotic mating barriers are present. Even if these barriers do not exist, the chance of physical contact between the A1 and A2 mating type is low as we only found both mating types in Xibei and

Southern parts of China, always with a big disparity of the mating type ratios. In addition, the high level of linkage disequilibrium indicates a strong preference for a clonal spread in China.

Complex virulence patterns are associated with the region of origin

The virulence pattern of 21 representative *P. infestans* isolates on the *R1-R11* differential set of potato lines shows a high complexity with regional differences. In this study, the virulence pattern fits well with the SSR genotyping of the clonal lineages, which contrasts to previous studies (Guo *et al.*, 2009, Knapova *et al.*, 2002). The resolution of the SSR markers in this study was much higher than in these previous studies and in our study the markers allowed the distinction in clear subgroups. Since virulence is genetically inherited (Van der Lee *et al.*, 2001) one would expect that with enough isolates and sufficient resolution in the genotyping, the clonal isolates identified would show identical or at least highly similar virulence patterns. Although, the correlation between a specific genotype and its virulence profile cannot be expected to be absolute, the relative stability of virulence of a clonal lineage could help in the virulence prediction of new isolates that belong to previously identified clonal lineages. The 21 isolates tested will not elucidate the correlation but it can provide evidence for a correlation between genotype and virulence profile. Unfortunately, the isolates from the previous study by Guo *et. al*., 2009 (Guo *et al.*, 2009) cannot be tested, as these isolates were not stored as viable cultures. Therefore, the discrepancy between our study and the study performed by Guo *et. al.,* 2009 (Guo *et al.*, 2009) on the correlation between genotype and virulence spectrum cannot be resolved.

A few *R* genes from *S. demissum* (*R*5, *R*6, *R*8 and *R*9) could still be effective within some regions. However as was found for isolates with the "Blue_13" genotype in Europe, the isolates with the CN002/"Blue_13" genotype have a highly complex virulence pattern. SC07-S0902 (CN02_01) is virulent on all *S. demissum R* genes tested and some isolates with complex virulence patterns were found previously in Yunnan and Inner Mongolia during earlier surveys (Zhijian *et al.*, 2007, Jun *et al.*, 2007). The race structure within Yunnan and Inner Mongolia was similar as previously reported (Yuqi *et al.*, 1996, Jun *et al.*, 2007, Zhijian *et al.*, 2007), especially in Northern China. For some regions deployment of *R*5, *R*6, *R*8, *R*9 and possibly *R2* could still be useful to exploit the regional differences in virulence for resistance management.

However, we suggest that resistance breeding should be primarily based on selection of new broad spectrum *R*-genes that can be selected based on their effectiveness to the isolates we collected from the most important potato provinces.

To conclude, the ten SSR markers have uncovered a spatial structure consistent with the model of a meta population for the Chinese *P. infestans* population. Using the current SSR set, it is now possible to combine and compare genotyping data from different laboratories. Our results show that such a global approach is useful; we found the "Blue_13" genotype in China using the genotyping data of three "Blue_13" variants from the Netherlands. To our knowledge this is the first report of "Blue_13" outside Europe, opening up new possibilities on tracking the world wide migration pattern of this aggressive strain. The survey described in this study provides a good basis for future studies to understand the dynamics of the national population structure in China. A national survey with deeper sampling could explore the spatial structure and gene flow of *P. infestans* in China in more detail. In addition our results imply that the transportation of potato and tomato commodities and in particular the transport of seed potato's from Sichuan to the Northern or Western part of China and vice versa will increase the risk of convening the two mating types, creating more possibilities for the sexual cycle. This potentially will boost the genetic variation and result in the formation of persistent oospores, which could have a dramatic impact on the epidemiology of late blight in China. Continued genotyping of *P. infestans* will be necessary to trace the movement and diversification of the lineages and to identify new dominant genotypes, newly introduced lineages, or recombinant genotypes.

MATERIALS AND METHODS

Sampling locations

Sampling intensity varied by year and regiondepending on late blight outbreaks and reporting from the farmers. Sichuan is a mountainous region and although the sampling position on the national map seem nearby, they are often separated by mountain barriers and the traveling distance between sampling locations is at least 40 km. Isolates from Yunnan Province each represent one infected field, whereas the samples from Kunming include multiple isolates per field. Samples from Fujian were collected from potato and tomato hosts. As reference, isolates collected before 2004 from potato in Fujian and other provinces, were included.

Isolate sampling and long-term storage

Single-zoospore isolates of *P. infestans* were obtained through local universities and research institutes. Local scientists visited the infected fields with farmer consent to collect infected potato leaves.

Sampling was generally done between the 1st of June and the 1st of October.

Potato leaves with a single lesion were collected from the fields and placed in plastic bags or in 9cm Petri dishes containing 1.5 % water agar and incubated until sporulation at 15°C at a light intensity of 12 Wm^{-2} for 16 hours. Infected leaves were sectioned into 0.5 cm^2 pieces that were individually placed underneath tuber slices inside an otherwise empty 9cm Petri dish. After 5-7 days at 20°C, mycelium emerging from the top of the tuber slice was transferred to Pea Agar (PA) with ampicillin (Shattock *et al.*, 1990, Goodwin *et al.*, 1992a). One isolate per infected leaf was maintained. In total, 188 new isolates were obtained from the field and cultured (Table S).

For long-term storage the isolates were grown for 1-2 weeks on PA at 20°C. Five agar plugs with fresh mycelium were transferred to a cryotube vial (1.8 ml) after which 1.5 ml 15% sterile Dimethylsuloxid (DMSO) was added. Within an hour, the filled vials were frozen at -80°C for 24 hours. After this pre-freezing step, the vials were quickly transferred to a liquid nitrogen storage tank.

Determination of Mating Types

An agar plug obtained from the edge of an actively growing colony of the test isolate was transferred to one side of a PA containing Petri dish and a mycelium plug of an A1 tester (isolate VK98014) or an A2 tester (isolate EC3425) was placed on the other side of Petri dish. Plates were incubated in the dark for 14-21 days at 18°C. After mycelial contact between both colonies was established, the contact zone was monitored for the presence of oospores regularly during 7 consecutive days using a microscope at 100x magnification. When oospores were found in the Petri dish with the A1 tester isolate the unknown isolate was classified to have the A2 mating type and vice versa. Due to the difficulties to preserve living isolates in hot summers under basic laboratory conditions in the local institutes, only part of the collection survived long enough to perform a mating type test (Table S).

Preparation of sporangial suspensions

An agar plug obtained from the edge of an active colonygrowing on PA medium was placed underneath a potato tuber slice inside an otherwise empty 9 cm Petri dish. Inoculated tuber slices were incubated for 6-8 days in a climate chamber (15°C at a light intensity of 12 Wm^{-2} for 16 hours a day). *P. infestans* mycelium, growing through the tuber slices, was transferred to a single drop of water, which was placed on the adaxial (upper) side of a potato leaf, cv Bintje, inside a 9cm Petri dish containing 1.5% water agar. Inoculated leaves were incubated for 6-8 days in a climate chamber using the conditions described above. Following incubation, sporulating potato leaves were rinsed gently in tap water to liberate sporangia. Sporangial suspensions were adjusted to 1×10^4- 2×10^4 sporangia per ml to serve as inoculum for the detached leaf virulence assays.

Virulence assays

To generate reliable virulence profiles for the isolates, virulence assays were replicated three times. A total of 21 isolates representing all available SSR genotypes and harboring isolates of six different geographic origins (Table 2) were selected for virulence assays. This representative selection included isolates within and among clonal subgroups to study the relationship between genotype and virulence pattern. Virulence phenotypes were determined using a detached leaf assay employing a combination of Black's and Mastenbroeks differential sets for *Solanum demissumR*-genes *R1* to *R11*; *R1* (Mastenbroek 43154-5), *R2* (Mastenbroek 44158-4), *R2* (Black 1512c), *R3b* (Mastenbroek 4642-1), *R4*

(Mastenbroek 4431-5), *R5* (Black 3053-18), *R6* (Black XD2-21), *R7* (Black 2182ef(7)), *R8* (Black 2424a(5)), *R9* (Black 2573), *R10* (Black 3618ad(1)), and *R11* (Black 5008ab(6)) (Black *et al.*, 1953) and potato cv Bintje as a susceptible control. Each experiment contained two leaflets per differential x isolate combination per Petri dish and two Petri dishes per replicate experiment. Leaflets were inoculated by spraying them with a sporangial suspension of $2x10^4$ sporangia per ml of the appropriate *P. infestans* isolate. Petri dishes containing the inoculated leaflets were incubated at 15°C and a 16h/8h light/dark regime. Infection was assessed of severity per leaflet visually after one week incubation as described by the protocol from Plant Research International in the Netherlands (Flier & Turkensteen, 1999).

In Planta production of oospores

Ten isolates from different provinces were selected for *in planta* crossing test (Table 3). Sporangial suspensions of an A1 and an A2 mating isolate ($1x10^4$ sporangia ml^{-1}), produced as described above, were prepared and mixed in equal amounts. This suspension was then cooled for at least 30 min at 4°C to allow liberation of zoospores. Meanwhile, leaflets or leaves of cv Bintje were placed abaxial (lower) side up in 140 mm Petri dishes containing 1% water agar (WA). The mixed sporangial suspension was sprayed onto the potato leaflets using a spraying nozzle (pressure is 0.5 kg.m^{-2}) until the leaves were completely covered with small droplets. Petri dishes were placed in a plastic tray on wet filter paper and wrapped in a transparent plastic bag to prevent dehydration. The inoculated leaves were incubated at 15°C in the dark for one day followed by at least two weeks at 15°C and a light intensity of 12 Wm^{-2} for 16 h per day. In case of dehydration, after one week the leaves were sprayed with tap water. Presence of oospores was assessed visually following incubation using a binocular microscope (Leica, Germany) at 100x magnification.

DNA extraction

Agar plugs of each individual *P. infestans* isolate were taken from the edge of a seven-day old actively growing colony on PA and transferred to liquid pea broth. After 3-4 days incubation at 20ºC in the dark, sufficient mycelium was available for DNA extraction. Genomic DNA was isolated from 20mg lyophilized mycelium using the DNeasy 96 Plant Kit (Qiagen, Germany) following the manufacturer's instructions and eluted in 200µl ultra-pure water. DNA extracts were stored at -20ºC until further use.

Haplotype test

Mitochondrial haplotypes were determined using the PCR-RFLP method of Griffith & Shaw (Griffith & Shaw, 1998). Restriction digestions of the amplified regions P2 (*MspI*) and P4 (*EcoRI*) allowed the differentiation of four mitochondrial (mtDNA) haplotypes Ia, Ib, IIa and IIb.

Microsatellite genotyping

Ten microsatellite markers were used in this study for the genotypic study. Markers used were Pi4B and G11 (Knapova & Gisi, 2002), Pi63 and Pi70 (Lees *et al.*, 2006) and PinfSSR2, 3, 4, 6, 8, 11 (Li *et al.*, 2010). The forward primers of 9 markers and one reverse primer of PinfSSR6 were labeled with VIC, FAM, NED and PET (Applied Biosystems, Table 1). Amplification reactions were as described previously (Li *et al.*, 2010), with some minor modifications. Amplifications were run in a PTC200 thermocycler (MJ Research, Waltham, Massachusetts, USA), with an initial denaturation at 95°C for 15 min, followed by 30 cycles of 95°C for 20 sec, 58°C for 90 sec, and 72°C for 60 sec, and a final

extension at 72°C for 20 min. 1-2 µl of the PCR product was added to 1 µl de-ionized formamide loading buffer and denatured at 92°C for 3 min. The resulting amplification products were sized by capillary electrophoresis on an automated ABI 3730 using the molecular standard GeneScan-500 ROX and scored using GeneMapper 3.7 software (Applied Biosystems). To facilitate scoring and set up of an international database, a total of 11 reference isolates were used, including eight regular reference isolates for SSR analysis (T30-4, 80029, 88133, VK1.4, 90128, IPO-0, IPO428-2, VK98014, (Li *et al.*, 2010)) from Europe and three "Blue_13" genotype variants (NL05246, NL05238 andNL05147).

Data analysis

Based on the SSR data, pair wise comparisons of genetic distances were calculated using Nei's formula (Nei, 1972). A genetic distance matrix was established and subsequently used to construct a dendrogram based on the Neighbour Joining (NJ) clustering procedure. The SSR data were analyzed using the phylogenetic software package TREECON® for Windows Version 1.3b (Van de Peer & De Wachter, 1994). Bootstrapping was performed by using 1,000 bootstraps. The phylogenetic tree was generated using 229 isolates of *P. infestans* (Table S).

To estimate the diversity, polymorphism information content (PIC), the number of alleles per locus (Na), the observed heterozygosity (Ho), the expected heterozygosity (He) and deviations from the Hardy-Weinberg equilibrium (HWE) were determined. The significance of the deviations from the HWE were calculated with exact P values estimated using the Markov chain algorithm with 10,000 dememorization steps 100 batches and 1,000 iterations by using GENEPOP version 4.0 (Raymond & Rousset, 1995). To examine the distribution of genetic variation, analysis of molecular variance (amova; (Excoffier *et al.*, 1992) was performed using WINAMOVA (Excoffier *et al.*, 1992). Co-dominant SSR data were first converted to a binary data matrix by treating absence as "0" and presence as "1" of a defined allele. Raw data is pre-processed by Miller, Mark P.'s AMOVA-PREP (Miller, 1998), to generate the AMOVA input files. An AMOVA analysis was then performed with significant tests for 1,000 permutations to determine how the genetic diversity is partitioned within and between populations.

To examine the genetic structure of Chinese *P.infestans* isolates, the clustering program STRUCTURE 2.2 (Pritchard *et al.*, 2000) was run after clone correction (each genotype has one representative isolate). We specified the number of clusters (K) from 1-15, five independent runs were conducted to assess the consistency of the results across runs, and all runs were based on 100,000 iterations after a burn-in period of 100,000 iterations. We calculated the statistic ΔK, which indicates the highest level hierarchical structure in the population (Evanno *et al.*, 2005). To perform ΔK calculations, we randomly assigned the likelihood from each of five Structure runs from each K into one of five groups, each containing a single likelihood from each K. To validate the genetic structure, principle component analysis (PCA) (Rohlf, 1987, Rohlf, 2008) was conducted to construct plots of the most significant axes for grouping pattern verification (Gower, 1966).

Identification of sub-clones and naming system

The set of SSR markers used is highly informative and allows the identification of sub-clonal lineages within a population. Sub-clones were defined as nearly identical genotypes only separated from the main lineage by the absence of specific alleles in some SSR loci which could be due to asexual mitotic

recombination (Chamnanpunt *et al.*, 2001) or chromosomal deletions (Van der Lee *et al.*, 2001). In addition SSR loci G11 and PinfSSR4 are extremely variable, resulting in sub-clonal variation showing new alleles within an otherwise identical genetic background (Lees *et al.*, 2009, Li *et al.*, 2010). The naming of the clonal and sub-clonal genotypes starts with the two-letter country abbreviation (CN), followed by the ranking number of the clonal lineage in the study. For example, one main clonal lineage found in this study is CN01. The sub-clonal genotypes within this clonal are named CN01_01, CN01_02, … and so on.

ACKNOWLEDGEMENTS

170 x 240 mm

SUPPLEMENTARY MATERIAL

Table S. The origin of the *Phytophthora infestans* isolates

Isolate	Year	Province	Sampling County	Latitude	Longitude	Host	Mating Type	Haplotype	Sub-clonal name	Cite
HLJ00--B15	2000	Heilongjiang	Keshan	48°02'	125°52'	Potato	A1	IIA	CN01_01	Guo et al, 2010
HLJ00-V1	2000	Heilongjiang	wangkui	46°69'	126°29'	Potato		IIA	CN01_12	Guo et al, 2010
HLJ01-220	2001	Heilongjiang	Huma	51°43'	126°39'	Potato	A1	IIA	CN01_01	Guo et al, 2010
HLJ04-DA04-3	2004	Heilongjiang	Jiagedaqi	50°25'	124°06'	Potato	A1	IIA	CN01_01	
HLJ04-H1	2004	Heilongjiang	Keshan	48°01'	125°52'	Potato	A1	IIA	CN01_07	
HLJ04-HL043	2004	Heilongjiang	Hailun	47°27'	126°58'	Potato	A1	IIA		
HLJ05-NL1	2005	Heilongjiang	Keshan	48°01'	125°52'	Potato	A1	IA		
HLJ05-W13	2005	Heilongjiang	Haerbin	45°48'	126°32'	Potato	A1	IIA	CN01_02	
HLJ05-W22	2005	Heilongjiang	Haerbin	45°48'	126°32'	Potato	A1	IIA	CN01_01	
HLJ05-W33	2005	Heilongjiang	Hegang	47°20'	130°17'	Potato	A1	IIA	CN01_01	
HLJ06-HH13*	2006	Heilongjiang	Heihe	50°14'	127° 31'	Potato	A1	IIA	CN01_01	
HLJ06-HH26	2006	Heilongjiang	Heihe	50°14'	127° 31'	Potato	A1	IIA	CN01_09	
HLJ06-HL110	2006	Heilongjiang	Hailun	47°27'	126°58'	Potato	A1	IIA	CN01_01	
HLJ06-KS622	2006	Heilongjiang	Keshan	48°01'	125°52'	Potato	A1	IIA		
HLJ06-KS632	2006	Heilongjiang	Keshan	48°01'	125°52'	Potato	A1	IIA		
HLJ06-NH71	2006	Heilongjiang	Nahe	48°29'	124°53'	Potato	A1	IIA	CN01_01	
HLJ06-QG51	2006	Heilongjiang	Qinggang	46°41'	126°06'	Potato	A1	IIA		
HLJ06-QG72	2006	Heilongjiang	Qinggang	46°41'	126°06'	Potato	A1	IIA	CN01_06	
HLJ06-SH50	2006	Heilongjiang	Suihua	46°38'	126°58'	Potato	A1	IIA	CN01_01	
HLJ08-219	2008	Heilongjiang	Hailun	47°27'	126°58'	Potato		IIA		
HLJ08-222	2008	Heilongjiang	Hailun	47°27'	126°58'	Potato		IIA		
HLJ08-223	2008	Heilongjiang	Hailun	47°27'	126°58'	Potato	A1	IIA		
HLJ08-230	2008	Heilongjiang	Suihua	46°38'	126°58'	Potato	A1	IIA	CN01_04	
HLJ08-244	2008	Heilongjiang	Mudanjiang	44°33'	129°37'	Potato	A2	IIA	CN01_01	
HLJ08-250	2008	Heilongjiang	Haerbin	45°48'	126°32'	Potato	A1	IIA	CN01_04	

170 x 240 mm

ID	Year	Province	Location	Latitude	Longitude	Host			Code	Reference
JL03-277	2003	Jilin	Changchun	43°49'	125°19'	Potato	A1	IIA	CN01_01	Guo et al, 2010
JL03-278	2003	Jilin	Yanbian	42°53'	129°30'	Potato	A1	IIA	CN01_01	Guo et al, 2010
IM02-253	2002	Inner Mongolia	Fengzhen	40°26'	113°06'	Potato	A1	IIA	CN01_01	Guo et al, 2010
IM02-262	2002	Inner Mongolia	Liangcheng	40°31'	112°30'	Potato	A1	IIA	CN01_01	Guo et al, 2010
IM02-265	2002	Inner Mongolia	Liangcheng	40°31'	112°30'	Potato	A1	IIA	CN01_01	Guo et al, 2010
IM02-267	2002	Inner Mongolia	Huhhot	40°50'	111°44'	Potato	A1	IIA	CN01_10	Guo et al, 2010
IM02-268	2002	Inner Mongolia	Huhhot	40°50'	111°44'	Potato	A1	IIA	CN01_10	Guo et al, 2010
IM02-270	2002	Inner Mongolia	Shangtuhai	41°12'	111°17'	Potato	A1	IIA	CN01_01	Guo et al, 2010
IM02-272	2002	Inner Mongolia	Huhhot	40°50'	111°44'	Potato	A1	IIA	CN01_10	Guo et al, 2010
IM03-274	2003	Inner Mongolia	Huhhot	40°50'	111°44'	Potato	A1	IIA	CN01_01	Guo et al, 2010
IM03-279	2003	Inner Mongolia	Huhhot	40°50'	111°44'	Potato	A1	IIA		Guo et al, 2010
IM03-281	2003	Inner Mongolia	Huhhot	40°50'	111°44'	Potato	A1	IIA		Guo et al, 2010
IM03-282	2003	Inner Mongolia	Huhhot	40°50'	111°44'	Potato	A1	IIA		Guo et al, 2010
IM03-283	2003	Inner Mongolia	Huhhot	40°50'	111°44'	Potato	A1	IIA		Guo et al, 2010
IM03-287	2003	Inner Mongolia	Huhhot	40°50'	111°44'	Potato	A1	IIA	CN01_10	Guo et al, 2010
IM07-1.3	2007	Inner Mongolia	Xingan	46°04'	122°04'	Potato	A1	IIA	CN01_05	
IM07-2.2	2007	Inner Mongolia	Ximeng	42°55'	114°02'	Potato	A1	IIA	CN01_08	
IM07-2.4	2007	Inner Mongolia	Ximeng	42°55'	114°02'	Potato	A1	IIA	CN01_08	
IM07-3.2	2007	Inner Mongolia	Xingan	46°04'	122°04'	Potato	A1	IIA	CN01_01	
IM07-3.4	2007	Inner Mongolia	Xingan	46°04'	122°04'	Potato	A1	IIA	CN01_01	
IM07-5.1	2007	Inner Mongolia	Xingan	46°04'	122°04'	Potato	A1	IIA	CN01_01	
IM07-6.1	2007	Inner Mongolia	Aershan	47°10'	119°56'	Potato	A1	IIA	CN01_03	
IM07-6.3	2007	Inner Mongolia	Aershan	47°10'	119°56'	Potato	A1	IIA	CN01_03	
IM07-6.5	2007	Inner Mongolia	Aershan	47°10'	119°56'	Potato	A1	IIA	CN01_03	
IM07-7.1	2007	Inner Mongolia	Wumeng	40°59'	113°07'	Potato	A1	IIA	CN01_10	
IM07-7.2	2007	Inner Mongolia	Wumeng	40°59'	113°07'	Potato	A1	IIA	CN01_10	
IM07-7.3	2007	Inner Mongolia	Wumeng	40°59'	113°07'	Potato	A1	IIA	CN01_10	
IM07-8.1	2007	Inner Mongolia	Xingan	46°04'	122°04'	Potato	A1	IIA	CN01_01	
IM07-9.1	2007	Inner Mongolia	Humeng	45°12'	116°13'	Potato	A1	IIA	CN01_01	
IM09-335	2009	Inner Mongolia				Potato	A1	IIA		

Isolate	Year	Province	Location	Latitude	Longitude	Host			Code	Reference
IM09-336	2009	Inner Mongolia				Potato	A1	IIA	CN01_01	Guo et al, 2010
IM09-337	2009	Inner Mongolia				Potato	A1	IIA	CN01_01	Guo et al, 2010
IM97-214	1997	Inner Mongolia	Zhalantun	48°00'	122°44'	Potato	A1	IIA	CN01_01	Guo et al, 2010
IM98-201	1998	Inner Mongolia	Huhhot	40°50'	111°44'	Potato	A1	IIA	CN01_10	Guo et al, 2010
IM98-202	1998	Inner Mongolia	Huhhot	40°50'	111°44'	Potato	A1	IIA	CN01_11	Guo et al, 2010
IM98-204	1998	Inner Mongolia	Huhhot	40°50'	111°44'	Potato	A1	IIA	CN01_10	Guo et al, 2010
IM98-207	1998	Inner Mongolia	Huhhot	40°50'	111°44'	Potato	A1	IIA	CN01_10	Guo et al, 2010
IM98-210	1998	Inner Mongolia	Huhhot	40°50'	111°44'	Potato	A1	IIA	CN01_10	Guo et al, 2010
IM98-212	1998	Inner Mongolia	Hailaer	49°12'	119°44'	Potato	A1	IIA	CN01_01	Guo et al, 2010
IM98-213	1998	Inner Mongolia	Hailaer	49°12'	119°44'	Potato	A1	IIA	CN01_01	Guo et al, 2010
IM98-222	1998	Inner Mongolia	Taiqibaochang	41°54'	115°17'	Potato	A1	IIA	CN01_01	Guo et al, 2010
IM98-223	1998	Inner Mongolia	Baotou	40°39'	109°50'	Potato	A1	IIA	CN01_01	Guo et al, 2010
IM98-224	1998	Inner Mongolia	Baotou	40°39'	109°50'	Potato	A1	IIA	CN01_01	Guo et al, 2010
IM98-225	1998	Inner Mongolia	Yimengdaqi	39°36'	109°46'	Potato	A1	IIA	CN01_01	Guo et al, 2010
IM98-226	1998	Inner Mongolia	Huhhot	40°50'	111°44'	Potato	A1	IIA	CN01_01	Guo et al, 2010
IM98-240	1998	Inner Mongolia	Wumeng	40°59'	113°07'	Potato	A1	IIA	CN01_01	Guo et al, 2010
IM99-216	1999	Inner Mongolia	Zhalantun	48°00'	122°44'	Potato	A1	IIA	CN01_01	Guo et al, 2010
IM99-217	1999	Inner Mongolia	Zhalantun	48°00'	122°44'	Potato	A1	IIA	CN01_01	Guo et al, 2010
IM99-231	1999	Inner Mongolia	Huhhot	40°50'	111°44'	Potato	A1	IIA	CN01_10	Guo et al, 2010
IM99-233	1999	Inner Mongolia	Huhhot	40°50'	111°44'	Potato	A1	IIA	CN01_10	Guo et al, 2010
IM99-234	1999	Inner Mongolia	Huhhot	40°50'	111°44'	Potato	A1	IIA	CN01_10	Guo et al, 2010
IM99-236	1999	Inner Mongolia	Huhhot	40°50'	111°44'	Potato	A1	IIA	CN01_10	Guo et al, 2010
IM99-237	1999	Inner Mongolia	Huhhot	40°50'	111°44'	Potato	A1	IIA	CN01_10	Guo et al, 2010
IM99-238	1999	Inner Mongolia	Huhhot	40°50'	111°44'	Potato	A1	IIA	CN01_01	Guo et al, 2010
HB01-V2	2001	Hebei	Weichang	41°56'	117°45'	Potato	A1	IIA	CN01_01	Guo et al, 2010
HB06-W0102	2006	Hebei	Weichang	41°56'	117°45'	Potato	A1	IIA	CN01_01	
HB06-W0201	2006	Hebei	Weichang	41°56'	117°45'	Potato	A1	IIA		
HB06-W0302	2006	Hebei	Weichang	41°56'	117°45'	Potato	A1	IIA	CN01_01	
HB07-Ch0101	2007	Hebei	Zhang Jiakou	40°46'	114°53'	Potato	A1	IIA	CN01_08	
HB07-Ch0301	2007	Hebei	Zhang Jiakou	40°46'	114°53'	Potato	A1	IIA	CN01_08	

ID	Year	Province	Location	Latitude	Longitude	Host	Mating type	Lineage	Code
HB07-Ch0604	2007	Hebei	Zhang Jiakou	40°46'	114°53'	Potato	A1	IIA	CN01_08
HB07-Ch1001	2007	Hebei	Zhang Jiakou	40°46'	114°53'	Potato	A1	IIA	CN01_08
HB07-W0101	2007	Hebei	Weichang	41°56'	117°45'	Potato	A1	IIA	CN01_08
HB07-W0301	2007	Hebei	Weichang	41°56'	117°45'	Potato	A1	IIA	CN01_08
HB07-W0501	2007	Hebei	Weichang	41°56'	117°45'	Potato	A1	IIA	CN01_01
HB07-W0602	2007	Hebei	Weichang	41°56'	117°45'	Potato	A1	IIA	CN01_07
HB07-W0702	2007	Hebei	Weichang	41°56'	117°45'	Potato	A1	IIA	CN01_01
HB07-W0704	2007	Hebei	Weichang	41°56'	117°45'	Potato	A1	IIA	CN01_01
HB08-169	2008	Hebei	Chengde	40°58'	117°56'	Potato	A1	IIA	CN01_01
HB08-170	2008	Hebei	Chengde	40°58'	117°56'	Potato	A1	IIA	CN01_01
HB08-171	2008	Hebei	Chengde	40°58'	117°56'	Potato	A1	IIA	CN01_01
HB08-173	2008	Hebei	Chengde	40°58'	117°56'	Potato	A1	IIA	CN01_01
HB08-176	2008	Hebei	Chengde	40°58'	117°56'	Potato	A1	IIA	CN01_01
HB08-277	2008	Hebei	Chengde	40°58'	117°56'	Potato	A1	IIA	CN01_01
HB08-278	2008	Hebei	Chengde	40°58'	117°56'	Potato	A1	IIA	CN01_01
GZ09-345	2009	Guizhou	Weining	26°51'	104°16'	Potato		IA	CN02_04
GZ09-346	2009	Guizhou	Weining	26°51'	104°16'	Potato		IA	CN02_04
SC07-M0102	2007	Sichuan	Wenchuan	31°28'	103°35'	Potato	A1	IA	
SC07-M0103	2007	Sichuan	Wenchuan	31°28'	103°35'	Potato	A1	IA	
SC07-P20128	2007	Sichuan	Pengzhou	30°11'	103°52'	Potato	A2	IA	CN02_01
SC07-P20216	2007	Sichuan	Pengzhou	30°11'	103°52'	Potato	A2	IA	CN02_01
SC07-P20225	2007	Sichuan	Pengzhou	30°11'	103°52'	Potato	A2	IA	CN02_01
SC07-Pb0104	2007	Sichuan	Pengshan	30°11'	103°52'	Potato	A2	IA	CN02_01
SC07-Pt0103	2007	Sichuan	Pengshan	30°11'	103°52'	Potato	A2	IA	CN02_01
SC07-Pt0104	2007	Sichuan	Pengshan	30°11'	103°52'	Potato	A2	IA	CN02_01
SC07-Pt0108	2007	Sichuan	Pengshan	30°11'	103°52'	Potato	A2	IA	CN02_01
SC07-Pt0109	2007	Sichuan	Pengshan	30°11'	103°52'	Potato	A2	IA	CN02_01
SC07-Pt0203	2007	Sichuan	Pengshan	30°11'	103°52'	Potato	A2	IA	CN02_01
SC07-S0401	2007	Sichuan	Shifang	31°07'	104°10'	Potato	A2	IA	CN02_01
SC07-S0902*	2007	Sichuan	Shifang	31°07'	104°10'	Potato	A2	IA	CN02_01

ID	Year	Province	Location	Latitude	Longitude	Host	Mating	Lineage	Genotype
SC07-X0103	2007	Sichuan	Chengdu	30°39'	104°03'	Potato	A2	IA	CN02_01
SC07-X0121	2007	Sichuan	Chengdu	30°39'	104°03'	Potato	A2	IA	CN02_01
SC07-X0123	2007	Sichuan	Chengdu	30°39'	104°03'	Potato	A2	IA	CN02_01
SC07-X0315	2007	Sichuan	Chengdu	30°39'	104°03'	Potato	A2	IA	CN02_01
SC09-297	2009	Sichuan	Jiangchuan	30°41'	105°25'	Potato	A2	IA	CN02_01
SC09-303	2009	Sichuan	Xindu	30°49'	104°09'	Potato		IA	CN02_09
SC09-306	2009	Sichuan	Chengdu	30°39'	104°03'	Potato	A2	IA	CN02_01
SC09-309	2009	Sichuan	Chengdu	30°39'	104°03'	Potato	A2	IA	CN02_01
SC09-320	2009	Sichuan	Chengduebian	30°38'	104°02'	Potato	A2	IA	CN02_01
SC09-321	2009	Sichuan	Chengduebian	30°38'	104°02'	Potato	A2	IA	CN02_01
SC09-323	2009	Sichuan	Chengduwanyuan	30°33'	104°15'	Potato	A2	IA	CN02_01
SC09-324	2009	Sichuan	Chengduebian	30°38'	104°02'	Potato	A2	IA	CN02_01
SC09-328	2009	Sichuan	Chengdudayi	30°35'	103°31'	Potato			CN02_01
SC09-391	2009	Sichuan	Liangshanxide	28°18'	102°24'	Potato		IA	CN02_12
SC09-392	2009	Sichuan	Liangshanxide	28°18'	102°24'	Potato		IA	CN02_07
SC09-393	2009	Sichuan	Liangshanxide	28°18'	102°24'	Potato		IA	CN02_01
SC09-394	2009	Sichuan	Liangshanxide	28°18'	102°24'	Potato		IA	CN02_01
SC09-395	2009	Sichuan	Liangshanxide	28°18'	102°24'	Potato		IA	CN02_01
SC09-397	2009	Sichuan	Liangshanxide	28°18'	102°24'	Potato		IA	CN02_01
SC09-398	2009	Sichuan	Xindu	30°47'	104°09'	Potato		IIA	
SC09-399	2009	Sichuan	Xindu	30°47'	104°09'	Potato		IIA	
SC09-400	2009	Sichuan	Xindu	30°47'	104°09'	Potato		IA	CN02_01
SC09-401	2009	Sichuan	Xindu	30°47'	104°09'	Potato		IIA	
SC09-402	2009	Sichuan	Liangshanzhaojue	28°00'	102°50'	Potato		IA	CN02_01
SC09-403	2009	Sichuan	Liangshanzhaojue	28°00'	102°50'	Potato		IA	CN02_11
SC09-404	2009	Sichuan	Liangshanzhaojue	28°00'	102°50'	Potato		IA	CN02_11
SC09-405	2009	Sichuan	Liangshanzhaojue	28°00'	102°50'	Potato		IA	CN02_06
SC09-407	2009	Sichuan	Liangshanzhaojue	28°00'	102°50'	Potato		IA	CN02_01
SC09-408	2009	Sichuan	Liangshanzhaojue	28°00'	102°50'	Potato		IA	CN02_01
SC09-409	2009	Sichuan	Liangshanzhaojue	28°00'	102°50'	Potato		IA	CN02_01

ID	Year	Province	Location	Latitude	Longitude	Host			
SC09-410	2009	Sichuan	Liangshanzhaojue	28°00'	102°50'	Potato		IA	CN02_01
SC09-412	2009	Sichuan	Liangshanmianning	28°32'	102°10'	Potato		IA	CN02_01
SC09-415	2009	Sichuan	Liangshanmianning	28°32'	102°10'	Potato		IA	CN02_01
SC09-416	2009	Sichuan	Pixian	30°37'	103°40'	Potato		IA	CN02_01
SC09-417	2009	Sichuan	Pixian	30°37'	103°40'	Potato		IA	CN02_02
SC09-419	2009	Sichuan	Pixian	30°37'	103°40'	Potato		IA	CN02_01
SC09-420	2009	Sichuan	Dazhou	31°12'	107°28'	Potato		IA	CN02_01
SC09-421	2009	Sichuan	Dazhou	31°12'	107°28'	Potato		IA	CN02_01
SC09-422	2009	Sichuan	Dazhou	31°12'	107°28'	Potato		IA	CN02_01
SC09-423	2009	Sichuan	Dazhou	31°12'	107°28'	Potato		IA	
SC09-424	2009	Sichuan	Liangshanpuge	27°22'	102°32'	Potato		IA	
SC09-425	2009	Sichuan	Liangshanpuge	27°22'	102°32'	Potato		IA	
SC09-426	2009	Sichuan	Pengzhou	30°59'	103°57'	Potato		IA	CN02_01
SC09-427	2009	Sichuan	Pengzhou	30°59'	103°57'	Potato		IA	CN02_01
SC09-428	2009	Sichuan	Bailu	31°12'	103°54'	Potato		IA	CN02_01
SC09-429	2009	Sichuan	Bailu	31°12'	103°54'	Potato		IA	CN02_10
SC09-430	2009	Sichuan	Bailu	31°12'	103°54'	Potato		IA	CN02_01
SC09-431	2009	Sichuan	Ganzizhou	30°02'	101°57'	Potato		IA	
SC09-432	2009	Sichuan	Ganzizhou	30°02'	101°57'	Potato		IA	
SC09-433	2009	Sichuan	Ganzizhou	30°02'	101°57'	Potato			
SC09-434	2009	Sichuan	Ganzizhou	30°02'	101°57'	Potato		IA	
SC09-CQ388	2009	Chongqing	Shizhu	29°59'	108°06'	Potato		IA	CN02_10
SC09-CQ389	2009	Chongqing	Shizhu	29°59'	108°06'	Potato		IA	CN02_01
SC09-CQ390	2009	Chongqing	Shizhu	29°59'	108°06'	Potato		IA	CN02_01
YN05-d5-2-2	2005	Yunnan	Diqing	27°49'	99°42'	Potato	A2	IA	CN02_08
YN05-LSX18	2005	Yunnan	Qujing	25°29'	103°47'	Potato	A1	IA	
YN05-XH05-5-4	2005	Yunnan	Xuanwei	26°13'	104°06'	Potato	A1	IA	
YN07--401204	2007	Yunnan	Kunming	25°02'	102°43'	Potato	A1	IA	
YN07-400407	2007	Yunnan	Dali	25°36'	100°16'	Potato	A1	IA	
YN07-4032072	2007	Yunnan	Dali	25°36'	100°16'	Potato	A1	IA	

170 x 240 mm

ID	Year	Province	Location	Latitude	Longitude	Host	Mating type	Lineage	GenBank
YN07-404906	2007	Yunnan	Qujing	25°29'	103°47'	Potato	A1	IA	
YN07-500504	2007	Yunnan	Kunming	25°02'	102°43'	Potato	A1	IA	
YN07-501805	2007	Yunnan	Wenshan	23°22'	104°15'	Potato	A1	IA	
YN07-5032052	2007	Yunnan	Kunming	25°02'	102°43'	Potato	A1	IA	
YN09-285	2009	Yunnan	Jiangchuan	24°17'	102°45'	Potato		IA	CN02_01
YN09-288	2009	Yunnan	Jiangchuan	24°17'	102°45'	Potato		IA	CN02_03
YN09-291	2009	Yunnan	Zhaotong	27°20'	103°43'	Potato		IIB	
FJ02-p76	2002	Fujian	Putian	25°27'	119°00'	Potato	A1	IIB	CN03_11
FJ02-p83	2002	Fujian	Yongtai	25°52'	118°55'	Potato	A1	IIB	CN03_22
FJ02-p86	2002	Fujian	Dehua	25°29'	118°14'	Potato	A1	IIB	CN03_10
FJ04-211	2004	Fujian	Longyanliancheng	25°42'	116°45'	Tomato		IIB	CN03_24
FJ05-179	2005	Fujian	Liancheng	25°42'	116°45'	Tomato	A1	IIB	CN03_25
FJ05-182	2005	Fujian	Liancheng	25°42'	116°45'	Tomato	A1	IIB	CN03_25
FJ05-188	2005	Fujian	Zhangping	25°17'	117°25'	Tomato	A1	IIB	CN03_23
FJ06-199	2006	Fujian	Longyanzhangping	25°17'	117°25'	Tomato		IIB	CN03_14
FJ06-201	2006	Fujian	Longyanzhangping	25°17'	117°25'	Tomato		IIB	CN03_24
FJ06-175	2006	Fujian	Zhangping	25°17'	117°25'	Tomato	A1	IIB	CN03_18
FJ06-177*	2006	Fujian	Zhangping	25°17'	117°25'	Tomato	A1	IIB	CN03_20
FJ06-180	2006	Fujian	Zhangping	25°17'	117°25'	Tomato	A1	IIB	CN03_02
FJ06-185	2006	Fujian	Liancheng	25°42'	116°45'	Tomato	A1	IIB	CN03_25
FJ07-183	2007	Fujian	Fuzhou	26°04'	119°17'	Potato	A1	IIB	CN03_14
FJ07-184	2007	Fujian	Fuzhou	26°04'	119°17'	Potato		IIB	CN03_13
FJ07-187	2007	Fujian	Fuzhou	26°04'	119°17'	Tomato		IIB	CN03_17
FJ07-190	2007	Fujian	Fuzhou	26°04'	119°17'	Tomato		IIB	CN03_13
FJ07-198	2007	Fujian	Longyanzhangping	25°17'	117°25'	Tomato		IIB	CN03_16
FJ07-203	2007	Fujian	Tanzhouzhangpu	26°08'	119°08'	Tomato		IIB	CN03_06
FJ07-209	2007	Fujian	Longyanliancheng	25°42'	116°45'	Tomato		IIB	CN03_05
FJ07-178	2007	Fujian	Liancheng	25°42'	116°45'	Tomato	A1	IIB	CN03_20
FJ07-181	2007	Fujian	Zhangping	25°17'	117°25'	Tomato	A1	IIB	CN03_21
FJ07-184	2007	Fujian	Liancheng	25°42'	116°45'	Tomato	A1	IIB	CN03_20

170 x 240 mm

ID	Year	Province	Location	Latitude	Longitude	Host	Mating type		Genotype
FJ07-187	2007	Fujian	Zhangping	25°17'	117°25'	Tomato	A1	IIB	CN03_19
FJ07-189	2007	Fujian	Zhangping	25°17'	117°25'	Tomato	A1	IIB	CN03_03
FJ08-178	2008	Fujian	Fuzhouxindian	26°07'	119°18'	Tomato		IIB	CN03_15
FJ08-181	2008	Fujian	Zhangzhou	24°30'	117°38'	Tomato		IIB	CN03_13
FJ08-182	2008	Fujian	Fuzhouxindian	26°07'	119°18'	Tomato			CN03_07
FJ08-186	2008	Fujian	Fuzhouxindian	26°07'	119°18'	Tomato		IIB	CN03_13
FJ08-197	2008	Fujian	Fuzhou	26°04'	119°17'	Tomato			CN03_04
FJ08-200	2008	Fujian	Longyanzhangping	25°17'	117°25'	Tomato		IIB	CN03_01
FJ08-205	2008	Fujian	Tanzhoulongchi	26°08'	119°08'	Tomato		IIB	CN03_07
FJ08-207	2008	Fujian	Tanzhoulongchi	26°08'	119°08'	Tomato		IIB	CN03_08
FJ09-193	2009	Fujian	Fuzhouchangle	25°57'	119°31'	Potato		IIB	CN03_14
FJ09-194	2009	Fujian	Fuzhouchangle	25°57'	119°31'	Potato			CN03_13
FJ09-195	2009	Fujian	Fuzhouchangle	25°57'	119°31'	Potato		IIB	CN03_13
FJ09-208	2009	Fujian	Tanzhoulongchi	26°08'	119°08'	Tomato		IIB	CN03_09
GS09-357	2009	Gansu	Gansu	36°03'	103°49'	Potato	A1	IIA	
NX09-365	2009	Ningxia	Guyuanjingyuan	35°29'	106°19'	Potato	A2	IA	CN02_01
NX09-367	2009	Ningxia	Guyuanjingyuan	35°29'	106°19'	Potato	A1	IIA	CN01_01
NX09-370	2009	Ningxia	Guyuanjingyuan	35°29'	106°19'	Potato	A2	IA	CN02_01
NX09-371	2009	Ningxia	Guyuanjingyuan	35°29'	106°19'	Potato	A2	IA	CN02_01
SX09-377	2009	Shanxi	Ankangpingli	35°29'	106°19'	Potato	A1	IIA	
SX09-378	2009	Shanxi	Ankanglangao	32°18'	108°54'	Potato	A2	IA	CN02_05
SX09-379	2009	Shanxi	Ankanglangao	32°18'	108°54'	Potato	A2	IA	CN02_01
SX09-381	2009	Shanxi	Ankanglangao	32°18'	108°54'	Potato	A2	IA	CN02_05
SX09-382	2009	Shanxi	Ankanglangao	32°18'	108°54'	Potato	A2	IA	CN02_01

* to define as the reference isolates for further studies on Chinese *P. infestans*

CHAPTER 5

Population dynamics of *Phytophthora infestans* in the Netherlands reveals expansion and spread of dominant clonal lineages and virulence in sexual offspring

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CHAPTER 5

SUMMARY

For a comprehensive survey of the structure and dynamics of the Dutch *Phytophthora infestans* population, 652 *P. infestans* isolates were collected from commercial potato fields in the Netherlands during a ten year period, 2000 - 2009. Genotyping was performed using twelve highly informative microsatellite markers and mitochondrial haplotypes. In addition, for each isolate the mating type was determined. STRUCTURE analysis grouped the 322 identified genotypes in three clusters. Cluster 1 consists of a single clonal lineage NL-001; all isolates in this cluster have the A2 mating type and the Ia mitochondrial haplotype. This lineage is also known as "Blue_13". Clusters 2 and 3 display a more elaborate substructure containing many unique genotypes. In Cluster 3 also several distinct clonal lineages were identified. Isolates of Cluster 2 predominantly originated from the starch potato region located in the North East of the Netherlands. This survey witnesses that the Dutch population was undergoing dramatic changes in the ten years under study. The most notable change was the emergence and spread of A2 mating type strain NL-001 or "Blue_13". The results emphasize the importance of the sexual cycle in generating genetic diversity and the importance of the asexual cycle as the propagation- and dispersal mechanism for successful genotypes. Isolates were also screened for absence of the *Avrblb1/ipiO* class I gene, which is indicative for virulence on *Rpi-blb1*.This therefore is also the first report of *Rpi-blb1* breakers in the Netherlands. Superimposing the virulence screening on the SSR genetic backbone indicates that lack the *Avrblb1/ipiO* class I gene only occurred in sexual progeny. So far the asexual spread of the virulent isolates identified has been limited.

INTRODUCTION

The oomycete *Phytophthora infestans* is the causal organism of late blight on potato and tomato, among other hosts. Globally, late blight carries multiple costs, including complete crop failures, economic losses due to decreased yields and fungicide applications with a potentially negative impact on human health and the environment (Anonymous, 2011). In the Netherlands, the total area under potato cultivation amounts to 165.000 ha and annually yields 7.9 million ton of potato representing a farm gate value of about M€790. The number of fungicide applications varies between 10 and 16 per season. Costs for potato late blight control (chemicals, application and losses) amount to 125M€ per year, almost 16% of the total farm gate value (Haverkort *et al.*, 2008).

From these figures it is clear that farmers, the potato industry, consumers and the environment could greatly benefit from more efficient and environmentally friendly ways to control late blight through e.g. the introduction and durable exploitation of host plant resistance. *P. infestans,* however, is renowned for its capacity for adaptation particularly with respect to emergence of virulence towards resistant cultivars and to a lesser extent fungicide resistance (Haas *et al.*, 2009). One of the prerequisites for durable management of late blight, therefore, is an up to date knowledge on characteristics of the local *P. infestans* population and a high level understanding of population dynamics in order to avoid erosion of cultivar resistance and development of fungicide resistance. Currently, one of the most promising *R* genes is the broad spectrum resistance gene *Rpi-blb1* (Van Der Vossen *et al.*, 2003, Song *et al.*, 2003) which is used in breeding programs.

P. infestans is heterothallic and both, A1 and A2 mating types are required for completion of the sexual cycle. Sexual reproduction results in high levels of genetic variation in the offspring and may lead to increased and more rapid evolution of the pathogen. In the Netherlands only the A1 mating type was found prior to the 1980's and all isolates grouped in a single (US1) clonal lineage (Drenth *et al.*, 1994) that was also found in many other parts of the world (Drenth *et al.*, 1993). During the 1980's, following a renewed global migration of both (A1 and A2) mating types, a new *P. infestans* population rapidly displaced the US1 clonal lineage (Spielman *et al.*, 1991, Drenth *et al.*, 1993) in the Netherlands. Isolates having the US1 genotype were not detected in the Netherlands ever since (Drenth *et al.*, 1994, Spielman *et al.*, 1991). One of the driving forces behind this displacement may have been the higher levels of aggressiveness and fitness in the new population as compared to the old

population (Flier & Turkensteen, 1999). The newly introduced *P. infestans* genotypes in combination with the occurrence of sexual reproduction also considerably raised the level of genetic diversity in the Dutch *P. infestans* population leading to a highly variable population (Drenth *et al.*, 1994) with a presumed higher level of adaptability as compared to the previous, purely asexually reproducing population.

More than two decades after the introduction of new genotypes in Europe, investigators from the UK reported that a single *P. infestans* genotype with A2 mating type, EU13_A2 or "Blue_13" is dominant in the UK (Lees *et al.*, 2009). "Blue_13's" dominant position was hypothesized to have emerged from superior levels of fitness in combination with resistance to the frequently used metalaxyl and a "Blue_13" favorable choice of commonly grown cultivars (White & Shaw, 2009).

Molecular markers provide the opportunity to track and trace individual genotypes and to study population diversity. In the past, genotypic characterization of *P. infestans* isolates included allozyme pattern, mitochondrial (mt) DNA haplotype, RG57 RFLP fingerprints (Fry *et al.*, 1991, Fry *et al.*, 1992, Drenth *et al.*, 1993, Drenth *et al.*, 1994) and AFLP fingerprinting (Flier *et al.*, 2003, Van der Lee *et al.*, 2004). Co-dominant markers such as microsatellites, also known as simple sequence repeats (SSRs), have previously been used to investigate the genetic structure and reproductive biology of numerous plant pathogens (Tenzer *et al.*, 1999). Particularly for diploid or aneuploid species such as *P. infestans* SSRs may provide a better understanding of the overall genetic structure. A recently developed and standardized set of twelve highly informative microsatellite markers (Li, *et.al*, in preparation) was exploited to perform high resolution genotyping. In addition, we developed a new assay for monitoring. *Avrblb1*, the avirulence gene matching *Rpi-blb1* (Song *et al.*, 2003, Van Der Vossen *et al.*, 2003), also known as *RB. Avrblb1*, which is also known as *ipiO* (Pieterse *et al.*, 1994, Vleeshouwers *et al.*, 2008) is genetically highly diverse in *P. infestans.* Absence of class I variants of *Avrblb1/ipiO* has been reported to be associated with virulence on potato plants carrying *Rpi-blb1* (Champouret *et al.*, 2009). In this study, we combine genotyping with both functional and neutral markers.

On the premise that understanding of the population genetics of plant pathogens will contribute to the development of more sustainable disease management strategies, the objective of this study was to describe and analyze the dynamics of the Dutch *P. infestans* population over the course of a decade from 2000 to 2009. Four major topics were addressed:

i) the distribution of genetic variation within the Dutch population associated with geographical levels (national versus regional versus local populations); ii) a description of the occurrence and dynamics of dominant clonal genotypes in the Netherlands; iii) monitoring for the virulence for the *Rpi-blb1* gene; iv) the origin of isolates virulent on *Rpi-blb1*.
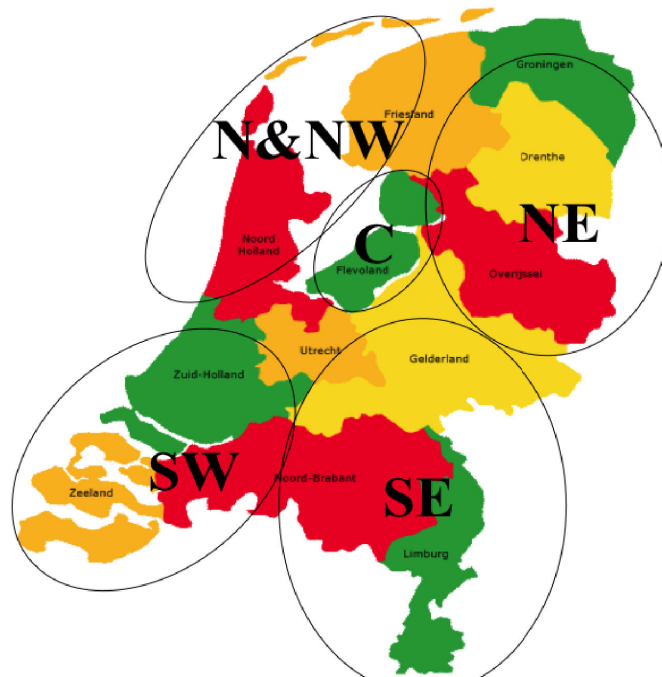
Fig. 1. Map of the Netherlands showing the sampling regions where the *P. infestans* isolates, analyzed in this study

RESULTS

Sampling

Isolates were collected from 207 different locations (Table 1) encompassing all five major Dutch potato-growing areas (Fig. 1). A total of 676 *P. infestans* isolates was analyzed in the phylogeny analysis, including 652 isolates from the 2000 to 2009 samplings, 16 isolates from the 1980's and 1990's collection representing older populations and eight isolates used as reference for the SSR markers.

Microsatellite polymorphism across loci

93

In 668 isolates (652 of the 2000 - 2009 set plus 16 older ones), the twelve microsatellite loci revealed high Polymorphism Information Content (PIC) values, ranging between 0.218 - 0.841, with an average value of 0.534 and different allele frequencies (Table 2). G11 is the most informative locus of the 12 SSRs, with the highest value for the Shannon's Information index (I=2.006). A total of 75 alleles was detected over 12 loci and the average number of alleles per locus was 6.250, ranging from 3 (Pi4B, Pi70, PinfSSR2 and PinfSSR6) to 17 (D13) alleles per locus. The effective number of alleles including rare alleles ranged from 1.155 (Pi70) to 6.151 (G11), with an average of 2.436. Locus D13 had the highest observed number of alleles (17 alleles), but its effective number of alleles (1.872) was under the mean number (2.436) for the whole population. SSR markers revealed 28 rare alleles with a frequency lower than 0.05. The frequency of the rare alleles within loci (Table 2) ranged from 0 (Pi4B, Pi63, PinfSSR6, PinfSSR11) to 0.429 (D13). Allele sizes ranged from 116 to 356 bp. Locus D13 had the largest range of allele sizes (58 bp), while PinfSSR2 and PinfSSR6 had the smallest range (4 bp).

The mean expected heterozygosity (He) was 0.524 (varying between 0.134 and 0.838) and the mean observed (Ho) heterozygosity was 0.577 (varying between 0.114 and 0.809). Five loci were not in HWE. After clone-correction, the loci displaying excess of heterozygotes were still significantly different from HWE (Table 2). The linkage disequilibrium tests revealed linkages between pairs of loci, but these linkages were not consistent across geographic populations. In each population, all loci behaved as neutral according to the Ewens-Watterson test indicating the absence of clear selection forces influencing the frequency of all SSR loci used in this study.

Genetic diversity in all years and all regions

The complete set of isolates shows a high genetic diversity with 322 unique genotypes among 652 field isolates. To examine the distribution of genetic variation, among and within populations as defined by the five geographical areas, AMOVA was performed. This showed that, 95% of the variance can be attributed to the regional stratum (within regions) whereas the remaining 5% was attributed to the national stratum (between regions). This strongly indicates the absence of separate regional populations. In testing the departure from mutation-drift equilibrium based on heterozygosity excess or deficiency, bottleneck analysis was conducted for the regional populations under the two-phased model of mutation of microsatellites. The five geographic populations displayed no significant excess of

heterozygosity (P>0.05) through Wilcoxon significant rank test, suggesting that none of these five populations has not experienced a recent bottleneck.

Table 1. Total numbers of collected isolates and "Blue_13" isolates (number after back slash) from different years and sampling regions

Region	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	Sum
C	8	0	4	10	6	34/25	28/16	16/12	10/5	34/6	150/64
N & NW	6	1	0	4	2	23/14	6/3	9/5	4/4	0	55/26
NE	18	22	20	21	28/4	30/6	18/4	23/11	9/7	20/3	209/35
SE	19	1	2	7	17/5	19/15	10/6	16/10	24/13	23/16	138/65
SW	4	0	3	10	3	46/5	10/4	16/12	2/2	6/6	100/29
Sum	55/0	24/0	29/0	52/0	56/9	152/65	72/33	80/50	49/31	83/31	652/219

For STRUCTURE analysis, clone corrected data were used. Thus, the genetic structure of 358 isolates was analyzed using the correction for STRUCTURE 2.2 outputs as described by Evanno *et al.* (2005) (Fig 2). For all K, memberships were consistent between all runs. The first ∆K peak for K=3 indicates to the presence of three main clusters. When individual isolates with a membership lower than 70% were not taken into account, nine of 358 isolates were misclassified. The AMOVA for K=3 indicated that 25% of the variance was attributed to variation between the three clusters, 75% of the variance was due to variation within clusters. Pair-wise estimates of FST indicated a high degree of differentiation varying from 0.18 between cluster 2 and 3 to 0.42 between cluster 1 and 2.

Based on a plot against the first two dimensions from the PCA, three populations were identified Pop 1, Pop2 and Pop3 that correspond to the three clusters identified by the STRUCTURE analysis (Fig. 3).

A phylogenetic tree of all 652 isolates from the survey plus the 16 older isolates and eight SSR reference isolates was constructed using the NJ method (Fig. 4A, B, D and F). In this tree, isolate VK1.4 a member of the US-1 clonal lineage served as an out-group. Isolate characteristics such as geographic origin, mating type and haplotype were subsequently superimposed on the phylogenetic tree using different colors (Fig. 4A, 4B, 4D).   When the isolate position on the two dimensional PCA was combined with the phylogenetic tree (Fig. 4C), isolates belonging to the phylogenetic NL-001 clade grouped similarly in Cluster 1 by STRUCTURE and Pop 1 by PCA (Fig. 2, 3). Cluster 1 contains only isolates with A2 mating

170 x 240 mm

Table 2. Microsatellite polymorphisms across loci and regions

	D13	G11	Pi04	Pi4B	Pi63	Pi70	PintSSR2	PintSSR3	PintSSR4	PintSSR6	PintSSR8	PintSSR11	Mean
Dye color	FAM	NED	VIC	PET	VIC	VIC	PET	NED	FAM	VIC	FAM	NED	
Size range	100-185	130-180	160-175	200-295	265-280	185-205	165-180	255-275	280-305	230-250	250-275	325-360	
PIC value	0.536	0.841	0.632	0.592	0.560	0.128	0.377	0.533	0.668	0.538	0.576	0.430	0.534
Freq of rare alleles	0.429	0.143	0.036	0.000	0.036	0.000	0.036	0.107	0.179	0.000	0.036	0.000	0.084
na[1]	17	13	4	3	4	3	3	6	11	3	4	4	6.25
ne[2]	1.872	6.151	2.722	2.447	2.218	1.155	1.614	2.086	2.733	2.120	2.364	1.752	2.436
I[3]	1.207	2.006	1.061	0.993	0.935	0.277	0.586	0.894	1.243	0.899	0.983	0.779	0.989
Ho[4]	0.209	0.662	0.772	0.766	0.720	0.114	0.419	0.713	0.809	0.638	0.671	0.436	0.577
He[4]	0.466	0.838	0.633	0.592	0.550	0.134	0.381	0.521	0.635	0.529	0.578	0.429	0.524
P[5]	1.000	1.000	0.972	0.000*	0.000*	0.993	0.005*	0.000*	0.008*	0.000*	0.843	0.176	/
P (clone corrected)[5]	1.000	1.000	0.99	0.000*	0.000*	0.993	0.001*	0.000*	0.442	0.000*	1.000	0.098	/
Fst	0.018	0.023	0.075	0.016	0.014	0.006	0.012	0.006	0.003	0.011	0.000	0.025	/
C L3[6]	10/151	9/151	0/151	1/151	6/151	0/151	0/151	5/151	16/151	3/151	1/151	4/151	/
N&NW L3	7/55	1/55	0/55	0/55	1/55	0/55	0/55	2/55	3/55	0/55	1/55	0/55	/
NE L3	12/209	4/209	0/209	0/209	0/209	0/209	0/209	1/209	7/209	4/209	0/209	0/209	/
SE L3	7/138	5/138	1/138	1/138	0/138	0/138	0/138	5/138	21/138	3/138	0/138	0/138	/
SW L3	1/100	10/100	0/100	0/100	1/100	0/100	0/100	9/100	17/100	6/100	0/100	0/100	/

[1] na = Observed number of alleles
[2] ne = Effective number of alleles (Kimura & Crow, 1964)
[3] I = Shannon's Information index (Lewontin, 1972)
[4] Ho=observed heterozygosity, He=expected heterozygosity
[5] Significant p<0.05 deviation of HWE; the deviation after Bonferroni correction is the same as before.
[6] L3=the number of individual isolates having the locus with 3 alleles per (/) total number of isolates sampled in that region.
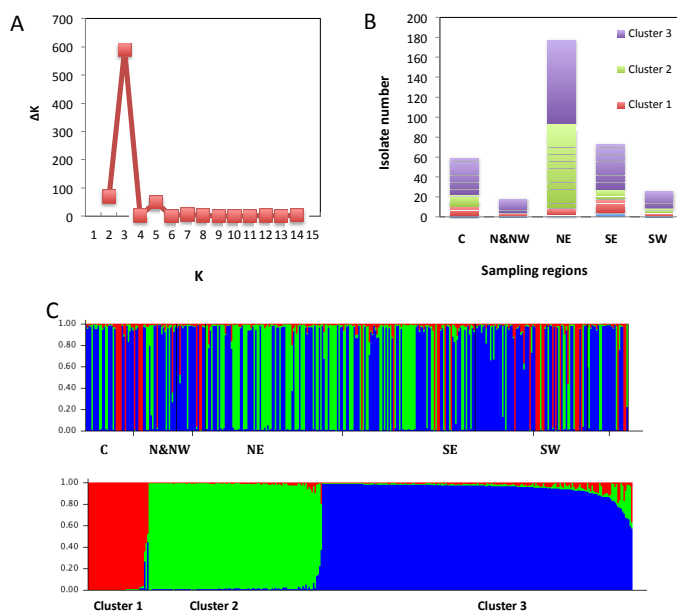
Fig. 2. STRUCTURE analysis of 12 microsatellite loci after clone correction. (A) Result of the ΔK calculation, the second order rate of change of LnP(D) with respect to K (1 to 15); ΔK for K=2-14 revealed a single distinct peak at K=3; (B) Summary of results for STRUCTURE analysis at K=3 showing the proportion of isolates from five sampling regions. The isolates were assigned in admixture when the membership is below 70%. (C) Plot example of the raw STRUCTURE output for one run (K=3) organized by geographic region (top) and by Q value.
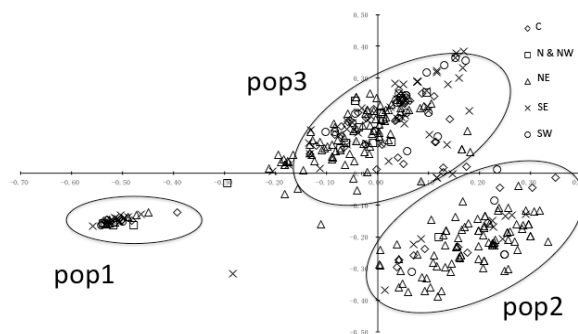
Fig. 3. Two-dimensional plot of the first two axes (X=PC1, Y=PC2) of a principal coordinate analysis. Symbols represent the five different sampling regions.
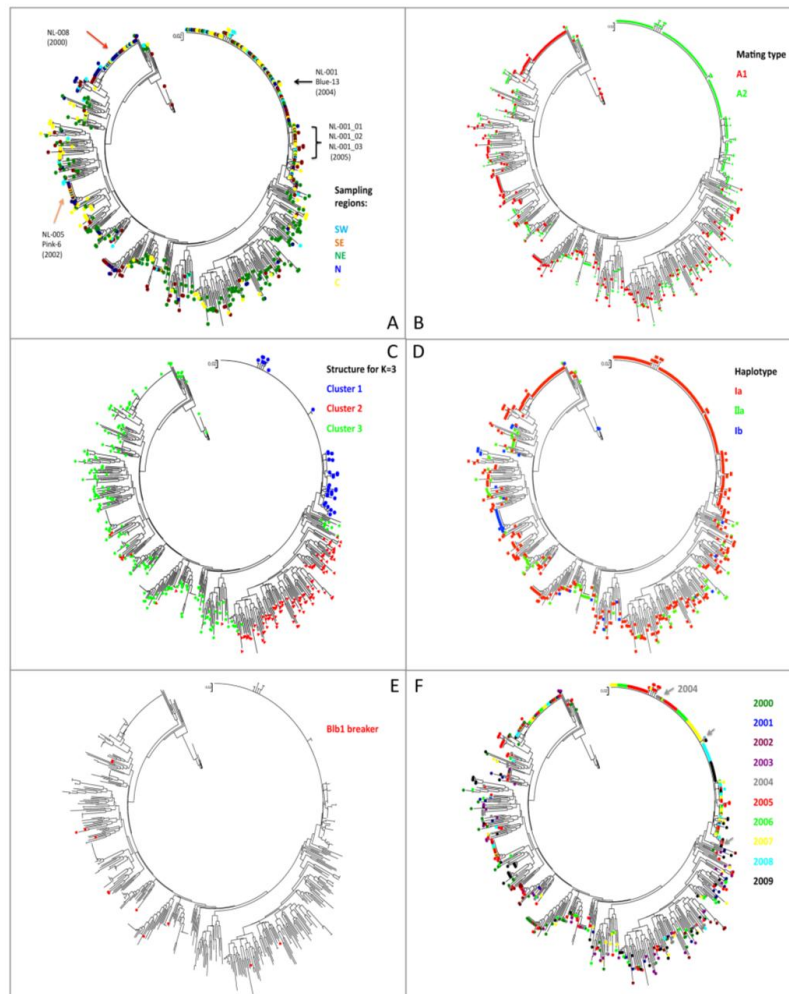
Fig. 4. Rooted phylogeny of Dutch *P. infestans* isolates over 10 years using NJ among 12 SSRs. The tree contains 668 isolates and eight reference isolates (see Materials and Methods). The sampling isolates were marked with colors, indicating different characters. A indicates five different sampling regions. B shows the mating types, the A2 mating type is in green, the rest isolates were identified with the A1 mating type in red. C is the result of STRUCTURE analysis. For K=3, three clusters were clearly identified in Fig. 4 and the isolates within three clusters are aligned on the phylogeny tree. D shows three haplotypes identified in the Dutch *P. infestans* population. E shows the isolates that lack *Avrblb1/ipiO* class I on the phylogeny tree. F shows the sampling years of isolates on the phylogeny tree.

type and mitochondrial haplotype Ia, and could be defined as a single clonal lineage with some sub-clonal variation. Cluster 2 predominantly originates from the North East starch potato region.

Within the three large clusters identified by STRUCTURE, three genotypes are dominant over the various temporal and spatial scales (Fig. 4A). One dominant genotype, called NL-001 (A2 and Ia) was retrieved 144 times from isolates collected between 2004 and 2009 covering all five sampling regions and representing 22.1% of the 652 samples. NL-001 has the same SSR genotype profile as the EU13_A2 (or "Blue_13") clonal lineage previously reported by Lees *et al*., 2009. NL-001_02 (7 isolates), NL-003 (11) and NL-004 (10) are sub-clonal lineages that show small but consistent differences to NL-001_01 in the phylogenetic tree and also have the A2 mating type and Ia haplotype. Out of 12 SSRs, only D13 and SSR4, respectively, showed two rare alleles in NL-001_02, NL-001_03 and NL-001_04. A second dominant genotype called NL-008 (A1 and Ia) was retrieved 43 times between 2000 and 2009. Genotype NL-005 (identical to the previously reported SSR genotype EU6_A1 or "Pink_6" (Lees *et al.*, 2009)) grouped 15 isolates collected in five different years (2002, 2005-2008) and was present in a low frequency (2.3%) over the 10 year period. Next to the three more dominant genotypes, some genotypes were identified at even lower frequency (less than 1%), less than 15 isolates per genotype some of which were found in multiple years and multiple regions.

Temporal dynamics of three major clonal lineages

Fig. 4F illustrates the temporal dynamics of the genotypes during the years 2000 - 2009. The vast majority of genotypes were found only once. Isolates with identical SSR genotypes always showed the same mt haplotype and the same mating type, further establishing the clonal identity of these isolates.

Three major clonal lineages (NL-001, NL-005 and NL-008) cover 31% of the entire isolate collection 2000 - 2009 with 144, 15 and 43 isolates, respectively. Isolates with the NL-008 genotype were found in 2000, the first year of sampling, in all sampling regions except for the NE (Fig. 4A, 4F). Genotype NL-005 was identified in 2002 and was also found between 2005 - 2008 with a low overall frequency of 2% (Fig. 4F). Also this genotype was retrieved from many regions of the Netherlands from the 2002 onwards, but not from the NE.

Genotype NL-001 was first found in two regions (NE and SE) in 2004 and dominated the population from the start (Fig. 4F). Since then NL-001 isolates were dominantly present in

all regions (N & NW in 2009 not sampled) from the very beginning to the very end of every blight season in 2004 - 2009 (Fig. 5) with a frequency roughly correlating to the total number of isolates collected in the Netherlands.
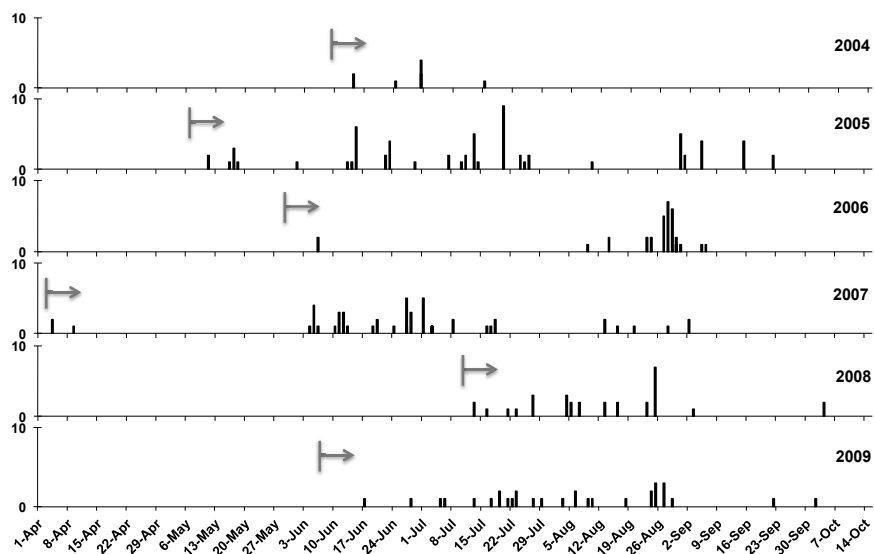
Fig. 5. The temporal dynamics of "Blue_13" lineage from 2004 to 2009. The arrow indicates the start time of the first isolate sampling in that season. Vertical bars indicate the day that isolate "Blue_13" was collected with the height representing the number.

Screening for isolates that lack *Avrblb1* (*ipiO* class 1)

To determine whether isolates contain class I *Avrblb/ipiO* variants, a TaqMan PCR procedure was developed and validated. All class I members of (ipiO-01, ipiO-02, ipiO-05, IpiO-07, IpiO-08) could be detected while the class II and Class III *ipiO* variants (IpiO-03 and IpiO-04 respectively) showed at least a factor 250 times lower specificity. This enabled clear differentiation between isolates PIC99177, PIC99189 that do not contain *ipiO* class I variants but do contain *ipiO* class II variants from isolates T30-4, 90128, 98014, VK1.4, PIC99183 that contain *ipiO* class I variants. When the DNA of the field isolates was tested, twelve of the 652 isolate samples (1,8%) lacked an *Avrblb1* class I gene. *Avrblb1* is also known as *ipiO*. Based on the SSR genotyping 10 unique genotypes were found among 12 virulent isolates. Isolates with identical SSR genotypes also showed identical results on the *Avrblb1/ipiO* class

I screening. No variation for the presence of *Avrblb1/ipiO* class I was found among (sub-clones of) any of the dominant lineages. Isolates lacking *Avrblb1/ipiO* class I and therefore virulent to *Rpi-blb1*, were collected in the NL in 2000, 2003, 2004, 2005, 2007, 2008 and 2009. Apart from two identical genotypes that will be discussed below, no particular genetic clustering was observed among isolates that lack *Avrblb1* (Fig. 4E). In two cases an identical SSR profile matched with the lack of *Avrblb1/ipiO* class I. Two virulent isolates isolated in 2004 had the same genotype and also originated from the same field, and the same potato variety. Two other isolates with an identical genotype were collected in 2005 and 2008, respectively. They were sampled in the same region (NE) but from different fields and different potato varieties. Apparently, this genotype was able to survive for several years but it has not been found since 2008. The other isolates that lack *Avrblb1/ipiO* class I represented unique genotypes, none of which seemed to have spread significantly by the asexual cycle.

DISCUSSION

A better understanding of *P. infestans* population dynamics will contribute to more durable disease management strategies, particularly if high resolution neutral markers are combined with the use of functional markers for e.g. virulence towards individual *R* genes. Here, we analyzed the Dutch *P. infestans* population over an entire decade, determined the overall population structure and described the emergence, dynamics and displacement of the main clonal lineages in correlation with regional cultural practices.

STRUCTURE analysis, using a set of 12 highly informative SSR markers, splits the Dutch *P. infestans* population into three major clusters, which, for Cluster 2, roughly corresponds to the NE geographical region of origin. Cluster 1 consists of a single clonal lineage, NL-001 with 27 sub-clones previously identified as genotype "Blue_13" (Lees *et al.*, 2009). The sub-clonal lineages showed small but consistent differences mostly by loss of alleles and by the presence of rare alleles in the SSR loci D13, G11 and PinfSSR4, that are known to be highly variable (Li *et al.*, 2012, Li *et al.*, 2010). The clonality of this cluster was further illustrated by the fact that all members have the A2 mating type, and the Ia mt haplotype. Also, all members of this cluster have a copy of *Avrblb1/ipiO* class I gene and are thus expected to be avirulent on potato lines carrying the *Rpi-blb1R* gene. This was confirmed by virulence assays (data not shown). Members of Cluster 1 were first found in 2004 on

multiple locations (NE and SE) and from 2005 onward NL-001 members were found in all regions of the Netherlands.

Cluster 2 isolates mainly originate from the NE, a region in which almost exclusively starch potatoes are cultivated. In this region higher levels of late blight infection are more easily tolerated, especially at the end of the growing season. Moreover crop rotation in the NE is generally shorter than in other regions, facilitating oospore driven epidemics. As a matter of fact during a survey to establish the relative importance of primary inoculum sources in the period 1999 - 2005 oospore initiated epidemics were only found in the North East of the Netherlands (Evenhuis *et al.*, 2007). In Cluster 2 the genetic variation is high and most of the genotypes are found only once (73.0%) or twice (27.0%), the latter often collected from the same field in the same year. This pattern is consistent with a more important role of the sexual cycle and the formation of oospores resulting in high levels of recombination in combination with a limited spread of these genotypes through the asexual cycle. The diversity of this cluster is further illustrated by the absence of clonal lineages and the presence of both mating types, A1 and A2, in a more or less equal ratio (A1/A2=161/197) and multiple mt haplotypes, Ia, IIa and Ib. Some of the isolates of this cluster lack the *Avrblb1/ipiO* class I gene and were indeed found to be virulent on potato plants carrying the *Rpi-blb1* resistance gene (data not shown).

Cluster 3 includes several (older) clonal lineages in combination with a large number of unique genotypes. The old clonal lineages, like NL-008 and NL-005 ("Pink_6"), are found in multiple regions and in multiple years, but remarkably none in the NE, despite intense sampling. Cultivars grown in the NE are mainly starch potatoes not cultivated in the other parts of the Netherlands. Vice versa cultivars grown in the other parts of the Netherlands are predominantly ware potatoes, including seed potato production. Furthermore, crop rotation in the NE is 1 in 2-3 years, compared to 1 in 4 for other regions, Such shorter crop rotations facilitates oospore driven epidemics, thus stimulating a more or less local *P. infestans* population in the North east of the Netherlands. The absence of most of the clonal lineages in the NE region may therefore be due to elevated levels of oospore derived infections masking the infections of the clonal lineages or may correlate to differences in resistance of the different cultivars used in this region. These differences are too small to consider the Dutch *P. infestans* population to be a geographically structured meta-population, as supported by the AMOVA analysis on the allele frequencies. However, it can be concluded that the sampled

populations reveal a distinct signature for the NE population which could potentially be associated with distinct regional agronomic practices and a slightly more tolerant attitude towards late blight infections in production crops, allowing for increased chances of formation of oospores.

Gene flow can be maintained by dispersal of sexual and/or asexual propagules. In the Dutch *P. infestans* population, re-isolation of clonal isolates was widespread and nationwide. It can only be the result of asexual reproduction. This survey witnesses that the Dutch population was undergoing dramatic changes in the ten years under study. The most notable change was the emergence and spread of A2 mating type strain NL-001 or "Blue_13". Since 2004, NL-001 or "Blue_13" was detected in many countries (Kildea *et al.*, 2010) but its origin is unclear (Li *et al.*, 2012). The isolates described in this study from 2004 on are the earliest reported for this genotype. This could indicate that NL-001/"Blue13" originates from the Netherlands. However, there are two remarkable features that may hint to another origin. Firstly, the SSR analysis of these isolates revealed the presence of 27 sub-clonal variations. Sub-clonal variants of NL-001 were found even in the first year (2004) and the number of sub-clonal variants does not show a clear increase over time. Also in other countries many sub-clonal variants were found (Lees *et al.*, 2009). This is in sharp contrast with other clonal lineages found in the Netherlands where little or no sub-clonal variation was found even though these appear to be present in the Netherlands for a longer period. Thus, it appears that NL-001/"Blue_13" was not introduced into the Netherlands as a single clone, but as a set of several sub-clonal variants. Interestingly, the summer of 2003 had exceptionally high temperatures which may have resulted in a population bottleneck for the *P. infestans* population and primed the partial displacement. The second remarkable fact that may hint to another origin is the spread of this genotype inside and outside the Netherlands. It was first found in 2004 on multiple locations in the Netherlands, but the genotype was absent in the surveys from 2000 to 2003. It seems unlikely that this dominant genotype would not have been picked up before 2004, if it was present. In addition, NL-001/"Blue_13" was found in multiple locations outside the Netherlands from 2005 onwards, ranging from various European countries to regions in China (Li *et al.*, in preparation). It is difficult to envisage how a genotype could spread so rapidly inside and outside the Netherlands, often in the absence of any recorded shipment of (seed) potatoes from e.g. the Netherlands.

CHAPTER 5

Although a large part of the Dutch *P. infestans* population has the A1 mating type and the members of the NL-001 clonal lineage generate large amounts of oospores when confronted with the A1 tester in the laboratory mating type test, no clear evidence was found for NL-001/"Blue_13" as parent in the sexual cycle in this study. In an unchanging environment sexual reproduction of well adapted genotypes is evolutionarily costly, meaning that most sexual progeny will have a lower fitness than the successful parent. This would promote a strictly asexual strategy until detrimental mutations in a clonal lineage result in reduced fitness.

Some studies indicated that the sexual reproduction plays a key role in some countries (Sujkowski *et al.*, 1994, Brurberg *et al.*, 1999, Gavino *et al.*, 2000, Nagy *et al.*, 2003), whereas in other studies the contribution of clonality to the population was significant (Elansky *et al.*, 2001, Le *et al.*, 2008, Runno-Paurson *et al.*, 2009, Blandón-Díaz, 2011). Although asexual dispersal appears to be important in the species, it was observed that gene flow between the populations depends on the dispersal of the sexual product. Pop 2 in the PCA showed a typical pattern of sexual recombination (Fig. 3). A previous study suggested that oospores may act as an infection source in the Netherlands since the 1990's (Zwankhuizen *et al.*, 2000). Our study clearly indicates involvement of an active sexual cycle through the production of oospores. A combined sexual-asexual reproduction/dispersal mechanism seems therefore characteristic for the *P. infestans* population in the Netherlands.

A fast and reliable TaqMan real-time PCR for screening isolates lacking *Avrblb1/ipiO* class I was developed in this study. Previously it was shown that isolates lacking this class of genetic variants are virulent towards *Rpi-blb1* and homologs (Champouret *et al.*, 2009). In the Netherlands, virulent isolates were identified and screening for *Avrblb1/ipiO* classes indeed showed the absence of this class I in these isolates. Application of this TaqMan real time PCR on additional, more recent *P. infestans* isolates (data not shown) yielded several additional isolates lacking the *Avrblb1/ipiO* class I variant. Virulence bio-assays demonstrated that also these isolates were virulent on potato lines carrying the *Rpi-blb1* gene (data not shown). This is the first time that such isolates are reported to occur outside Mexico. Superimposing the virulence screening on the SSR genetic backbone shows that lack of the *Avrblb1/ipiO* class I variant occurred in 12 isolates, representing 10 unique genotypes that do not show any particular genetic association. In most cases these unique genotypes did not demonstrate successful dispersal through the asexual cycle. In all but one case the specific genotype was not retrieved in following years. The one case where the genotype managed to survive for

several years may be of special interest. Isolates with this genotype should be studied in more detail for phenotypic traits as it may have compensatory mutations or recombined genes compensating the loss of *Avrblb1/ipiO* class I gene. None of the clones and sub-clones of the clonal lineages showed lack of *Avrblb1* indicating the region is stable during mitosis. The lack of recombination between *ipiO* class I and *ipiO* class II suggests that these classes are not allelic. Therefore isolates that lack *ipiO* class I variants may have lost this region, possibly during meiosis. In meisosis translocation can lead to null alleles as can non-disjunction previously found to occur in *P. infestans* (Carter *et al.*, 1999, Van der Lee *et al.*, 2004). In this study, the first *Rpi-blb1* breakers were found in 2000, long before this gene was cloned (Song *et al.*, 2003, Van Der Vossen *et al.*, 2003). Until today no varieties have been released with this particular *R* gene. Therefore, the occurrence of isolates which lack the *Avrblb1/ipiO* class I variant could be regarded as a random genetic "accident" beneficial in case potato lines with *Rpi-blb1* or its homologs are grown. Bouwmeester *et.al.* recently reported that the *ipiO* gene may be important for virulence (Bouwmeester *et al.*, 2011), so the reduced spread of genotypes that lack the *Avrblb1/ipiO* class I gene could be in line with this. Nevertheless, the lack of spread of virulent isolates observed should be treated with care, as selection pressure was mostly absent and most of the other sexual progeny also do not spread and seem to be lost in next year. Fortunately, the frequency of virulence towards the *Rpi-blb1 R* gene in the Dutch *P. infestans* population is still low. Therefore *Rpi-blb1* and similar *R* genes, particularly in combination, may provide effective protection in an integrated pest management strategy where monitoring of the *P. infestans* population for virulence and preventive fungicide is applied during periods of high disease pressure. Also preventive action towards the sexual cycle could be of key importance to durable introduction of the *Rpi-blb1* resistance gene and possibly other *R* genes.

MATERIALS AND METHODS

Isolate sampling

Sampling areas (Fig. 1) were categorized according to geographical location and type of potato cultivation. The North East (NE) of the Netherlands is characterized by starch potato crops grown on sandy and peat soils. The North (N), North West (NW) and South West (SW), as well as the Central (C) areas are dominated by ware and seed potatoes grown on clay soils. The regions N and NW are close to each other, therefore treated as one region (N & NW) in this study. The South East (SE) is characterized by ware potato crops on sandy soils.

CHAPTER 5

Production fields with potato late blight outbreaks were reported by the extension services of private companies (Cebeco Agrochemie, Agrifirm, CropSolutions, Agrarische Unie, Profyto, Nestlé, Syngenta, Bayer, HLB, DLV, Dacom and Agrovision) and farmers. Infected leaves were predominantly sampled from production fields but also from allotment gardens, potato dumps and volunteer potato plants. The samples were collected during the whole growing season, generally between the 1st of April and the end of September. Location, sampling date and, if known, cultivars were recorded. A maximum of two samples per field were collected. Samples were never taken from the same plant, and preferably from distinct foci.

Isolation of *Phytophthora infestans*

Infected potato leaflets containing a single lesion were hand-picked and positioned upside down in a Petri dish containing 1.5% water agar. Petri dishes were closed, sealed and sent to the laboratory. A small tissue sample from the edge of the lesion was then placed under a potato tuber disc (approximately 0.5 cm thick) inside an otherwise empty Petri dish and incubated for one week at 15°C at a light intensity of 12 Wm^{-2} for 16 hours. Mycelium emerging from the top of the tuber slice was then transferred to ampicillin contained (1.6 mg/L) pea agar (PA) (Goodwin *et al.*, 1992a). All isolates (Table 1, Appendix Table) were stored in liquid nitrogen and maintained as part of the *P. infestans* collection at Plant Research International, the Netherlands.

Determination of Mating Types

An agar plug, obtained from the edge of an actively growing colony of the test isolate, was transferred to PA on one side of the Petri dish and a similar mycelium plug of an A1 (isolate VK98014) or A2 (isolate EC3425) tester isolate was placed on the other side. Plates were incubated in the dark for 14 - 21 days at 18°C. After mycelial contact between both colonies was established, the contact zone was monitored for the presence of oospores during 7 days using a microscope at 100x magnification. When oospores were found in the Petri dish with the A1 tester isolate the unknown isolate was classified to have the A2 mating type and vice versa.

DNA extraction

Agar plugs of the individual *P. infestans* isolates, taken from the edge of a seven-day old, actively growing colony on PA, were transferred to liquid pea broth. After 3 - 4 days of incubation at 20ºC in the dark, mycelium was collected for lyophilization and subsequent DNA extraction. Genomic DNA was isolated from 20 mg of lyophilized mycelium using the DNeasy 96 Plant Kit (Qiagen, Hilden, Germany). The procedure followed the detailed manufacturer's instructions. Elution was done with 200 µl ultra-pure water. DNA extracts were stored at -20ºC until further use.

Haplotype test

Mt haplotypes were determined using the PCR-RFLP method of Griffith & Shaw (Griffith & Shaw, 1998). Restriction digestions of the amplified regions P2 (*Msp*I) and P4 (*Eco*RI) allowed for differentiation of the four mt haplotypes Ia, Ib, IIa and IIb.

SSR amplification

Twelve microsatellite markers were used (Table 2). Amplification of the SSR markers was carried out as described by Li *et. al.* (in preparation). To facilitate scoring and data entry in the international Euroblight database, eight reference isolates were used (T30-4, 80029, 88133, VK1.4, 90128, IPO-0, IPO428-2, VK98014). Amplifications were run in a PTC200 thermocycler (MJ Research, Waltham, Massachusetts, USA), with an

initial denaturation at 95°C for 15 min, followed by 30 cycles of 95°C for 20 sec, 58°C for 90 sec, and 72°C for 60 sec, and a final extension at 72°C for 20 min. Electrophoresis was performed on a ABI3730 DNA analyzer (Applied Biosystems, USA). 5 µl PCR volume was diluted 1000 times of which 1 µl was added to 9 µl of deionised formamide containing 0.045µl of GeneScan-500LIZ standard (Applied Biosystems, USA). The mixture was capillary electrophoresed on an automated ABI 3730 according to the manufacturer's instructions. SSR allele sizing was performed using GeneMapper v3.7 software (Applied Biosystems, USA).

Genetic data analysis

Clone correction - To eliminate the bias imposed by the large asexual reproductive capacity and avoid redundancy in the collection (Kumar *et al.*, 1999, Chen & McDonald, 1996), a "clone-corrected data set" was constructed by including only one representative isolate of each genotype. Thus, a data subset containing 358 isolates remained. This data subset was used for the analysis of the Hardy-Weinberg equilibrium (HWE), linkage disequilibrium (LD) and served as input to the STRUCTURE software and the Principal Coordinate Analysis (PCA). The full data set, containing 652 isolates, was used for analysis of genetic diversity and distance-based phylogeny.

Ploidy level - In analyzing the variation in microsatellite loci, most *P. infestans* isolates showed a maximum of two alleles per locus as expected for a diploid organism. However, for several cases at least one of the loci showed more than two alleles and these isolates should thus considered to be aneuploid or polyploid. This complicates the analysis since most of the analysis tools assume haploid or diploid data. In previous studies, loci with three alleles were modified to resemble diploid loci by setting one of the alleles to a null allele (Li *et al.*, 2010). In this study, alleles were scored in two ways: 1) by assigning specific allele sizes and 2) by a binary representation of the presence (1) or absence (0) of specific alleles. To avoid the factitious error of heterozygote reduction, a "diploid" data set was created by including only the minimum and maximum alleles for loci with three stable alleles to assign specific loci (Chen *et al.*, 2008). This so-called special diploid data set was then used to calculate basic measures of genetic diversity described below. The complete binary dataset with "0", "1" was used to analyze the genetic structure of the Dutch *P. infestans* population as described below.

Basic measures of genetic diversity - To analyze the variation in microsatellite loci, the observed number of alleles (na), the effective number of alleles (ne) and Shannon's Information index (I) per locus in all populations were estimated using the special diploid data set described above with 652 isolates in POPGENE (49). The significance of deviations from HWE, using Bonferroni corrections, was determined using the special diploid dataset with 358 isolates and exact P values estimated by GENEPOP version 4.0 (Raymond & Rousset, 1995) and the Markov chain algorithm with 10,000 dememorization steps, 100 batches and 1,000 iterations. GENEPOP 4.0 was also used to calculate the observed heterozygosity (Ho), expected heterozygosity (He), the polymorphism information content (PIC) value and the level of LD applied to the special diploid dataset with 652 isolates to determine the extent of distortion from independent segregation of loci. To examine the distribution of genetic variation among and within populations, analysis of molecular variance (AMOVA) using Arlequin version 3.5 (Excoffier *et al.*, 2005) was employed with the binary dataset of 652 isolates. Arlequin 3.5 was also used to perform two versions of the neutral tests, the Ewens-Watterson test and the Ewens-Watterson-Slatkin test to check whether an actual allele frequency deviates significantly from a probability distribution for allele frequencies under the infinite-alleles model in a neutrally evolving population

by the special diploid dataset with 652 isolates. BOTTLENECK software version 1.2.02 (Cornuet & Luikart, 1996) was employed to test the bottleneck hypothesis under a two-phased model of mutation (TPM) using the same dataset as in Arlequin. BOTTLENECK tests for testing the departure from mutation-drift equilibrium based on heterozygosity excess or deficiency.

Genetic structure analysis - Two types of clustering methods were used: a model-based method to detect spatial sub-structures, and a genetic distance-based method to explore the phylogeny by multi-locus genotype data (Excoffier & Heckel, 2006). The spatial genetic structure was analyzed using the Bayesian clustering program STRUCTURE 2.2 (Pritchard *et al.*, 2000) with the binary dataset of 358 isolates. The range for the number of clusters (K) was specified from 1-15. For each run, we examined the output for consistency of clustering assignments and checked parameters for convergence. To infer K values and determine the best level of structure supported by the data, a more formal method developed by Evanno *et al.* (Evanno *et al.*, 2005), calculates the ΔK statistic, the modal value of which can be a useful *ad hoc* indicator for the level of uppermost hierarchical structure (Evanno *et al.*, 2005). To perform ΔK calculations, we randomly assigned the likelihood from each of five STRUCTURE runs from each K into one of five groups, each containing a single likelihood from each K. Within each of these five groups, we then calculated the necessary differences from Ln'(K) and |Ln''(K)|. |Ln''(K)| was averaged over the five groups, and divided by the standard deviation of the likelihood for the ultimate calculation of ΔK.

To validate the genetic substructure, PCA using NTSYS software (Rohlf, 1987, Rohlf, 2008) and the same dataset as in STRUCTURE was conducted to construct plots of the most significant axes for grouping pattern verification.

The genetic distance between individual isolates was calculated using the binary data with all isolates including the references, PowerMarker software (Liu & Muse, 2005) and the Neighbor-Joining (NJ) method based on the proportion of shared alleles distance. PowerMarker is specifically designed to analyze genetic marker data, especially SSR/SNP data. Phylogenetic trees were created by MEGA 4.0 (Tamura *et al.*, 2007) using the distance matrix generated by Powermarker and 1,000 bootstrap replications.

Screening for virulence on potato lines carrying the *Rpi-blb1* gene

A TaqMan PCR was designed to differentiate between *Rpi-blb1* virulent and *Rpi-blb1* avirulent *P. infestans* isolates through amplification of the *Avrblb1/ipiO* class I region. *P. infestans* isolates lacking class I *ipiO* variants were shown to be virulent on *Rpi-blb1* (Champouret *et al.*, 2009). The PCR was performed in a total volume of 30 µl in a AB mastermix (Applied Biosystems, Veenendaal, the Netherlands) using the 250 nM primers of FW_145 (gaagagcgggcgttttct) and RV_227 (gtcttggactgagtgc) to amplify the *Avrblb1/ipiO* class I region and 83.3 nM of the FAM labeled probe Ipio_1_LNA4 (ctttatgGattcaaACTtgga) to visualize the amplification. As a positive control the ITS region was amplified using 50 nM FITS1-15ph (tgcggaaggatcattaccacacc) and RITS1-279ph (gcgagcctagacatccactg) visualized by an 83.3 nM of a VIC labeled probe (cggcTACtgctggc). The PCR profile consisted of 10 min at 95°C followed by 40 cycles of alternation between 95°C (15 sec) and 60°C (60 sec). The specificity of the TaqMan was validated on avirulent isolates T30-4, 90128, 98014, VK1.4, PIC99183, and virulent isolates PIC99177, PIC99189 as well as of cloned fragments of the *Avrblb1* gene/class I *ipiO* gene of these isolates.

CHAPTER 5

170 x 240 mm

CHAPTER 6

Large sub-clonal variation in
Phytophthora infestans populations associated
to Ecuadorian potato landraces

R.A. Delgado, A. Monteros-Altamirano, Y. Li, R.G.F. Visser, T.A.J. van der Lee, B. Vosman

CHAPTER 6

SUMMARY

The population of *Phytophthora infestans* on potato landraces in three provinces (Carchi, Chimborazo and Loja) of Ecuador was analysed. All isolates were of the A1 mating type. SSRs were used to assess the genetic diversity of the isolates. The *P. infestans* isolates from the potato landraces grouped in a single clade together with reference isolates belonging to the clonal lineage EC-1. Of the 66 SSRs profiles obtained, 31 sub-clonal genotypes were identified. The Shannon diversity indexes calculated from the SSRs data showed that the diversity in Carchi (2.44) was higher than the diversity in Chimborazo (1.94) and Loja (2.04). The latter two were not significantly different from each other. From the Evenness values for SSRs profiles, a high value was observed in Loja (0.93), were most isolates were different. Nevertheless all isolates belonged to the EC-1 clonal lineage. The 66 isolates, constituted 49 different races on the *S. demissum* differential set (*R1* to *R11*). The *P. infestans* population observed was complex, and virulent on 4 to 11 *R-genes*. Significant differences were observed in the Shannon diversity indexes among the three provinces, Carchi being the largest (3.15) and Loja the lowest (1.89). The Evenness values showed that there was no predominance of a particular race. This single ancient clonal lineage shows large sub-clonal variation and has developed virulence for all *R-genes* tested even in the absence of a clear selection pressure. In addition it is able to spread among the partial resistant landraces of potato grown in small acreages. In combination this signifies the big challenges to deploy *R*-gene management strategies to obtain durable control of late blight in potato.

INTRODUCTION

Studies on the oomycete *Phytophthora* associated with *Solanaceae* in Ecuador, showed the presence of two closely related species, *P. infestans* and *P. andina*. *P. infestans* is the causal agent of late blight attacking potato and tomato, which is the most important potato disease in Ecuador (Oyarzún *et al.*, 2002). Two clonal lineages, EC-1 and US-1, both of the A1 mating type have been identified (Forbes *et al.*, 1997). The EC-1 lineage, is predominant in potatoes and US-1 in tomatoes (Forbes *et al.*, 1997, Oyarzun *et al.*, 1998). *P. andina* which has been found only in Ecuador, exists in two clonal lineages, EC-2 and EC-3, and causes disease in non-tuber bearing hosts such as *Solanum betaceaum*, *Solanum quitoense*, *Solanum hispidum*, and *Solanum muricatum*(Adler *et al.*, 2004, Ordoñez *et al.*, 2000, Oliva *et al.*, 2010). For *P. andina* the two mating types (A1 and A2) were found in Ecuador (Oliva *et al.*, 2010).

Studies on the race structure of *P. infestans* populations isolated from cultivated potatoes in Ecuador showed an increase in the complexity from a predominance of avirulent isolates (INIAP, 1974, INIAP, 1975, INIAP, 1976) to complex races, which was suggested to have resulted from the displacement of the US-1 on potatoes by the EC-1 lineage (Forbes *et al.*, 1997). Additionally, EC-1 was found to be more aggressive on potato than the US-1 clonal lineage (Oyarzun *et al.*, 1998). Forbes *et al.* (1997) found 24 races infecting potatoes in Ecuador, 14 in Carchi, 14 in Chimborazo and 8 in Loja. More recently, in commercial varieties and selected clones from the INIAP's national potato breeding program, 27, 17 and 37 races were found in Carchi, Cotopaxi and Pichincha provinces, respectively (Tello, 2008). In Perú, three clonal lineages, including EC-1 were identified that infect potato landraces (Garry *et al.*, 2005).

There are more than 400 potato landraces in Ecuador (Cuesta *et al.*, 2005). These landraces are the result of selection and conservation carried out by small scale farmers in the highlands (Cuesta *et al.*, 2005). In contrast to conventional potato cultivation these potatoes are cultivated on small acreage, with low input of pesticides and often several landraces together (Cuesta *et al.*, 2005, Monteros & Reinoso, 2010). They constitute a potential source of genetic variation for breeding purposes, like quality, earliness and resistance to biotic and abiotic stresses.

Despite the importance of *P. infestans* and the fact that much survey work on population structure has been carried out, studies of its molecular diversity have been limited by the power of the genetic markers and difficulties in comparing results between laboratories. Work has revealed the presence of a standard microsatellite (SSR) set for analyzing the population

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diversity and comparable results between laboratories (Li *et al.* preparation). SSRs are tandemly repeated motifs of one to six bases found in the nuclear genomes of all eukaryotes tested and are often abundant and evenly dispersed (Tautz & Renz, 1984, Lagercrantz *et al.*, 1993). Microsatellite sequences are usually characterized by a high degree of length polymorphism, and are ideal single-locus co-dominant markers for genetical studies. In this study, the standard SSR set will be applied to genotypically dissect the *P. infestans* in Ecuador.

Despite all previous studies on *Phytophtora* species, there are no specific studies on populations of *P. infestans* associated with Ecuadorian potato landraces and it is unknown if there are any other lineages or species present on them. The aim of this research was to characterize the *P. infestans* populations associated with Ecuadorian potato landraces, compare these to the populations previously reported on commercial potatoes to assess the impact of small scale farming on the *P. infestans* population and study the genetic diversity among the isolates collected.

RESULTS

Isolate collection

A total of 66 *P. infestans* isolates were collected from 16 farms (Table 2). Thirty-seven isolates from Carchi, 20 from Chimborazo and 9 from Loja, during 2009-2010. The reason that the number collected in Loja was low, was because of a severe drought that occurred in that province at the time of collection, making it difficult to obtain samples.

Isolate characterization

All isolates were of the A1 type mating type. The number of races collected and associated virulence factors is shown in Table 3. There were 49 different races in total. Twenty seven races were identified in Carchi, 18 in Chimborazo and seven in Loja. Seventy-five percent of the isolates carried 7 to 11 avirulence genes in Carchi. Eighty-five and fifty-five percent of isolates carried 7 to 10 avirulence genes in Chimborazo and Loja, respectively. One race (1,3,4,5,6,7,8,9,19,11) was present in all three provinces. The rest was restricted to one site. Race 1,3,4,5,6,7,10,11 was the more frequent (6 %) among all the isolates and restricted to Carchi (Table 3).

The percentage of virulence on the differential set of the isolates from the different provinces is shown in Fig.   1. From this, it can be seen that all isolates are virulent on *R4*, except for one isolate originating from Carchi. Virulence on the *R2* differential was the less frequent one and that virulence was absent in isolates from Loja. When we analyzed the

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complexity of the isolates from the three areas we observed that all isolates were virulent on at least 4 differentials in all regions (Fig. 2). In the province Carchi and Chimborazo, isolates were able to overcome from 4 to 11 *R* genes and in Loja from 5 to 10.

Table 1. Reference isolates used in the genetic study

Isolate	Host	Species	RG57	Mating Type	Year	Province	Country
1917	*S. muricatum*				1995	Pichincha	Ecuador
2946					1996		Ecuador
3166	*S. brevifolium*	*P. andina*	EC2	A2	1998	Pichincha	Ecuador
3236	*Solanum spp.*		EC2	A2	1999	Pichincha	Ecuador
3337	*S. paucijugum*				2001	Cotopaxi	Ecuador
3351	*S. tuberosum*	*P. infestans*	EC1	A1	2001	Tungurahua	Ecuador
3355	*S. tuberosum*	*P. infestans*	EC1	A1	2001	Tungurahua	Ecuador
3364	*S. betaceum*	*P. andina*	EC3		2001	Tungurahua	Ecuador
3365	*Anarrhichomenum*	*P. andina*	EC2				Ecuador
3374	*S. brevifolium*				2001	Tungurahua	Ecuador
3380	*S. betaceum*	*P. andina*	EC3		2001	Tungurahua	Ecuador
3381	*S. lycopersicum*	*P. infestans*	US1	A1	2001	Tungurahua	Ecuador
3383	*S. phureja*	*P. infestans*	EC1	A1	2001	Pichincha	Ecuador
3390	*S. solisii*	*P. infestans*	EC1	A1	2001	Tungurahua	Ecuador
3401	*S. betaceum*	*P. andina*	EC3		2001	Tungurahua	Ecuador
3404	*S. caripense*	*P. infestans*	US1	A1	2001	Tungurahua	Ecuador
3420	*S. minutifoliolum*				2001	Tungurahua	Ecuador
3421	*S. muricatum*	*P. andina*	EC2	A2	2001	Tungurahua	Ecuador
3424	*S. muricatum*	*P. andina*	EC2	A2	2001	Tungurahua	Ecuador
3425	*S. brevifolium*	*P. andina*			2001	Tungurahua	Ecuador
3447	*S. phureja*	*P. infestans*	EC1	A1	2001	Cotopaxi	Ecuador
VK1.4	*S. tuberosum*	*P. infestans*	US-1	A1	1958		Netherlands
88133	*S. tuberosum*	*P. infestans*		A2	1988		Netherlands
80029	*S. tuberosum*	*P. infestans*		A1	1980		Netherlands
90128	*S. tuberosum*	*P. infestans*		A2	1990		Netherlands
IPO428-2	*S. tuberosum*	*P. infestans*		A2	1992		Netherlands
T30-4	*S. tuberosum*	*P. infestans*		A1	1998		Netherlands
98014	*S. tuberosum*	*P. infestans*		A1	1998		Netherlands
IPO Complex	*S. tuberosum*	*P. infestans*		A2	1982		Belgium

Table 2. Isolates associated to potato landraces in Ecuador

Isolate	Variety Name	Accesion ID	Race	Place	Province	Altitude (masl)	Latitude	Longitude	Collection Date	SSR Multilocus Genotype
IC1.3	Chaucha Ratona	AXC-1	1,3,4,5,6,7,8,10,11	Ipueran, Julio Andrade, Tulcán	Carchi	3229	N00°40'394"	W77°40'446"	2/Jun/2009	EC-1_001
IC1.4	Chaucha Ratona	AXC-1	1,2,4,6,7,9,10	Ipueran, Julio Andrade, Tulcán	Carchi	3229	N00°40'394"	W77°40'446"	2/Jun/2009	EC-1_003
IC2.2	Amarilla		1,4,5,7,8,10,11	Ipueran, Julio Andrade, Tulcán	Carchi	3229	N00°40'394"	W77°40'446"	2/Jun/2009	EC-1_001
IC2.3	Amarilla		1,3,4,6,7,10,11	Ipueran, Julio Andrade, Tulcán	Carchi	3229	N00°40'394"	W77°40'446"	2/Jun/2009	EC-1_001
IC3.3	Sulipamba	AXC-3	1,4,5,7,10,11	Agua Fuerte, parroquia El Carmelo, Tulcán	Carchi	2985	N00°39'578"	W77°36'560"	2/Jun/2009	EC-1_005
IC3.4	Sulipamba	AXC-3	1,3,4,5,7,8,10,11	Agua Fuerte, parroquia El Carmelo, Tulcán	Carchi	2985	N00°39'578"	W77°36'560"	2/Jun/2009	EC-1_005
IC3.5	Sulipamba	AXC-3	1,4,6,7,8,10,11	Agua Fuerte, parroquia El Carmelo, Tulcán	Carchi	2985	N00°39'578"	W77°36'560"	2/Jun/2009	EC-1_001
IC4.2	Violeta		1,2,3,4,5,6,7,9,10	Agua Fuerte, parroquia El Carmelo, Tulcán	Carchi	2985	N00°39'578"	W77°36'560"	2/Jun/2009	EC-1_017
IC4.3	Violeta		2,3,4,5,7,10	Agua Fuerte, parroquia El Carmelo, Tulcán	Carchi	2985	N00°39'578"	W77°36'560"	2/Jun/2009	EC-1_018
IC5.1	Chaucha Amarilla	AXC-14	1,3,4,5,6,7,10,11	Troya, comunidad Virgen de Fátima, Urbina, Tulcán	Carchi	3362	N00°44'451"	W77°42'129"	2/Jun/2009	EC-1_001
IC6.1	Chaucha Ratona	AXC-1	1,2,3,4,5,7,9,10,11	Troya, comunidad Virgen de Fátima, Urbina, Tulcán	Carchi	3362	N00°44'451"	W77°42'129"	2/Jun/2009	EC-1_001
IC6.2	Chaucha Ratona	AXC-1	1,3,4,7,10	Troya, comunidad Virgen de Fátima, Urbina, Tulcán	Carchi	3362	N00°44'451"	W77°42'129"	2/Jun/2009	EC-1_004
IC8.3	Sabanera	AC-34	1,4,7,9	Chulamuez, Tulcán	Carchi	3316	N00°44'913"	W77°46'389"	2/Jun/2009	EC-1_001
IC8.4	Sabanera	AC-34	1,4,5,7,8,9	Chulamuez, Tulcán	Carchi	3316	N00°44'913"	W77°46'389"	2/Jun/2009	EC-1_004
IC9.1	Chaucha Amarilla		1,3,4,7,10,11	Tufiño, Tulcán	Carchi	3396	N00°47'806"	W77°52'126"	2/Jun/2009	EC-1_020
IC9.2	Chaucha Amarilla		1,4,5,7,8,10,11	Tufiño, Tulcán	Carchi	3396	N00°47'806"	W77°52'126"	2/Jun/2009	EC-1_016
IC9.3	Chaucha Amarilla		1,4,5,7,8,10,11	Tufiño, Tulcán	Carchi	3396	N00°47'806"	W77°52'126"	2/Jun/2009	EC-1_009
IC9.4	Chaucha Amarilla		5,6,7,8,10,11	Tufiño, Tulcán	Carchi	3396	N00°47'806"	W77°52'126"	2/Jun/2009	EC-1_009
IC10.1	Pamba Roja	AXC-17	1,3,4,5,6,8,10,11	Tufiño, Tulcán	Carchi	3396	N00°47'806"	W77°52'126"	2/Jun/2009	EC-1_011
IC10.2	Pamba Roja	AXC-17	1,2,3,4,5,6,7,8,10,11	Tufiño, Tulcán	Carchi	3396	N00°47'806"	W77°52'126"	2/Jun/2009	EC-1_012
IC10.3	Pamba Roja	AXC-17	1,2,3,4,5,7,8,9,10,11	Tufiño Tulcán	Carchi	3396	N00°47'806"	W77°52'126"	2/Jun/2009	EC-1_001
IC10.5	Pamba Roja	AXC-17	1,2,3,4,5,6,7,8,9,10,11	Tufiño, Tulcán	Carchi	3396	N00°47'806"	W77°52'126"	2/Jun/2009	EC-1_005
IC11.2	Curipamba	AXC-16	2,4,5,6,7,9	Tufiño, Tulcán	Carchi	3396	N00°47'806"	W77°52'126"	2/Jun/2009	EC-1_013
IC11.3	Curipamba	AXC-16	1,2,3,4,5,7,9,10	Tufiño, Tulcán	Carchi	3396	N00°47'806"	W77°52'126"	2/Jun/2009	EC-1_001
IC12.1	Gualcalá	AXC-22	1,3,4,5,6,7,8,10,11	Tufiño, Tulcán	Carchi	3396	N00°47'806"	W77°52'126"	2/Jun/2009	EC-1_006
IC12.3	Gualcalá	AXC-22	1,3,4,5,7,10,11	Tufiño, Tulcán	Carchi	3396	N00°47'806"	W77°52'126"	2/Jun/2009	EC-1_005

IC13.4	Parda Suprema	AXC-21	1,3,4,5,6,7,8,10,11	'Tufiño, Tulcán	Carchi	3396	N00°47'806"	W77°52'126"	2/Jun/2009	EC-1_012
IC14.2	Chaucha Amarilla		1,3,4,5,6,7,10,11	Talla/Chapuez Chico, Tulcán	Carchi	3001	N00°46'789"	W77°41'522"	3/Jun/2009	EC-1_031
IC14.4	Chaucha Amarilla		1,3,4,7,8,10,11	Talla/Chapuez Chico, Tulcán	Carchi	3001	N00°46'789"	W77°41'522"	3/Jun/2009	EC-1_006
IC15.3	Rosada		1,4,7,10,11	Talla/Chapuez Chico, Tulcán	Carchi	3001	N00°46'789"	W77°41'522"	3/Jun/2009	EC-1_016
IC16.3	Violeta		1,2,3,4,5,6,7,8,9,10,11	Talla/Chapuez Chico, Tulcán	Carchi	3001	N00°46'789"	W77°41'522"	3/Jun/2009	EC-1_001
IC17.2	Pura Sangre	AXC-13	1,3,4,5,6,7,8,9,10,11	Casa Fría, Julio Andrade, Tulcán	Carchi	3296	N00°42'218"	W77°44'647"	3/Jun/2009	EC-1_021
IC17.3	Pura Sangre	AXC-13	n.e.	Casa Fría, Julio Andrade, Tulcán	Carchi	3296	N00°42'218"	W77°44'647"	3/Jun/2009	EC-1_003
IC18.1	Curipamba		1,3,4,6,7,8,10,11	Casa Fría, Huaca, Montufar	Carchi	3487	N00°43'034"	W77°45'728"	3/Jun/2009	EC-1_001
IC18.2	Curipamba		1,2,3,4,5,6,7,8,910,11	Casa Fría, Huaca, Montufar	Carchi	3487	N00°43'034"	W77°45'728"	3/Jun/2009	EC-1_030
IC18.4	Curipamba		1,3,4,5,6,7,10,11	Casa Fría, Huaca, Montufar	Carchi	3487	N00°43'034"	W77°45'728"	3/Jun/2009	EC-1_004
IC19.4	Rosada		1,3,4,5,6,7,10,11	Casa Fría, Huaca, Montufar	Carchi	3487	N00°43'034"	W77°45'728"	3/Jun/2009	EC-1_001
IL4.1	Chaucha Negra	MPG-26	1,4,7,8,9,10,11	Ciudadela, San Lucas, Loja	Loja	2556	S03°43'261"	W79°14'788"	2/Jul/2009	EC-1_014
IL4.3	Chaucha Negra	MPG-26	1,4,7,8,9,10,11	Ciudadela, San Lucas, Loja	Loja	2556	S03°43'261"	W79°14'788"	2/Jul/2009	EC-1_001
IL4.4	Chaucha Negra	MPG-26	1,4,7,8,9,11	Ciudadela, San Lucas, Loja	Loja	2556	S03°43'261"	W79°14'788"	2/Jul/2009	EC-1_029
IL11.9	Semibolona		1,3,4,5,6,7,8,9,10,11	Huangara, Taquil, Loja	Loja	2402	S03°55'563"	W79°16'281"	13/Aug/2009	EC-1_007
IL22.3	Hualcala	MOPG-011	1,4,7,8,9	Llaco, Tenta, Saraguro	Loja	2552	S03°38'523"	W79°17'334"	30/Sep/2009	EC-1_028
IL18	Negra Carrizo	MOPG-002	1,4,7,8,9,10	Nauchin, Manu, Saraguro	Loja	2840	S 3°32'518"	W79°23'067"	30/Sep/2009	EC-1_025
IL18T1	Negra Carrizo	MOPG-002	1,3,4,5,6,7,9,10,11	Nauchin, Manu, Saraguro	Loja	2840	S 3°32'518"	W79°23'067"	30/Sep/2009	EC-1_026
IL18T2	Negra Carrizo	MOPG-002	1,4,7,9,11	Nauchin, Manu, Saraguro	Loja	2840	S 3°32'518"	W79°23'067"	30/Sep/2009	EC-1_027
IL21	Bodeguera Blanca	MOPG-009	1,3,4,5,6,7,9,10,11	Chorro Blanco, Manu, Saraguro	Loja	2820	S03°31'589"	W79°23'193"	30/Sep/2009	EC-1_007
ICh2.4	Chaucha Blanca	AMA-301	1,3,4,6,7,8,10,11	Utuñag, Penipe	Chimborazo	3032	S01°33'115"	W78°29'379"	1/Dec/2009	EC-1_002
ICh2.5	Chaucha Blanca	AMA-301	1,3,4,5,6,8,9,10,11	Utuñag, Penipe	Chimborazo	3032	S01°33'115"	W78°29'379"	1/Dec/2009	EC-1_001
ICh2.7	Chaucha Blanca	AMA-301	1,3,4,5,7,9,10,11	Utuñag, Penipe	Chimborazo	3032	S01°33'115"	W78°29'379"	1/Dec/2009	EC-1_023
ICh2.9	Chaucha Blanca	AMA-301	1,2,4,5,7,8,9,10,11	Utuñag, Penipe	Chimborazo	3032	S01°33'115"	W78°29'379"	1/Dec/2009	EC-1_008
ICh2.10	Chaucha Blanca	AMA-301	1,3,4,5,6,7,8,9,10,11	Utuñag, Penipe	Chimborazo	3032	S01°33'115"	W78°29'379"	1/Dec/2009	EC-1_022
ICh3.2	Chaucha Negra Pera	AMA-302	1,4,7,8	Utuñag, Penipe	Chimborazo	3032	S01°33'115"	W78°29'379"	1/Dec/2009	EC-1_002
ICh3.3	Chaucha Negra Pera	AMA-302	1,4,6,7,8,9,11	Utuñag, Penipe	Chimborazo	3032	S01°33'115"	W78°29'379"	1/Dec/2009	EC-1_010
ICh3.4	Chaucha Negra Pera	AMA-302	1,4,5,8,9,10,11	Utuñag, Penipe	Chimborazo	3032	S01°33'115"	W78°29'379"	1/Dec/2009	EC-1_002
ICh3.5	Chaucha Negra Pera	AMA-302	1,3,4,5,6,8,9,10,11	Utuñag, Penipe	Chimborazo	3032	S01°33'115"	W78°29'379"	1/Dec/2009	EC-1_010
ICh3.7	Chaucha Negra Pera	AMA-302	1,3,4,5,6,7,8,9,11	Utuñag, Penipe	Chimborazo	3032	S01°33'115"	W78°29'379"	1/Dec/2009	EC-1_008
ICh4.6	Semiuvilla		1,4,5,7,9,10,11	Matus, Penipe	Chimborazo	2679	S01°33'316"	W78°30'429"	1/Dec/2009	EC-1_019
ICh4.8	Semiuvilla		1,3,4,5,6,7,8,9,10	Matus, Penipe	Chimborazo	2679	S01°33'316"	W78°30'429"	1/Dec/2009	EC-1_002
ICh4.9	Semiuvilla		1,2,4,7,9,10,11	Matus, Penipe	Chimborazo	2679	S01°33'316"	W78°30'429"	1/Dec/2009	EC-1_019
ICh4.11	Semiuvilla		1,3,4,7,8,10	Matus, Penipe	Chimborazo	2679	S01°33'316"	W78°30'429"	1/Dec/2009	EC-1_002

ICh21.3	Curipamba	1,4,5,6,7,8,9,10,11	Bacun, Chunchi	Chimborazo	3121	S02°17'294"	W78°53'464"	8/mar/2010	EC-1_003
ICh21.4	Curipamba	1,4,5,6,7,8,9,10,11	Bacun, Chunchi	Chimborazo	3121	S02°17'294"	W78°53'464"	8/mar/2010	EC-1_003
ICh21.6	Curipamba	1,3,4,6,7,9,10,11	Bacun, Chunchi	Chimborazo	3121	S02°17'294"	W78°53'464"	8/mar/2010	EC-1_003
ICh21.7	Curipamba	1,4,5,7,8,9,10,11	Bacun, Chunchi	Chimborazo	3121	S02°17'294"	W78°53'464"	8/mar/2010	EC-1_003
ICh21.8	Curipamba	4,5,7,8,9,10,11	Bacun, Chunchi	Chimborazo	3121	S02°17'294"	W78°53'464"	8/mar/2010	EC-1_024
ICh21.9	Curipamba	1,4,7,9,10,11	Bacun, Chunchi	Chimborazo	3121	S02°17'294"	W78°53'464"	8/mar/2010	EC-1_003

170 x 240 mm

Table 3. Races of *P. infestans* associated to potato landraces in Ecuador

No.	Race	Carchi No.	Chimborazo No.	Loja No.	All sites No.
1	1,4,7,8		1		1
2	1,4,7,9	1			1
3	1,3,4,7,10	1			1
4	1,4,7,10,11	1			1
5	1,4,7,8,9			1	1
6	1,4,7,9,11			1	1
7	1,3,4,7,10,11	1			1
8	1,3,4,7,8,10		1		1
9	1,4,5,7,10,11	1			1
10	1,4,5,7,8,9	1			1
11	1,4,7,8,9,10			1	1
12	1,4,7,8,9,11			1	1
13	1,4,7,9,10,11		1		1
14	2,3,4,5,7,10	1			1
15	2,4,5,6,7,9	1			1
16	5,6,7,8,10,11	1			1
17	1,2,4,6,7,9,10	1			1
18	1,2,4,7,9,10,11		1		1
19	1,3,4,5,7,10,11	1			1
20	1,3,4,6,7,10,11	1			1
21	1,3,4,7,8,10,11	1			1
22	1,4,5,7,8,10,11	3			3
23	1,4,5,7,9,10,11		1		1
24	1,4,5,8,9,10,11		1		1
25	1,4,6,7,8,10,11	1			1
26	1,4,6,7,8,9,11		1		1
27	1,4,7,8,9,10,11			2	2
28	4,5,7,8,9,10,11		1		1
29	1,2,3,4,5,7,9,10	1			1
30	1,3,4,5,6,7,10,11	4			4
31	1,3,4,5,6,8,10,11	1			1
32	1,3,4,5,7,8,10,11	1			1
33	1,3,4,5,7,9,10,11		1		1
34	1,3,4,6,7,8,10,11	1	1		2
35	1,3,4,6,7,9,10,11		1		1
36	1,4,5,7,8,9,10,11		1		1
37	1,2,3,4,5,6,7,9,10	1			1
38	1,2,3,4,5,7,9,10,11	1			1
39	1,2,4,5,7,8,9,10,11		1		1
40	1,3,4,5,6,7,8,10,11	3			3
41	1,3,4,5,6,7,8,9,10		1		1
42	1,3,4,5,6,7,8,9,11		1		1
43	1,3,4,5,6,7,9,10,11			2	2
44	1,3,4,5,6,8,9,10,11		2		2
45	1,4,5,6,7,8,9,10,11		2		2
46	1,2,3,4,5,6,7,8,10,11	1			1

47	1,2,3,4,5,7,8,9,10,11	1			1
48	1,3,4,5,6,7,8,9,10,11	1	1	1	3
49	1,2,3,4,5,6,7,8,9,10,11	3			3
	Total of isolates	36	20	9	65

Table 4. Number of races, complexity, Shannon index and Evenness of *Phytophthora infestans* populations associated to potato landraces collected in three provinces in Ecuador

Provinces	No. of farms	No. of isolates	No. of races	C_i^a	C_p^b	$H_s^{c,d}$	E^e
Carchi	8	36	27	7.72	7.11	3.15 a	0.88
Chimborazo	3	20	18	7.80	7.67	2.86 b	0.95
Loja	5	9	7	7.11	6.86	1.89 c	0.86
All sites	16	65	49	7.66	7.31	3.78	0.91

[a] C_i: mean number of avirulence genes per isolate.

[b] C_p: mean number of avirulence genes per race.

[c] H_s: Shannon Index.

[d] Values followed by same letter are not significantly different (α=0.05) according to the t-test of Hutcheson for pairwise comparisons.

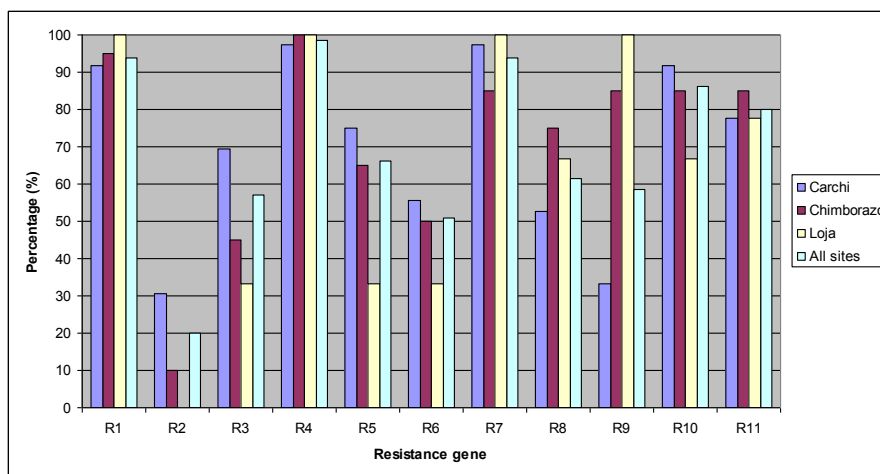[e] Evenness is based on Shannon index.

Fig. 1. Virulence percentage to potato *R*-genes of the differential set among isolates of *P. infestans* associated to potato landraces in Ecuador.

170 x 240 mm

Table 5. SSR multilocus genotypes of *P. infestans* isolates associated to potato landraces in Ecuador

Genotype	Marker												Isolates No.			
	D13	G11	Pi04	Pi4B	Pi63	Pi70	SSR2	SSR3	SSR4	SSR6	SSR8	SSR11	Carchi	Chimborazo	Loja	Overall
EC-1_001	134	162	170/174	206/214/218	279	191	173/175	268	283/291	242/244	264/266	330/356	12	1	1	14
EC-1_002	134	162	170/174	206/214/218	279	191	173/175	268	283/291/293	242/244	264/266	330/356		5		5
EC-1_003	132/134	162	170/174	206/214/218	279	191	173/175	268	283/291/293	242/244	264/266	330/356	2	5		7
EC-1_004	132/134	162	170/174	206/214/218	279	191	173/175	266/268	283/291	242/244	264/266	330/356	3			3
EC-1_005	132/134	162	170/174	206/214/218	279	191	173/175	268	283/291	242/244	264/266	330/356	3			3
EC-1_006	134	162	170/174	206/218/222	279	191	173/175	264/268	283/291	242/244	264/266	330/356	2			2
EC-1_007	134	162	170/174	206/214/218	279	191	173/175	268	283/291/295	242/244	264/266	330/356			2	2
EC-1_008	134	162	170/174	206/214/218	279	191	173/176	268	283/291	242/244	264/266	330/356		2		2
EC-1_009	134/136	162	170/174	206/214/218	279	191	173/175	268	283/291/295	242/244	264/266	330/356	2			2
EC-1_010	134	162	170/174	206/214/218	279	191	173/175	268	283/293	242/244	264/266	330/356		2		2
EC-1_011	134	162	170/174	214/218	279	191	173/175	268	283/291	242/244	264/266	356	1			1
EC-1_012	134/136	162	170/174	206/214/218	279	191	173/175	268	283/291	242/244	264/266	330/356	2			2
EC-1_013	132/134	162	170/174	206/214/218	279	191	173/175	268	283/291	244	264/266	330/356	1			1
EC-1_014	134/138	162	170/174	206/214/218	279	191	173/175	268	283/291/295	242/244	264/266	330/356			1	1
EC-1_015	134/138	162	170/174	206/214/218	279	191	173/175	268	283/293	242/244	264/266	330/356	1			1
EC-1_016	134	162/166	170/174	206/214/218	279	191	173/175	268	283/289/291	242/244	264/266	330/356	2			2
EC-1_017	134	162	170/174	206/214/218	279	191	173/175	268	283/291	242/244	264/266	356	1			1
EC-1_018	134	162	170/174	206/214/218	279	191	173/175	268	283/291/293	242/244	264/266	356	1			1
EC-1_019	134/138	162	170/174	206/214/218	279	191	173/175	268	283/291/293	242/244	264/266	330/356		2		2
EC-1_020	134	162	170/174	206/214/218	279	191	173	266/268	283/291/293	242/244	264/266	330/356	1			1
EC-1_021	132/134	162	170/174	206/214/218	279	191	173/175	266/268	283/289/293	242/244	264/266	330/356	1			1
EC-1_022	134	158/162	170/174	206/214/218	279	191	173/175	266/268	283/293	242/244	264/266	330/356		1		1
EC-1_023	130/134	162	170/174	206/214/218	279	191	173/175	268	283/291/293	244	264/266	330/356		1		1
EC-1_024	132/134	162	170/174	206/214/218	279	191	173/175	266/268	283/291/295	242/244	264/266	330/356		1		1
EC-1_025	134/142	162	170/174	206/214/218	279	191	173/175	268	283/291/295	242/244	264/266	330/356			1	1
EC-1_026	134/142	162	170/174	206/214/218	279	191	173/175	266/268	283/291/295	242/244	264/266	330/356			1	1
EC-1_027	134	162	170/174	206/214/218	279	191	173/175	266/268	283/291/295	242/244	264/266	330/356			1	1
EC-1_028	134	162	170/174	214/218	279	191	173	268	283/291/293	242/244	264/266	330/356			1	1
EC-1_029	134	160/162	170/174	206/214/218	279	191	173/175	268	283/291	242/244	264/266	330/356			1	1
EC-1_030	134	162	170/174	206/214	279	191	173/175	268	283/291	242/244	264/266	330/356	1			1
EC-1_031	134	162	170/174	206/218/222	279	191	173/175	264/268	283/291	244	264/266	330/356	1			1

Table 6. SSR genotypes observed in reference isolates

		D13	G11	Pi04	Pi4B	Pi63	P170	SSR2	SSR3	SSR4	SSR6	SSR8	SSR11
EC3337	EC-1	134/136	162	170/174	206/214/218	279	191	173/175	264/268	283/291	242/244	264/266	330/356
EC3351	EC-1	134	162	170/174	206/214/218	279	191	173/175	268	283/291/295	242/244	264/266	330/356
EC3355	EC-1	134	162	170/174	206/216/218	279	191	173/175	268	283/291/293	242/244	264/266	330/356
EC3383	EC-1	134	162	170/174	206/214/218	279	191	173/175	268	283/291/295	242/244	264/266	330/356
EC3390	EC-1	134	162/174	170/174	206/214/218	279	191	173/175	268	283/291/295	242/244	264/266	330/356
EC3420	EC-1	134	162	170/174	206/214/218	279	191	173/175	268	283/291	242/244	264/266	330/356
EC3447	EC-1	134	162	170/174	206/214/218	279	191	173/175	268	283/291/295	242/244	264/266	330/356
EC3404	US-1	134/136	158/168	170/174	214/218	270/273/279	188/191	173/177	266	287/289/299	244	266	340/356
EC3381	US-1	136/140	158	174	214/222	270/273/279	188/191	173/177	266	289/297	244	266	340/356
EC1917	US-1	134	158/168	170/174	214/218	270/273/279	188/191	173	266	287/289/299	244	266	340/356
VK1.4	US-1	138	158/162	170/174	214/218	270/273/279	188/191	173/177	266	287/289	244	266	340/356
428.2		134	162	170/174	214/218	273/279	191/194	173	258/268	293	242	260/266	330/356
80029		116/134		172	214	279	191	173	268	283/293	244	260/266	340
88133		152/154	162/168	174	214	279	191/194	173/175	268/270	287/297	242/244	260/264	330/340
90128			162	172	214/218	273/279	191	173/175	268	293	242/244	260/266	340/356
98014		134	160/162	170/174	218	270/273/279	191/194	173	258/268	287/289/291	242/244	260/264/266	340/356
IPO complex			148/162	172	218	279	191/194	173	268	287/293	244	260/266	340
T30-4		116	162	174	214	279	191	173/175	268/270	283/287	244	260/264	340
EC2946	*P. andina*	147/149	137	174	210/248/268	273		173/176	266	281/287	240/244	260/266	340
EC3166	*P. andina*	147/149	130/140	174	210/248/264/268	273		173/176	264/266	281/287	240/244	260/266	340
EC3236	*P. andina*		130/140	174	210/248/268	273	191	173/176	266	281/287	240/244	260/266	340
EC3364	*P. andina*	142	137	174	210/248/268	273	191	173/176	266	281/287	240/244	260/266	340
EC3365	*P. andina*	105	135	170/174	210/244/297	273		173/176	266	281	240	260/266	356
EC3374	*P. andina*	105	135/166	174	210/248/264/284	273	191	173/176	266	281/287	240/244	260/266	356
EC3380	*P. andina*	105	137/160	174	214/244/256/270	273	191	173/176	266	280	240/244	260/266	340
EC3401	*P. andina*	105	137/172	174	214/244/256/270	273	191	173/176	264	280	240/244	260/266	340
EC3421	*P. andina*	105	135	170/174	210/244/297	273		173/176	266	281	240	260/266	356
EC3424	*P. andina*	105	135	170/174	210/244/297	273		173/176	266	281	240	260/266	356

170 x 240 mm

EC3425   P. andina   108   135   170/174   210/248/289   273   194   173/176   266   281   258/260   356

Table 7. Percentage of isolates containing an allele of the 12 SSR multilocus fingerprint of *P. infestans* and *P. andina*

D13

	N	105	108	116	130	132	134	136	138	140	142	147	149	152	154	162
Landraces isolates	66			2	24	100	6	6			3					
EC-1	7					100	14		50	25	25					
US-1	4							43								
P. infestans Europe	7			29												
P. andina	11	55	9									9	18	18	14	14

G11

	130	135	137	140	148	158	160	162	166	168	172	174
Landraces isolates					2	2	100	3				
EC-1							100		25			14
US-1				14		100	25	86	14	50		
P. infestans Europe						14	9	9	14			9
P. andina	18	45	36	18			9	9	9			

P04

	170	172	174	206	210	214	216	218
Landraces isolates	100		100	97		95		98
EC-1	100	14	100	100		86	14	100
US-1	75		100			100		75
P. infestans Europe		43	57			71		57
P. andina	36		100		82	18		

P4B

	222	244	248	256	264	268	270	284	289	297
Landraces isolates	5									
EC-1	25									
US-1										
P. infestans Europe										
P. andina		45	55	18	18	36	18	9	9	27

Pi63

	N	270	273	279
Landraces isolates	66		100	
EC-1	7		100	
US-1	4	100	100	100
P. infestans Europe	7	14	43	100
P. andina	11	100		

P70

	188	191	194
Landraces isolates		100	
EC-1		100	
US-1	100	100	
P. infestans Europe		100	57
P. andina		100	9

SSR2

	173	175	176	177
Landraces isolates	100	94	3	
EC-1		100		
US-1	100	43		
P. infestans Europe				
P. andina	45			100

SSR3

	258	264	266	268	270
Landraces isolates		5	14	100	
EC-1		14	100		
US-1				100	29
P. infestans Europe		29			
P. andina	18	18	91		

SSR4

	280	281	283	287	289	291	293	295	297	299	301
Landraces isolates		100				94	33	14			
EC-1		100				100	14	57			
US-1				75	100				25	50	25
P. infestans Europe				57	14		14	57			
P. andina	18	82		45					14		

SSR6

	240	242	244
Landraces isolates		95	100
EC-1			100
US-1			
P. infestans Europe		57	
P. andina	100		

SSR8

	258	260	264	266	330	340	356
Landraces isolates			100	100	94	100	
EC-1					100	100	
US-1	100	100	100	100			
P. infestans Europe	43	100	43	71	29	71	
P. andina	9	100	91		91		

SSR11

	356
Landraces isolates	100
EC-1	100
US-1	100
P. infestans Europe	86 / 43
P. andina	55 / 45

Table 8. Number of *P. infestans* genotypes found in each province, Shannon index (H_s) and Evenness (E) observed with 12 SSRs on potato landraces in Ecuador

Sites	Sample size	Genotypes	H_s[a]	E	SSRs	
					Alleles/Isolate	Alleles/Genotype
Carchi	37	17	2.44 a	0.68	21	20
Chimborazo	20	9	1.94 b	0.66	21	21
Loja	9	8	2.04 b	0.93	21	21
All	66	31	3.12	0.74	21	21

[a] Values followed by same letter are not significantly different ($\alpha=0.05$) according to the t-test of Hutcheson for pairwise comparisons.

CHAPTER 6

Carchi had the highest Shannon diversity index for race phenotype (3.15) and it was significantly more diverse than Chimborazo (t_H=23.28, df=46.29, p<0.0001) and Loja (t_H= 7.31, df=15.32, p<0.001) (Table 4). Chimborazo, with a Shannon Index of 2.86, was more diverse than Loja (t_H=17, df= 17.13, p<0.0001), which showed the lowest diversity value (1.89). The Evenness among provinces showed slight, non-significant differences. The virulence complexity, expressed by the mean number of avirulence genes per isolate (C_i) and the mean number of avirulence genes per race (C_p), indicates that the most common races were the most complex. This is reflected in the C_i value, which is higher than C_p (Table 4). When analyzing the changes in races over the time in the Ecuadorian population of *P. infestans*, there is increase in diversity, evenness and number of races (Table 11).

Population diversity

Eight of the twelve SSRs were polymorphic with the *P. infestans* isolates from potato landraces. Markers Pi04, Pi63, Pi70 and SSR8 were monomorphic (Table 5). For some markers we observed three or even four alleles in a particular isolate (Fig. 3). In the *P. infestans* isolates from potato landraces, 3 alleles were observed with Pi4B and SSR4 markers (Table 5). In the reference *P. infestans* isolates, 3 alleles were detected with markers Pi4B, Pi63, SSR4 and SSR8 (Table 6). In the case of *P. andina*, three to four alleles were detected with Pi4B marker (Table 6). Several rare alleles were detected. Allele 279 from locus Pi63 was detected exclusively in all *P. infestans* isolates. Allele 188 from locus Pi70 was present in all US-1 reference isolates and absent in the rest of *P. infestans* isolates. In addition, allele 240 from locus SSR6 was just observed in *P. andina* isolates (Table 7).

To dissect the sub-variation within the clonal lineage of *P. infestans*, the absolute ratio of max. and min. peak height at all 8 polymorphic loci was calculated, comparing between the whole sampling collection and the clonal lineage. Three ratio situations were found, Max/Min = 1/1, 2/1, 1/0. The comparison result showed the ratio situation either kept the same or turned to one ratio when only including the clonal isolates except of SSR2 (Fig. 4). At SSR2, of the clonal lineage one isolate have two peaks with equal height and three isolates with only one peak. It indicates the loss of alleles cause the sub-variation in the clonal lineage (Fig. 4).
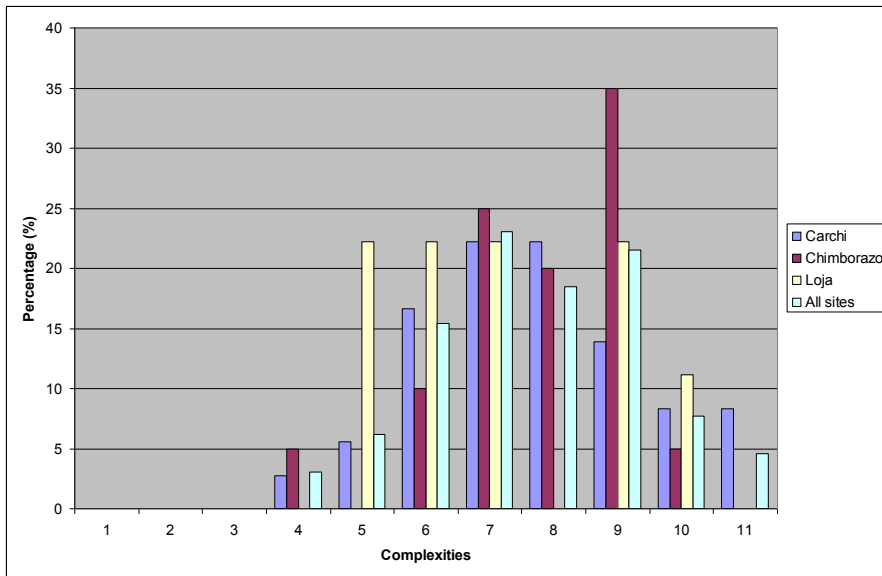
Fig. 2. Number of *R*-genes defeated by the *P. infestans* populations associated to potato landraces in Ecuador.
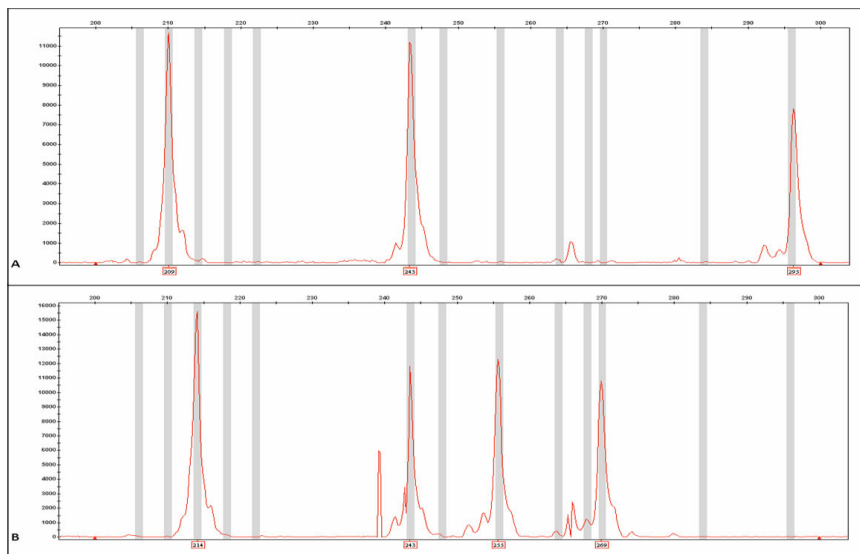
Fig. 3. Electropherograms of the fluoresceinated amplification products for locus Pi4B in isolates of *P. andina*. A: EC3365; B: EC3380.

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The phylogenetic tree obtained from the SSR profiles, showed that the *P. infestans* isolates from potato landraces grouped together in a large clade. This clade included the reference isolates known to belong to the clonal lineage EC-1. They were clearly distant from the reference isolates identified as US-1 and *P. andina* (Fig. 5). Based on the twelve SSR markers, seventeen, nine and eight multilocus genotypes were observed in Carchi, Chimborazo and Loja, respectively (Table 8). In total, 31 different multilocus genotypes were identified. From all, just one (EC-1_001) occurred in the three provinces, but predominated in Carchi. Another one (EC-1_003) was found in two provinces (Carchi and Chimborazo). The rest was restricted to one province (Table 5). There was a clear relation between genotype and geographic origin, but we did not find a relation among the multilocus genotypes and race phenotype. Isolates that belonged to the same race showed different multilocus genotypes and isolates with the same genotype showed different virulence spectra. Even isolates obtained from the same landrace and sharing the same virulence phenotype, could belong to different multilocus genotypes (Tables 9 and 10).

Based on the Shannon index, the *P. infestans* population in Carchi (H_s =2.44) was genetically more diverse than the population in Chimborazo (t_H=18.56, df= 30.39, p<0.001) and Loja (t_H=5.48, df= 9.43, p<0.001). The populations in Chimborazo and Loja were not significantly different from each other (t_H=-0.80 df= 10.73, p=0.372) (Table 8). The total race diversity (H_s= 3.78, Table 4) was significantly higher than the genotypic diversity (H_s = 3.03, Table 8) (t_H=12.66, df= 66.28, p=0.007).
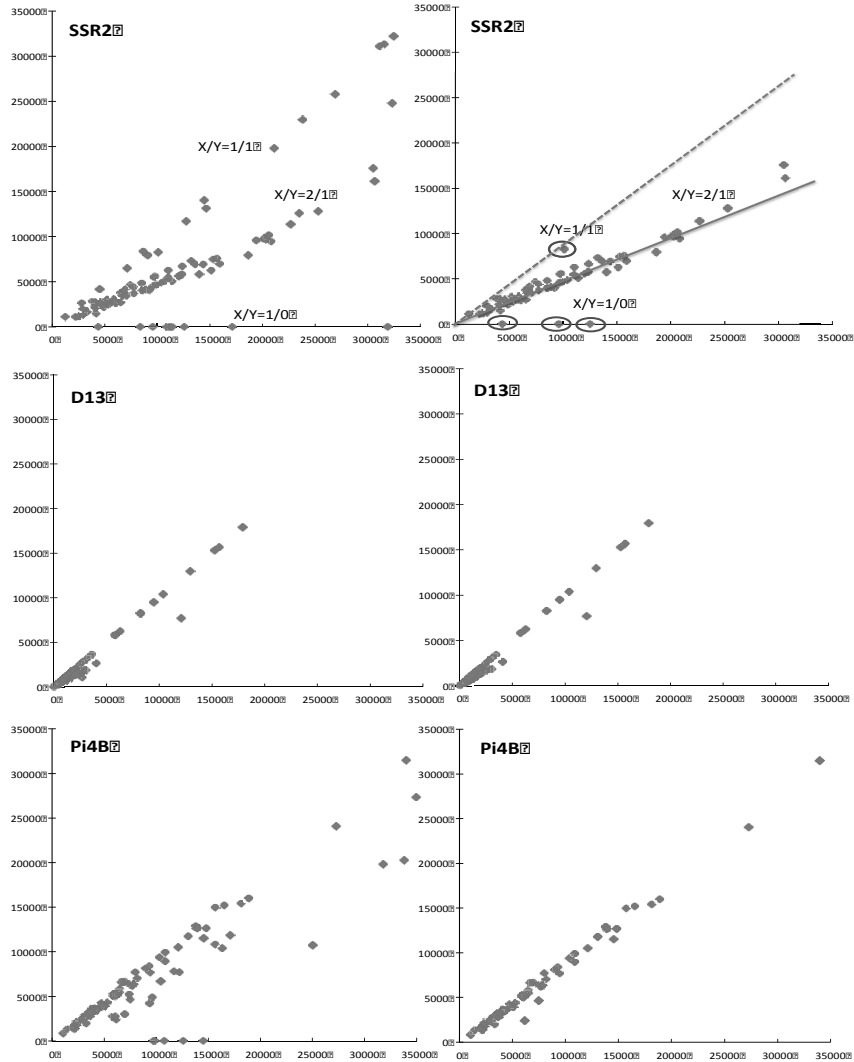
Fig. 4. The absolute ratio of max. and min. peak height at the three loci

Left includes all isolates in this study; Right only includes the clonal lineage of *P. infestans* identified in this study.
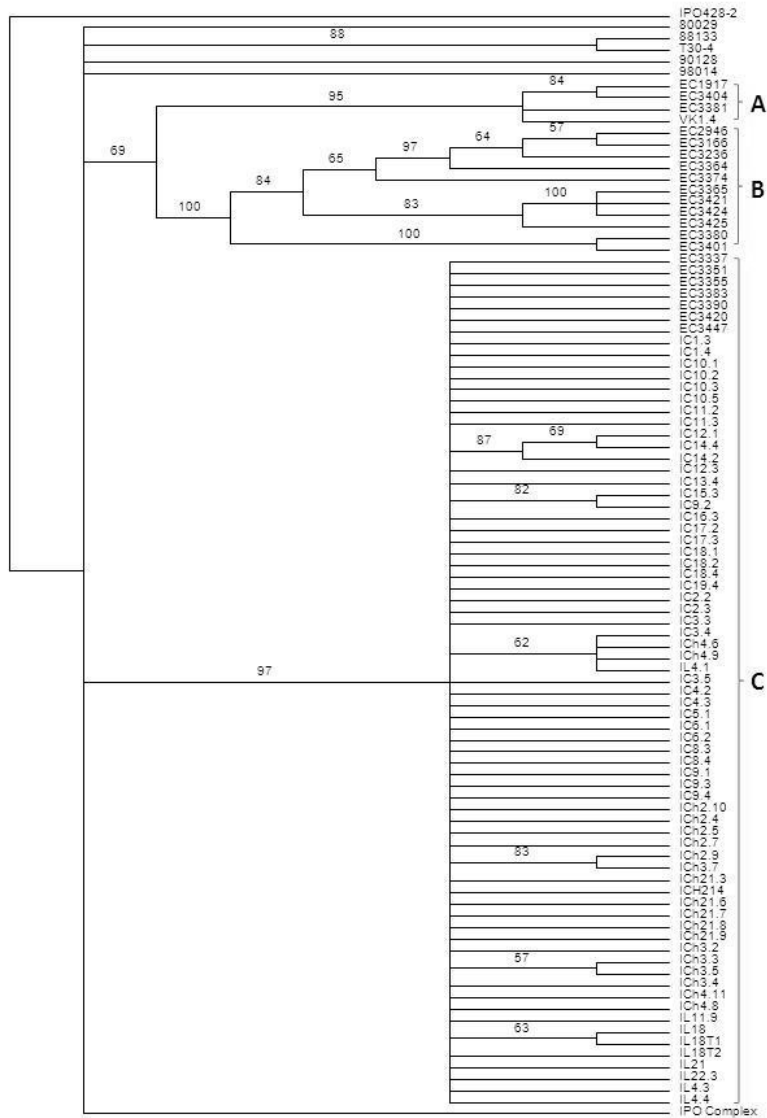
Fig. 5. Neighbour Joining tree of the Ecuadorian *P. infestans* populations associated to potato landraces based on 12 SSRs. Numbers at the nodes are Jacknife values supporting the branches based on 10.000 replicates. A: *P. infestans* isolates, clonal lineage US-1; B: *P. andina* isolates; *C*: *P. infestans* isolates, clonal lineage EC-1.

Table 9. Most abundant SSR Multilocus Genotypes and their race phenotypes of the *P. infestans* isolates associated to potato landraces in Ecuador

SSR Multilocus Genotype	Isolate	Race	Place	Province
	IC1.3	1,3,4,5,6,7,8,10,11	Ipueran, Julio Andrade, Tulcán	Carchi
	IC2.2	1,4,5,7,8,10,11	Ipueran, Julio Andrade, Tulcán	Carchi
	IC2.3	1,3,4,6,7,10,11	Ipueran, Julio Andrade, Tulcán	Carchi
	IC3.5	1,4,6,7,8,10,11	Agua Fuerte, parroquia El Carmelo, Tulcán	Carchi
	IC5.1	1,3,4,5,6,7,10,11	Troya, comunidad Virgen de Fátima, Urbina, Tulcán	Carchi
	IC6.1	1,2,3,4,5,7,9,10,11	Troya, comunidad Virgen de Fátima, Urbina, Tulcán	Carchi
	IC8.3	1,4,7,9	Chulamuez, Tulcán	Carchi
EC-1_001	IC10.3	1,2,3,4,5,7,8,9,10,11	Tufiño, Tulcán	Carchi
	IC11.3	1,2,3,4,5,7,9,10	Tufiño, Tulcán	Carchi
	IC16.3	1,2,3,4,5,6,7,8,9,10,11	Talla/Chapuez Chico, Tulcán	Carchi
	IC18.1	1,3,4,6,7,8,10,11	Casa Fría, Huaca, Montufar	Carchi
	IC19.4	1,3,4,5,6,7,10,11	Casa Fría, Huaca, Montufar	Carchi
	IL4.3	1,4,7,8,9,10,11	Ciudadela, San Lucas, Loja	Loja
	ICh2.5	1,3,4,5,6,8,9,10,11	Utuñag, Penipe	Chimborazo
	ICh2.4	1,3,4,6,7,8,10,11	Utuñag, Penipe	Chimborazo
	ICh3.2	1,4,7,8	Utuñag, Penipe	Chimborazo
EC-1_002	ICh3.4	1,4,5,8,9,10,11	Utuñag, Penipe	Chimborazo
	ICh4.8	1,3,4,5,6,7,8,9,10	Matus, Penipe	Chimborazo
	ICh4.11	1,3,4,7,8,10	Matus, Penipe	Chimborazo
	IC1.4	1,2,4,6,7,9,10	Ipueran, Julio Andrade, Tulcán	Carchi
	ICh21.3	1,4,5,6,7,8,9,10,11	Bacun, Chunchi	Chimborazo
EC-1_003	ICh21.4	1,4,5,6,7,8,9,10,11	Bacun, Chunchi	Chimborazo
	ICh21.6	1,3,4,6,7,9,10,11	Bacun, Chunchi	Chimborazo
	ICh21.7	1,4,5,7,8,9,10,11	Bacun, Chunchi	Chimborazo
	ICh21.9	1,4,7,9,10,11	Bacun, Chunchi	Chimborazo

Table 10. Most frequently observed *P. infestans* races and their Multilocus SSR genotype

Race	Isolate	Place	Province	SSR Multilocus Genotype
1,3,4,5,6,7,8,9,10,11	IC17.2	Casa Fría, Julio Andrade, Tulcán	Carchi	EC-1_021
	ICh2.10	Utuñag, Penipe	Chimborazo	EC-1_022
	IL11.9	Huangara, Taquil, Loja	Loja	EC-1_007
1,3,4,5,6,7,8,10,11	IC1.3	Ipueran, Julio Andrade, Tulcán	Carchi	EC-1_001
	IC12.1	Tufiño, Tulcán	Carchi	EC-1_006
	IC13.4	Tufiño, Tulcán	Carchi	EC-1_012
1,3,4,5,6,7,10,11	IC5.1	Troya, comunidad Virgen de Fátima, Urbina, Tulcán	Carchi	EC-1_001
	IC14.2	Talla/Chapuez Chico, Tulcán	Carchi	EC-1_031
	IC18.4	Casa Fría, Huaca, Montufar	Carchi	EC-1_004
	IC19.4	Casa Fría, Huaca, Montufar	Carchi	EC-1_001
1,4,5,7,8,10,11	IC2.2	Ipueran, Julio Andrade, Tulcán	Carchi	EC-1_001
	IC9.2	Tufiño, Tulcán	Carchi	EC-1_016
	IC9.3	Tufiño, Tulcán	Carchi	EC-1_009
1,2,3,4,5,6,7,8,9,10,11	IC10.5	Tufiño, Tulcán	Carchi	EC-1_005
	IC16.3	Talla/Chapuez Chico, Tulcán	Carchi	EC-1_001
	IC18.2	Casa Fría, Huaca, Montufar	Carchi	EC-1_030
1,4,5,6,7,8,9,10,11	ICh21.3	Bacun, Chunchi	Chimborazo	EC-1_003
	ICh21.4	Bacun, Chunchi	Chimborazo	EC-1_003
1,3,4,5,6,8,9,10,11	ICh2.5	Utuñag, Penipe	Chimborazo	EC-1_001
	ICh3.5	Utuñag, Penipe	Chimborazo	EC-1_010
1,4,7,8,9,10,11	IL4.1	Ciudadela, San Lucas, Loja	Loja	EC-1_014
	IL4.3	Ciudadela, San Lucas, Loja	Loja	EC-1_001
1,3,4,5,6,7,9,10,11	IL18T1	Nauchin, Manu, Saraguro	Loja	EC-1_026
	IL21	Chorro Blanco, Manu, Saraguro	Loja	EC-1_007

Table 11. Changes in number of races, Shannon index (H_s) and Evenness (E) of *P. infestans* populations over the years in Ecuador

	Year	Provinces		
		Carchi	Chimborazo	Loja
H_s	1990-1993[a]	2.33	2.12	1.55
	2007[b]	2.81	n.e.[d]	n.e.
	2009-2010[c]	3.15	2.86	1.89
E	1990-1993[a]	0.64	0.58	0.45
	2007[b]	0.67	n.e.	n.e.
	2009-2010[c]	0.88	0.95	0.86
Races	1990-1993[a]	14 (n= 39)	14 (n=38)	8 (n=31)
	2007[b]	27 (n= 68)	n.e.	n.e.
	2009-2010[c]	27 (n=36)	18 (n= 20)	7 (n= 9)

[a] Calculated from data of Forbes *et al* (1997).
[b] Calculated from data of Tello (2008).
[c] Data from this research.
[d] Not evaluated.

170 x 240 mm

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DISCUSSION

Phenotypic relationships among isolates

On 16 farms from 3 provinces of Ecuador we isolated 66 races of *P. infestans* of which 49 were different, which means that 75% of the isolates sampled were phenotypically different. A similar high frequency of different races among isolates was also found for Costa Rica (37 out of 40) (Barquero *et al.*, 2005). However, in other studies, lower frequencies were observed, e.g. Nepal 30 out of 251, Estonia 86/432, Finland 66/269, Norway 38/105 and China 61/125 races (Ghimire *et al.*, 2001, Hermansen *et al.*, 2000, Runno-Paurson *et al.*, 2010, Li *et al.*, 2009). It is unclear what the actual reason is, but the high race diversity may be related to a high mutation frequency and lack of selection for a particular race. Also the fact that we found 12 different races on one farm in Carchi points in that direction (Table 2). It may be expected that many more races will be found when more isolates are collected. Simple races capable of overcoming one to three *R* genes of the differential set were not observed. This is similar to observations made by Forbes et al (1997), who studied race composition of *P. infestans* isolates from Ecuador collected on potato cultivars.

Based on the Shannon index and the Evenness, there was no prevalence of a particular race. This may be related to the heterogeneity among the potato landraces, which are planted on small plots and often with more than one landrace together. In contrast to observations by Andrivon et al. reporting, adaptation of the *P. infestans* population to the most dominant varieties was observed in France and Morocco (Andrivon *et al.*, 2007), our results show no selection to certain races and even the most frequent race represented just 6 % of the samples (Table 3).

Genetic diversity among isolates

All *P. infestans* isolates from potato landraces grouped in a clade together with known EC-1 isolates, separated from US-1 and *P. andina* isolates (Fig. 5). This observation confirms that the *P. infestans* that attacks potato in the Ecuadorian highlands is from the EC-1 clonal lineage. Notwithstanding the fact that all isolates from potato landraces were clonal, genetic variation was detected using the SSRs. Thirty-one multilocus genotypes were detected among the 66 isolates. This means that about 47% of the isolates were different in their SSR profiles (Table 5). This variability is large for a clonal lineage. Clonal lineages have been identified in Northern China (Guo *et al.*, 2009), Europe (Lees *et al.*, 2009), and the Netherlands (Y. Li, unpublished) using microsatellites. Guo et al (2009) identified a single clonal lineage using just two SSRs. The use of the eight informative markers we used in this study will surely

enhance the identification of subclonal lineages. Nevertheless the variation in the clonal lineage named 'Blue_13' was assessed with the same markers (Y. Li, unpublished) and showed only a few subclonal lineages among hundreds of isolates genotyped.   The high diversity observed may be explained by the fact that in Ecuador potatoes are grown all year around, so several generations of the pathogen can occur, giving the chance of the appearance of new genotypes. The subclonal variation that was observed may partly be explained by loss of chromosome regions or mitotic recombination. Nevertheless also new alleles were detected that can only be explained by changes in the number of repeat units during mitosis. Changes in virulence spectrum have been attributed to (partial) chromosomal deletions (van der Lee *et al.*, 2001). Also, mutations in avirulence genes have been found to cause changes in the virulence phenotype (Armstrong *et al.*, 2005).

Another explanation may be related to the small scale farming system used for the potato landraces where fungicides are rarely used. Grünwald et al (2006) reported that unexposed populations of *P. infestans* showed more genetic diversity than those exposed to the fungicide metalaxyl.

The presence of more than two peaks in some of the SSR profiles (Fig. 3) supports the hypothesis that polyploidization or gene duplication followed by mutations may explain the genetic diversity observed in the *P. infestans* population of Ecuador (Tooley & Therrien, 1991). The occurrence of more than two alleles at a specific locus of *P. infestans* has been reported before (Knapova & Gisi, 2002, Lees *et al.*, 2006, Chacon, 2007, Akino *et al.*, 2009). The same mechanism seems to be active in *P. andina* as well, there we observed even four alleles (Fig. 3, Tables 5 & 7).

When we look at the Shannon index (3.03) and the Evenness (0.72) for all the SSRs genotypes in our study, than they are lower than those recorded in the Nordic countries (5.01 and 0.95, respectively (Brurberg et al 2011)) were oospores are main drivers of the epidemic and sexual recombination is high. In Ecuador, the small scale production and growth of several landraces in the same farm seems to support diverse pathogen populations even in absence of sexual recombination.

There was no correlation between the phenotypic and genotypic diversity. Isolates of a particular race showed different multilocus genotypes. These occurred even in isolates obtained from the same landrace or farm (Table 9).   This is remarkable and could indicate that part of the generation of virulence may be attributed to epigenetic changes for instance in expression rather than to changes in the DNA. An example is EC-01 where virulence is caused by loss of expression of *AvrVnt1*. Pel (2010) demonstrate that all alleles of *AvrVnt1*

identified are able to trigger the resistance response but a virulent EC-01 isolate does not express the gene during the early phases of infection.

The high race diversity observed in the *P. infestans* populations associated to Ecuadorian potato landraces is similar to earlier reports on populations from USA, France and China, where clonal lineages were shown to possess a wide diversity in races, but a low genetic diversity (Abu-El Samen *et al.*, 2003, Montarry *et al.*, 2006, Guo *et al.*, 2009a). The fact that such a large phenotypic and genetic diversity was observed in the Ecuadorian *P. infestans* population suggests that this clonal lineage is already very old.

Geographical distribution of isolates

The genetic diversity of the *P. infestans* population was highest in the Carchi province and significantly different from Chimborazo and Loja (Table 8). This difference in diversity observed in Carchi, could be due to the higher number of isolates and farms sampled. Among the Evenness values, the highest value was measured in Loja (0.93), although the number of samples was small (Table 8). This probably means that we have, as already expected, under sampled in Loja.   We also observed one genotype that occurs in the three provinces, which implies that the isolate migrated through the Ecuadorian highlands, perhaps due to the exchange of landrace seeds among the farmers (Monteros-Altamirano, 2011). The microsatellite analysis also showed some *P. infestans* lineage and species specific alleles (Table 7). These may be used to distinguish clonal lineages (Akino *et al.*, 2009). The SSR markers used in this study showed the genetic diversity within *P. infestans* population in Ecuador. In future they may be used to monitor changes in the population and displacement of pathogen genotypes across the country.

Diversity in the past and present

When we compare the race diversity of the *P. infestans* populations of our study with previous studies, we observed an increase in the Shannon index and Evenness over time. Also an increase in the number of races is evident (Table 11). The differences in Carchi in the years 2007 and 2009 - 2010 may be due to the fact that in the present study, the survey included 8 different farms, whereas Tello (2008) collected the entire samples just on one farm in that province and some races were much more frequent than others. The Evenness values are close to 1 for all provinces, which was not the situation in 1990-1993, where in Carchi 2 races were presented in 40% of the isolates. For Chimborazo and Loja, the situation was similar, 2 races at each location representing 55 and 70 % of the isolates sampled (Forbes *et al.*, 1997).

Consequences for potato cultivation and breeding in Ecuador

For potato growing in Ecuador, there is a need for monitoring the pathogen populations in order to monitor changes in race and genotype frequencies and complexity of virulence. The frequency of avirulence factors should be monitored in order to predict if *R-genes* still will be effective, and to adjust the potato varieties planted according to this.

From the observed avirulence spectrum of the Ecuadorian *P. infestans* population, it is evident that there is an urgent need for new *R-genes* not belonging to the *S. demissum* group. Other sources (Wang *et al.*, 2008, Jacobs *et al.*, 2010, Lokossou *et al.*, 2010) may still be useful for potato breeding against *P.infestans* in Ecuador, except *Rpi-vnt1.1* from *S. venturii* which is already frequently broken in this country(Foster *et al.*, 2009).

MATERIALS AND METHODS

Isolate collection

P. infestans isolates were collected from potato landraces present in three regions of Ecuador: in the provinces of Carchi in the north, Chimborazo in the centre and Loja in the south (Monteros *et al.*, 2008). Of each potato landrace present on a farm, five to ten leaves with a single lesion were sampled. Leaves were kept at 4 °C until isolation (Forbes, 1997). The name of the landrace sampled, farm owner, location, GPS-coordinates and altitude were recorded. The pathogen was isolated either from infected leaves or from small pieces of necrotic leaves, which were placed between potato slices of the susceptible variety Superchola, in both cases incubated in a humid chamber at 16 °C with a 12 hours photoperiod. Once mycelium was visible on leaves or slices, it was transferred to Petri dishes with Rye B Agar medium (Caten & Jinks, 1968) with antibiotics (Oyarzun *et al.*, 1998). Purified isolates of *P. infestans* were maintained on Rye A medium (Caten & Jinks, 1968).

Isolate characterization

The mating type was determined for each isolate by pairing it with known A1 (EC3090 or EC3690) and A2 (EC3260) isolates (provided by International Potato Center (CIP), Lima, Peru) on 10% clarified V8 agar (Forbes, 1997) at 18 °C. After 2 to 3 weeks each plate containing the paired isolates was assessed for the presence of oospores. Isolates that produced oospores in the presence of a known tester A2 were designated as the A1 mating type, and vice versa (Forbes, 1997).

Virulence was determined using a differential set of potato clones containing 11 major *P. infestans* resistance genes from *S. demissum* (Malcolmson & Black, 1966). R1 (CIP 801038), R3 (CIP 801041), R4 (CIP 801042), R5 (CIP 801043), R7 (CIP 801045), R8 (CIP 801046) and R9 (CIP 800994) differentials were provided by CIP. R2 (CIP 800987), R6 (CIP 800991), R10 (CIP 800995) and R11 (CIP 800996) differentials, and the cv. Bintje which has no known *R* genes (Monteros & Reinoso, 2010) were obtained from Wageningen University. Each isolate was inoculated on the differentials. From each plant, three leaflets were taken and placed in inverted Petri dishes containing Water-Agar. On the abaxial surface of each leaflet, two 20 μL drops containing 25×10^3 sporangia/mL were placed at each side of the mid vein. The inoculated leaves were placed in a climate chamber at 16 °C for six days with 12 hours of photoperiod, after which the reaction was scored.

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The reaction was considered compatible, when a necrotic lesion and/or sporulation was observed on a leaflet, and as incompatible when hypersensitive reaction was seen or no lesion was visible.

DNA extraction

All the *P. infestans* isolates obtained from potato landraces were grown from 10 – 14 days on Pea broth (Forbes, 1997). The mycelium was harvested and lyophilized. DNA was extracted from each sample with AGOWA sbeadex ® Maxi Plant kit on a KingFisher96 robot (Thermo Fisher Scientific).

Phylogenetic analysis

The *P. infestans* isolates collected were analyzed using the standard SSR set including markers Pi04, Pi63, Pi70, G11, D13, Pi4B (Lees et al, 2006), PinfSSR2, PinfSSR3, PinfSSR4, PinfSSR6, PinfSSR 8 and PinfSSR11 (Li *et al.*, 2010). In the analysis we included a number of isolates from previous surveys carried out in Ecuador for reference (Table 1). Amplified DNA fragments were detected using the GeneMapper® 4.0 software. The detected fragments were converted to binary data as presence (1) or absence (0) of each allele. From this a Neighbour Joining tree was produced using PAUP* 4.0 Beta software. Robustness of the phylogram branches was inferred from Jacknife values after 10.000 replicates (Swofford, 2002).

Diversity indexes

Race and genotypic diversity were estimated using the Shannon index (H_s) as $H_s = -\sum(p_i.\ln p_i)$, where p_i is the frequency of the race or genotype. The Evenness (E) was estimated by the formula: $E = H_s /\ln(n)$, where n is the total number of isolates of the sample and H_s is the Shannon index. E varies 0 from to 1 with 1 representing a situation in which all races or genotypes are equally abundant (Magurran, 1988).

The significance of different Shannon indices was assessed with the t-test of Hutcheson (t_H) (Hutcheson, 1970). It was calculated as follows: $t_H = (H_1-H_2)/[S^2_{H1}+S^2_{H2}]^{1/2}$, where H_1-H_2 is the difference among the Shannon indexes between two samples. The variance of each sample (S^2) was estimated with the formula: $S^2 = [\sum p_i.(\ln p_i)^2 – (\sum p_i.\ln p_i)^2/n]/n^2$, p_i and n are as described above.

The degrees of freedom (df) for each pairwise comparison were calculated using the formula: $df=[S^2_{H1}+S^2_{H2}]^2/[((S^2_{H1})^2/n_1) + ((S^2_{H2})^2/n_2)]$. Virulence complexity of the samples was estimated as: $C_i = \sum (p_i.v_j)$ and $C_p = (\sum v_j)/n$, where v_j is the number of avirulence genes present in a race and p_i and n are as described previously. C_i is the mean number of avirulence genes per isolate, whereas C_p represents the mean number of avirulence genes per race (Andrivon, 1994).

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CHAPTER 7

Genetic diversity of *Phytophthora infestans* population collected from potato and tomato crop regions during 2006-2008 in Tunisia

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CHAPTER 7

SUMMARY

In Tunisia, late blight caused by *Phytophthora infestans* is a serious threat on both potato and tomato. The Mediterranean weather conditions can be conducive for infection in all seasons when tomato and potato are grown year round. The consequences of these agricultural practices on the epidemiology and genetic variation of *P. infestans* are largely unknown. In the study a survey is performed on 165 *P. infestans* isolates collected from the North, North-East, North-West, Tunis area and Coastal zone of Tunisia between 2006 and 2008. Although mitochondrial genotyping shows little genetic diversity, 12 SSR markers reveal high genetic diversity. The presence of a major clonal lineage (NA-01, A1 mating type, Ia mtDNA haplotype) consists of relatively simple physiological races compared to populations in Algeria, Europe and USA. Another highly genetic diverse group contains more complex races and isolates with both mating types. Season clustering indicates that new genotypes generated by sexual reproduction overlaps between seasons and the sexual progeny may play an important role in the next season epidemics. Thepopulation on potato and tomato seems to be under different selection pressures. On tomato, asexual progeny is with two mtDNA haplotypes but less nuclear genotypes, compared to potato. Our data shows that the *P. infestans* population is currently changing, and the old clonal lineage is being replaced by a more complex, genetically diverse and sexually propagating population in two sub-regions in Tunisia. Despite the massive import of potato seeds from Europe, the *P. infestans* population in Tunisia is still clearly distinct. Strict management on late blight control may avoid the spread of aggressive *P. infestans* genotypes such as "Blue_13" from Europe or neighbouring countries.

INTRODUCTION

The late blight pathogen *Phytophthora infestans* (Mont.) de Bary, is classified as the plant destroyer number one (Fry, 2008, Govers & Gijzen, 2006) ever since its appearance in the dramatic epidemic leading to the potato Irish famine in 1845 (Woodham-Smith, 1962). Continued efforts worldwide to characterize this pathogen (Haas *et al.*, 2009) led to the understanding of its special ability to interfere with host cell responses (Birch *et al.*, 2006, Morgan & Kamoun, 2007) including host resistance responses. Most resistance genes introgressed from wild *Solanum* species to cultivated potato were quickly defeated due to adaptations of *P. infestans* population. Therefore, potato producing countries in the world still suffer from late blight damages. In Tunisia where potato is cultivated in three to four partly overlapping seasons, late blight epidemics can result in complete crop loss. The main season is from March to July, the late season from September to December, the extra-early season is from November to February and the early season is from December to March. The common main and late seasons could be found in major potato production regions but the extra and early season crops are cultivated in the North-East and the Coastal regions of Tunisia. On tomato, this pathogen can damage either greenhouses crop or field crop in the most producing regions. In field, culture conditions could play an important role in the spread of the disease. Indeed, drip and sprinkler irrigation system adopted in sampling regions could be the major factors of epidemic's propagation from basal leaves when temperatures rise-up. First focus is detected at low levels of soak land that could be due to either zoospores moving by water irrigation or presence of oospores in soil. In Tunisia, commercial production of tomato and potato can be found in adjacent plots in many regions. Sometimes potato and tomato are grown on the same farm or even in the same field. The damages caused by the pathogen are highly linked to the weather conditions. Rainy years correlate strongly with the index of late blight damages and a reduction of the annual crop production. On potato, *P. infestans* threats can occur in any seasons and on any stage of plant development. However, during the late season crop, the climate is the most conducive to the development of late blight epidemics due to the occurrence of clouded and rainy weather. Furthermore, tuber exchange between farmers in the same region or in contiguous regions is common. As in most other regions in the world, late blight is controlled by the use of chemicals. However, this strategy may not be durable as resistant strains are identified in many regions worldwide (Gisi & Cohen, 1996, Mukalazi *et al.*, 2001, Śliwka *et al.*, 2006, Cooke *et al.*, 2011). Recently, several studies report the damages caused by late bight all over the world and the correlation between

genetic variation of the pathogen and degree of severity in fields (Bouws & Finckh, 2007, Jaime-Garcia *et al.*, 2001). These studies dissect the biologic, physiologic, genotypic and epidemiologic aspects of the pathogen in order to understand its behavior in fields. However, in Tunisia, little is known about the genetic diversity and epidemiologic aspects of *P. infestans*. Few years ago, Jmour and Hamada (Jmour & Hamada, 2006) described the first report of the presence of A2 mating type in Tunisia and in 2008. Ferjaoui *et al*. reported on screening resistance levels on 16 potato varieties against *P. infestans* based on a national survey on potato. With the goal to characterize the Tunisian population of *P. infestans,* we use molecular markers (SSR, mtDNA haplotype, CAPS) to determine the genotypic and phenotypic diversity of this pathogen for assessing the importance of several epidemiological aspects.

RESULTS

Sampling Diversity

In this study, we focus on a wide sampling from major potato crop regions in Tunisia collected during 2006, 2007 and 2008. Four isolates (TU0501, TU0502, TU0503 and TU0504) collected at the end of December 2005 are analyzed together with 2006 population. Samples were collected from two hosts: potato and tomato in different times in one season, in different seasons, from different fields and also from the same field (Table 1). In the same field, two sampling strategies are used. The first one is to collect one sample from each of the four corners in a field with the same culture condition (cultivar, fungicide treatment, irrigation). The second strategy is to collect from varieties cultivated in the same field that differ in the level of resistance to *P. infestans*. Not for all infected tissue an isolate could be obtained. In total, 165 isolates from over 500 tissue samples were successfully purified. One hundred and twelve isolates were collected from potato field and tomato greenhouses and fields from the North-East region (Cap-Bon) of Tunisia (Fig. 1). In the North (Bizerte) of Tunisia, 29 isolates were collected only from potato fields. In the North-West, 20 isolates were collected from potato fields. Furthermore, from the Coastal Zone we collected two isolates (TU0604 and TU0772) from late season crop of potato in 2006 and season in 2007 respectively. Two isolates only (TU0501 and TU0809) were collected from two sub-regions in Tunis area: one potato isolate from Manouba (TunisM) in 2005 and one tomato isolate from Khoulaydia (TunisKh) in 2008.
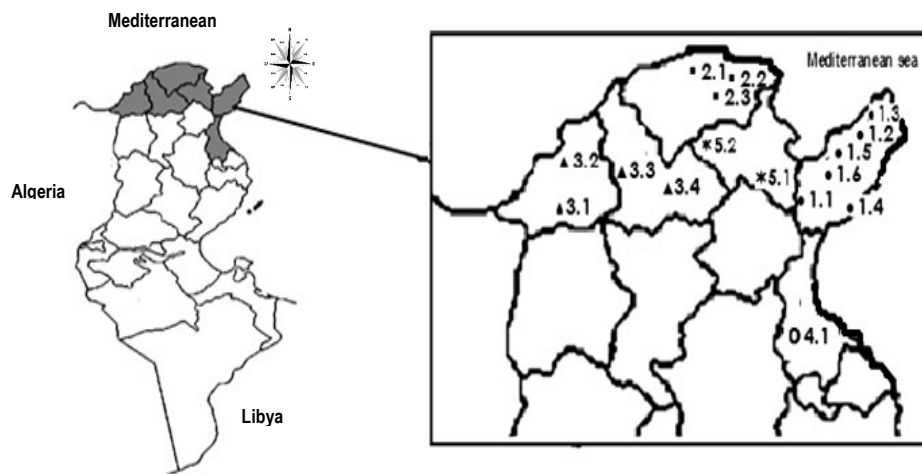
Fig. 1. Tunisian map show different sampling sub-regions, ●: North-East (1); 1.1: North-EastS; 1.2: North-EastDa; 1.3: North-EastH; 1.4: North-EastK; 1.5: North-EastT; ■: North (2); 2.1: NorthA; 2.2:NorthRJ; 2.3: NorthGM; ▲: North-West (3); 3.1: North-West BS; 3.2: North-West J; 3.3: North-West B; 3.4: North-West G; and **o**: Costal Zone (4); 4.1: CoastalCM; ✱Tunis (5)5.1: Tunis areaM; 5.2: Tunis areaKh.

Mating type distribution

Within the set of 165 individuals, 141 (85 %) had the A1 mating type and 24 (15%) had the A2 mating type. Isolates with A1 were foundin all sampling regions and in three sampling years (2006, 2007, and 2008). The A1:A2 frequency ratios were 9:1 in the North-East area (112 isolates), 8:2 in the North (29 isolates). In the North-West area, the Coastal Zone and in area of Tunis only the A1 mating type was found. The A1:A2 frequency ratios in different years were 9:1 (2006), 9:1 (2007) and 8:2 (2008). Among all sampled regions over three sampling years, there were highly significant differences in the proportions of A1 and A2 (North-East: Chi^2=50.42 DF=1; P<0.0001; North: Chi^2= 26.02, DF=1; P<0.0001; North-West, Coastal Zone and Tunis area: Chi^2= 98.02; DF=1; P<0.0001). After clonal correction, within 72 multilocus genotypes, 55 (76%) had the A1 mating type and 17 (24%) had the A2 mating type.

mtDNA haplotyping analysis

mtDNAs of all isolates (165) were amplified with P2 and P4 primer pairs. Nearly all isolates (161) had mtDNA haplotype Ia, only four isolates from tomato greenhouses in the North-EastT region (TU0613, TU0614, TU0621 and TU0622) had IIa, which also had the same multilocus genotype and the A1 mating type.

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170 x 240 mm

Table 1. Tunisian isolates used in this study

Locations	No	Samples ID	Crop season	Host	Mating /haplotype	SSR	Virulence
North-EastB	1	TU0775	L. season	Potato	1	1	
North-EastDA	3	TU0612, TU0625, TU0626	Season	Potato	3	3	1
North-EastH	10	TU0501, TU0636, TU0637, TU0638, TU0639, TU0640, TU0641, TU0642, TU0643, TU0644	L. season	Potato	10	6	1
North-EastK	69	TU0605, TU0606, TU0607, TU0608, TU0609, TU0610, TU0615, TU0616, TU0617, TU0619, TU0620, TU0623, TU0624, TU0627, TU0756, TU0757, TU0767, TU0768, TU0769, TU0770, TU0776, TU0777, TU0778, TU0779, TU0780, TU0781, TU0782, TU0783, TU0789, TU0790, TU0791, TU0804, TU0805, TU0806, TU0807, TU0810, TU0811, TU0812, TU0813, TU0814, TU0815, TU0816, TU0817, TU0818, TU0819, TU0820, TU0821, TU0822, TU0823, TU0824, TU0825, TU0826, TU0827, TU0828, TU0829, TU0830, TU0831, TU0832, TU0833, TU0834, TU0835, TU0836*, TU0839*, TU0840*, TU0862, TU0863, TU0864, TU0865, TU0866	E. season / Season / L. season	Potato / Tomato	69	51	11
North-EastT	25	TU0502, TU0613*, TU0614*, TU0618, TU0621*, TU0622*, TU0646*, TU0647*, TU0649, TU0751*, TU0752*, TU0753*, TU0754*, TU0755*, TU0759*, TU0760*, TU0761*, TU0762*, TU0763*, TU0764*, TU0765*, TU0766*, TU0771*, TU0773*, TU0774*	L. season / E. season	Potato / Tomato	25	18	4
North-EastS	4	TU0648, TU0750, TU0758*, TU0808*	L. season / E. season	Potato, Tomato	4	4	1
NorthA	1	TU0611	Season	Potato	1	1	
NorthRJ	1	TU0837	Season	Potato	1	1	
NorthGM	27	TU0628, TU0629, TU0630, TU0631, TU0632, TU0633, TU0634, TU0635, TU0792, TU0793, TU0794, TU0795, TU0796, TU0848, TU0849, TU0850, TU0851, TU0852, TU0853, TU0854, TU0855, TU0856, TU0857, TU0858, TU0859, TU0860, TU0861	Season / L. season	Potato	27	22	7
North-West J	8	TU0645, TU0897, TU0898, TU0899, TU0800, TU0801, TU0802, TU0803	L. season	Potato	8	8	2
North-West BS	7	TU0841, TU0842, TU0843, TU0844, TU0845, TU0846, TU0847	L. season	Potato	7	7	2
North-WestG	4	TU0785, TU0786, TU0787, TU0788	L. season	Potato	4	4	2
North-West MB	1	TU0784	L. season	Potato	1	1	1

				L. season, Season	Potato		
CoastalCM	2	TU0604, <u>TU0772</u>			Potato	2	2
Tunis areaM	1	<mark>TU0503</mark>		L. season	Potato	1	1
Tunis areaKh	1	**TU0809***		E. season	Tomato	1	1
Total	165					165 130	31

-: Name meaning: TUXXYY: TU: Tunisia, XX: year of sampling, YY: chronologic number of the isolate.

*: from tomato; underlined: from midseason crop; highlighted: from late season crop; bold: from early season crop.

Note: TU0814-TU0835, TU0848-TU0860, TU0862-TU0866 were sampled based on the second strategy of sampling and the others were sampled by first strategy of sampling both described in results section.

Table 2. Different SSR markers used in the study

Marker	Size range	Label	Primer sequence (5' to 3')	References
D13	100-184	6-FAM	F:TGCCCCCTGCTCACTC; R:GCTCGAATTCATTTTACAGA	32
G11	125-220	NED	F: TGCTATTTATCAAGCGTGGG; R: TACAATCTGCAGCCGTAAGA	32
Pt4B	200-300	PET	F: AAAATAAAGCCTTTGGTTCA; R: GCAAGCGAGGTTTGTAGATT	32
Pt04	160-176	VIC	F:AGCGGCTTACCGATGG; R:CAGCGGCTGTTTCGAC	32
Pt63	265-285	VIC	F: ATGACGAAGATGAAAGTGAGG; R: CGTATTTTCCTGTTTATCTAACACC	32
Pt70	185-200	VIC	F: ATGAAAATACGTCAATGCTCG; R: CGTTGGATATTTCTATTTCTTCG	32
PinfSSR2	165-180	PET	F: CGACTTCTACATCAACCGGC; R: GTTT GCTTGGACTGCGTCTTTAGC	34
PinfSSR3	254-274	NED	F: ACTTGCAGAACTACCGCCC; R: GTTT GACCACTTTCCTCGGTTC	34
PinfSSR4	280-305	6-FAM	F: TCTTGTTCGAGTATGCGACG; R: GTTTCACTTCGGGAGAAAGGCTTC	34
PinfSSR6	230-260	VIC	F: GTTTTGGTGGGCTGAAGTTTT; R: TCGCCACAAGATTTATTCCG	34
PinfSSR8	256-274	6-FAM	F: AATCTGATCGCAACTGAGGG; R: GTTT ACAAGATACACACGTCGCTCC	34
PinfSSR11	325-360	NED	F: TTAAGCCACGACATGAGCTG; R: GTTTAGACAATTGTTTTGTGGTCGC	34

170 x 240 mm

Table 3. Diversity of SSR loci used in this study (allele's number, gene diversity and genetic diversity).

Loci	Locations All. Total (Private)	N-E	N-ET	N	N-W	Ht^a	Hs^b	Mating types All. Total (Private)	A1	A2	Ht^a	Hs^b	Hosts All. Total (Private)	Pot.	Tom.	Ht^a	Hs^b	Season crops All. Total (Private)	LS	ES	SC	Ht^a	Hs^b
DI3	4(1)	0,05	0,41	0,00	0,11	0,12	0,14	4(3)	0,11	0,18	0,12	0,15	4(1)	0,05	0,33	0,12	0,19	4(1)	0,04	0,30	0,10	0,12	0,14
G11	9(4)	0,77	0,74	0,72	0,57	0,76	0,70	9(4)	0,75	0,71	0,76	0,73	9(5)	0,76	0,69	0,76	0,72	9(3)	0,72	0,79	0,75	0,76	0,75
Pi04	3(0)	0,54	0,31	0,41	0,05	0,44	0,33	3(0)	0,40	0,57	0,45	0,49	3(1)	0,48	0,20	0,45	0,34	3(0)	0,42	0,40	0,52	0,45	0,44
Pi4B	3(0)	0,62	0,57	0,60	0,50	0,60	0,57	3(0)	0,59	0,64	0,60	0,62	3(0)	0,61	0,52	0,60	0,57	3(0)	0,58	0,59	0,65	0,60	0,61
Pi63	3(0)	0,46	0,59	0,47	0,50	0,51	0,50	3(0)	0,52	0,35	0,51	0,44	3(0)	0,47	0,57	0,51	0,52	3(0)	0,51	0,52	0,48	0,51	0,50
Pi70	2(0)	0,48	0,28	0,33	0,15	0,41	0,31	2(0)	0,39	0,45	0,40	0,42	2(0)	0,43	0,20	0,40	0,32	2(0)	0,27	0,47	0,47	0,40	0,40
PinfSSR2	2(0)	0,34	0,42	0,47	0,50	0,42	0,43	2(0)	0,42	0,41	0,42	0,42	2(0)	0,41	0,46	0,42	0,44	2(0)	0,48	0,39	0,28	0,42	0,38
PinfSSR3	4(0)	0,32	0,55	0,43	0,50	0,44	0,45	4(1)	0,47	0,10	0,44	0,29	4(0)	0,38	0,56	0,44	0,47	4(0)	0,51	0,40	0,31	0,44	0,41
PinfSSR4	8(1)	0,76	0,69	0,66	0,65	0,75	0,69	8(2)	0,75	0,70	0,75	0,72	8(4)	0,76	0,67	0,75	0,71	8(1)	0,73	0,72	0,77	0,75	0,74
PinfSSR6	4(1)	0,48	0,29	0,35	0,15	0,42	0,32	4(2)	0,42	0,45	0,42	0,43	4(1)	0,44	0,24	0,42	0,34	4(1)	0,37	0,44	0,47	0,42	0,43
PinfSSR8	4(1)	0,52	0,50	0,57	0,50	0,53	0,52	4(0)	0,51	0,62	0,53	0,57	4(2)	0,53	0,50	0,53	0,52	4(1)	0,53	0,49	0,57	0,53	0,53
PinfSSR11	3(0)	0,56	0,54	0,44	0,50	0,57	0,51	3(0)	0,57	0,52	0,58	0,55	3(0)	0,58	0,51	0,58	0,54	3(0)	0,59	0,52	0,56	0,58	0,56
Means^c	4(0.7)	0,49	0,49	0,46	0,39	0,50	0,46	4,1(1)	0,49	0,48	0,50	0,48	4(1)	0,49	0,45	0,50	0,47	4(0,6)	0,48	0,50	0,49	0,50	0,49
Allele Nb	49(8)	45(6)	37(1)	36(0)	31(1)			49(12)	47(10)	39(2)			49(14)	48(13)	36(1)			49(7)	43(2)	40(1)	41(4)		

^{a} Ht : Genetic diversity measured as expected heterozygosity in the whole population (pooled samples).

^{b} Hs: Genetic diversity averaged from expected heterozygosity in different subpopulations.

^{c} Monomorphic loci were excluded in the computation of the average values.

All data in this table were computed in POPGENE V 1.31 (56) and Convert v. 1.31(19)

(**N-E**:North-East; **N-ET**: North-EasT; **N**:North; **N-W**: North-West; **Pot**: Potato; **Tom**: Tomato; **LS**: Late Season; **ES**: Early Season; **SC**: Season Crop)

Table 4. Genotypic diversity (A) and pairwise Fst (above diagonal) and Nm (below diagonal) (B) in *P. infestans* Tunisian sub-populations

Locations	A						B**			
	Ni[a]	Ng[b]	Nmf[c]	G.D[d]	Ia[e]	CF[f]	North	North-East	North-East T	North-West
North	24	20	3	0.98	2,85	17	0,000	0.053*	0.028*	0.036*
North-East	65	45	4	0.99	1,06	31	8.947	0,000	0.143*	0.160*
North-EastT	18	7	8	0.77	5,02	61	17.686	3.002	0,000	-0.015*
North-West	19	12	4	0.94	1,39	37	13.409	2.641	inf	0,000
All samples	126	72	20	0.97	2,43	43	-	-	-	-

[a] Number of individuals in each population

[b] Number of genotypes

[c] Number of most frequent genotype

[d] Genotypic diversity

[e] The index of association I_A, in all data r_d coefficient is significant ($0.1 < r_d < 0.4$).

[f] Clonal Fraction (%)

** Fst = 0.05994*, Nm= 0.25 (1 - Fst)/Fst =3.92 (in all samples)

* Significant at P<0.05

CHAPTER 7

Genotypic diversity

The isolates are grouped in four geographic regions, North, North-East, North-EastT and North-West. In contrast to the mating type analysis, isolates from the North-EastT are regarded as a separate group as they represent the widest tomato sampling region in this study. Both the Tunis area and the Coastal Zone are eliminated from the genetic study depending on geographic distribution and STRUCTURE analysis because of the low number of individuals within each population, but they are kept in other analysis (for host, mating type and season) and the phylogenic clustering. After eliminating the similar genotypes and samples with too much missing data due to PCR failure, the total number of isolates is reduced from 157 to 130 individuals (considering four individuals from Coastal and Tunis areas).

The 12 SSR markers yielded 49 different alleles in 126 individuals ranging from 2 (Pi07 and PinfSSR2) to 9 (G11) alleles per locus with a mean of 4 alleles per locus (Table 3). In the whole population, the gene diversity varied from 0.12 for D13 to 0.76 for marker G11 with an average value of 0.50. The lowest value of gene diversity was detected in North area (0 for D13) and the highest value was detected in North-East (0.77 for G11). Within 126 isolates, 72 different multilocus genotypes were detected including 24 genotypes which were sub-clones of a dominant clonal lineage (Table 4). High genotypic diversity ranging between 0.77 and 0.99 was detected in all sub-populations tested. Low clonal fraction values were detected in most sub-populations, except for North-EastT (61%), which had the lowest genotypic diversity (0.77), the highest proportion of the most frequent genotype and the highest value of index of association (I_A). Otherwise, the clonality ranged from 17 to 37% for the remainders and the overall clonality was about 43%, which was less than the intermediate level (50%).

The phylogenic tree constructed by Neighbor-joining method using Nei's genetic distances showed high genetic variability (Fig. 2). The isolates were splited into two clusters. Cluster I consisted of a dominant clonal lineage called NA-01 (Cluster I in Fig. 2) and included 57 isolates and 24 multilocus genotypes from all major sub-regions (Fig. 1). All isolates had the A1 mating type and the Ia haplotype. Isolates originating from tomato and potato were found in this group. Remarkably, the North-EastK, the deepest sampled region, none of 66 potato isolates belonged to this clonal lineage. However, three tomato isolates from this region were found in this cluster (TU0836, TU0839 and TU0840). Cluster II consisted of a highly diverse group comprising isolates with A1 or A2 mating type. In this second cluster, we identified two mtDNA haplotypes (Ia and IIa) and a limited

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Fig. 2. Phylogenic tree of 130 individuals and one reference (VK 1.4) computed by the Neighbor Joining algorithm.

number of clonal isolates often originating from the same location, except for some isolates discussed in detail below. Almost all isolates in this cluster (68 of 73) were from potato. Exceptly one isolate TU0646 (A1,Ia) and a distinct group of four clonal isolates (TU0613, TU0614, TU0621 and TU0622,A1 and IIa) were from the tomato greenhouse.
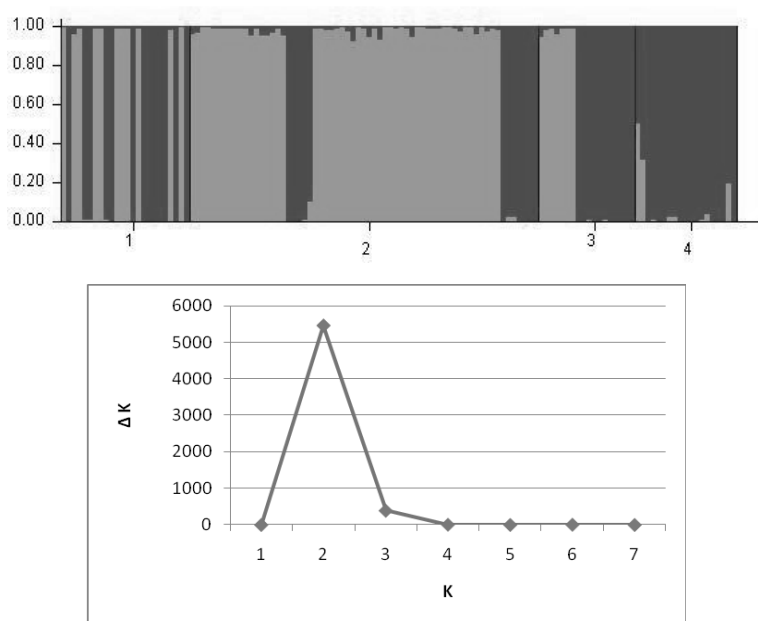
Fig.3. Upper: Model-based ancestry of 126 *P. infestans* isolates using STRUCTURE 2.2 (43). Colors represent model-based subpopulations: green, clonal lineage NA-01; red, genetically diverse group (cluster II). 1, 2, 3 and 4 represent sampling regions as following: 1, North; 2, North-East; 3, North-EastT; 4, North-West; Bottom: ΔK values of 7 populations computed by CorrSieve program.

Sub-clonal variation and genotype distribution during sampling years

Within the genetic clonal lineage NA-01, many sub-clonal genotypes were identified. These sub-clonal genotypes showed minor differences from the dominant clonal lineage by loss of particular alleles or by changes in the repeat number of some hypervariable loci. For instance sub-clonal genotype NA-01_02 (TU0635) differed from the NA-01_01 (TU0640) by the absence of allele 266 for PinfSSR3 and the absence of allele 295 for PinfSSR4. Similarly, allele 158 at G11 was lost for NA-01_17 genotype (TU0763). Alleles 175 at PinfSSR2, 217 at Pi4B marker and 142 at G11 marker were lost in genotypes NA-01_15 (TU0760), NA-01_20 (TU0845) and NA-01_21(TU0843) respectively in addition with the occurrence of insertions or deletions in PinfSSR4. For instance

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NA-01_06 (TU0846) had allele 287 from PinfSSR4 whereas most other isolates of NA-01 had allele 289 for PinfSSR4. Difference between NA-01_11 (TU0840) and NA-01_12 (TU0796) was due to the deletion of 2 base pairs at the original allele 244 at PinfSSR6. A deletion found at allele 266 of PinfSSR3 gave rise to a new sub-clonal genotype NA-01_18 (TU0837). Meanwhile, the insertion at allele 270 in PinfSSR3 locus generated a new sub-clonal genotype NA-01_19 (TU0853).

Some sub-clonal lineages in NA-01 were dominant. The most important one was NA-01_16 composing 21 isolates collected from almost all sub-regions. Of Cluster II, genotypes withboth mating types were identified in the same year either from the North area (TU0629, TU0630, and TU0634) or from the North-EastK (TU0608 and TU0620). Three genotypes (TU0793, TU0794 and TU0795) differed with each other by few mutations (loss and insertion) at two loci PinfSSR4 and Pi04. The A2 isolates (TU0848, TU0856, and TU0858) represented new genotypes genetically different from the A2 genotypes collected during 2007. The clonal group of A2 genotype identified during 2006 (TU0619, TU0623...) was found in the same sub-cluster with two A2 genotypes (TU0776, TU0779) identified in the next year (2007). Then, in the same cluster (II), the appearance of new genotypes (TU0804, TU0821, TU0825...) extremely different from them during 2008 were noted. Although the number of isolates collected from the Tunis area and coastal region was low (2 isolates from each site) we identified a member of the clonal lineage in each site (TU0809, TU0772 respectively) and one from each (TU0305, TU0504 respectively) clustered with the genetically diverse group.

Population genetic structure

An analysis of the population genetic structure using 126 *P. infestans* isolates was carried out with model-based STRUCTURE approach. When ΔK had the highest value (Fig. 3), two distinct gene pools (K=2) were identified (Fig. 3). The first inferred group (red colour in Fig. 3) represented the clonal lineage NA-01 detected in all sampling regions. The second inferred group (green colour in Fig. 3) represented Cluster II. 121 of 126 (96%) computed isolates had more than 90% membership, whereas 5 isolates (TU0645, TU0787, TU0897, TU0847 and TU0643) were categorized as admixture forms with less than 90% membership. North area included both clonal lineage group and diverse group. Almost all genotypes from North-East area belonged to Cluster II except for a few NA-01 sub-clonal genotypes originating from tomato and few others from potato and collected from sub-regions (North-EastH, North-EastS) other than North-EastK. Interestingly, almost all isolates collected from the North-West and North-EastT belonged to NA-01 clonal lineage,

A

Isolate	Location	Host	Virulence pattern	Frequency
TU0846	North-West	Potato	0	0,097
TU0784	North-West	Potato	0	"
TU0849	North area	Potato	0	"
TU0630	North area	Potato	1	0,097
TU0806	North-East K	Potato	1	"
TU0862	North-East K	Potato	1	"
TU0614	North-East T	Tomato	7	0,032
TU0645	North-West	Potato	1.2	0,032
TU0786	North-West	Potato	1.7	0,065
TU0765	North-EastT	Tomato	1.7	"
TU0779	North-East K	Potato	1.4	0,065
TU0827	North-East K	Potato	1.4	"
TU0619	North-East K	Potato	4.8	0,032
TU0818	North-East K	Potato	3.7	0,032
TU0756	North-East K	Potato	1.6.7	0,032
TU0625	North-East DA	Potato	1.4.6	0,097
TU0646	North-EastT	Tomato	1.4.6	"
TU0816	North-East K	Potato	1.4.6	"
TU0858	North area	Potato	1.3.7	0,032
TU0899	North-West	Potato	1.2.7	0,032
TU0785	North-West	Potato	1.2.3.7	0,097
TU0843	North-West	Potato	1.2.3.7	"
TU0850	North area	Potato	1.2.3.7	"
TU0618	North-EastT	Potato	1.3.6.7	0,032
TU0758	North-EastS	Tomato	1.4.6.7	0,032
TU0793	North area	Potato	1.2.4.6.7	0,032
TU0629	North area	Potato	1.3.6.7.11	0,032
TU0789	North-East K	Potato	1.3.4.6.10.11	0,032
TU0790	North-East K	Potato	1.3.4.7.10.11	0,032
TU0856	North area	Potato	1.2.3.7.10.11	0,032
TU0609	North-East K	Potato	1.2.3.4.6.10.11	0,032
total=31			total= 21 races	total =1.00

B

C

Fig. 4. A: Table of virulence patterns and race frequencies of 31 Tunisian isolates collected from different sampling regions. B: Histogram of avirulence proportions within the set of 31 isolates tested for virulence characterisation. C: Phylogenic tree based on SSR patterns sorted by of 30 isolates used in virulence analysis rooted by reference isolate VK 1.4. virulence pattern was linked to the isolate's name in the tree (e.g. TU0785-1.2.3.7- North-West: TU0785 from North-West that has 1.2.3.7 virulence pattern).

except one genotype from North-EastT (green panel in sub-population 3, Fig. 3) that grouped four tomato isolates and belonged to the highly diverse group.

Virulence analysis

To investigate the virulence diversity in the population, we selected a set of 31 isolates based on genetic and geographic origin. These isolates were subjected to virulence assays on detached leaves of the potato differential set. The virulence assays showed reliable results that were fully confirmed in replicated tests. The virulence spectrum in Tunisian isolates was highly variable (Fig. 4A). In total, 21 races were found in the set of 31 *P. infestans* isolates and more than a half of them displayed a unique race. The majority of the tested isolates were avirulent on most of the tested R1-R11 differentials. For example, 25 of 31 isolates tested (80 %) contained less than 5 virulence factors. Difference between virulence frequencies were noted between different *R* genes. For example, as displayed in Fig. 4B, all isolates were avirulent on the R5 and R9 differentials. Also the R8 differential (96%), R10 and R11 (87%) were resistant to most isolates. The R1 differential was resistant to only 6 out of 31 isolates (19%). Differences between geographic regions were also found. Among isolates collected from the North-East, virulence to all R differentials except R5 and R9 was noted. Similar results were found for the North area, from which isolates could infect all *R* differentials, except R5 and R8 differentials. In North-EastT and North-West areas, *P. infestans* isolates were generally less complex and could infect less R differentials. Phylogenic tree (Fig. 4C) based on SSR markers and sorted by virulence factor names showed the presence of two clusters (I and II). Isolates from Cluster I have from 0 to 4 virulence factors. Remarkably, the race 1.2.3.7 in Cluster I was detected at North-West and North areas and represented high frequency among all races detected (0.097). In this group no virulence was found for the *R* genes 5, 8, 9 10 and 11. In contrast isolates found in Cluster II showed a more diverse virulence pattern.

DISCUSSION

The comprehensive dataset generated from this survey illustrates the genetic changes of late blight population in Tunisia. In two regions both mating types are present while in other regions only the A1 mating type is detected. When they coexist, frequency is usually high for the A1 mating type and low for the A2 mating type either in sampling regions, or in sampling years and also in the same field (data not shown). Still the genotypic diversity, linkage disequilibrium and differentiation analysis indicate that in the North and

North-East areas the sexual cycle plays an important role in adaptation and epidemiology, while in other regions asexual propagules driving the epidemics and sub-clonal variation is the main source of genetic variation. From our analysis it is also clear that potato is the host where the sexual cycle is most prominent. Although the crops are often grown side by side the populations on potato and tomato are completely different as was found by others (Berg, 1926). The Tunisian population shows low diversity in mtDNA haplotyping analysis. As our data indicates that our survey performs at the start of a new wave of genotypes, we exploit the high resolution genotyping and the short sampling intervals. Recordings of weather data and late blight indexes to infer the migration of the A2 mating types and their direct consequences of gene flow and local migration, the genetic diversity could be the result of i) the weather conditions highly conductive to the spray of the pathogen, ii) the seed exchanges between Tunisian farmers and iii) the relative broad geographic distances between crop regions (maximum geographic distance=250 km).

The SSR markers allow the discrimination of homozygotes and heterozygotes since a single allele at a particular locus is detected in the former whereas both alleles are clearly resolved in the latter (Duncan *et al.*, 1998, Lees *et al.*, 2006). SSR markers are classified as ideal markers for rigorous genetic analysis since they are robust, flexible, reproducible, and offer the greatest potential to study the genetic diversity of *P. infestans* (Cooke & Lees, 2004, Guo *et al.*, 2009, Knapova & Gisi, 2002, Li *et al.*, 2010). We used fluorescence-based SSR detection on the automated sequencer in a one-step multiplex PCR method described by Li *et al.* (Chapter 3). In our study, each marker yields at least 2 alleles. However, the high level of discrimination and the ability to detect all alleles of a particular locus also causes difficulties in the standard genotypic analysis as a number of isolates show three alleles at a particular locus. This is found both in the clonal lineage NA-01 and in the sexual recombination in Cluster II. The presence of three alleles can be the result of translocations, non-disjunction during meiosis or polyploidization followed by insertions or deletions in repeat units at the SSR loci. Currently no program can cope with these uncertainties therefore we used a 1/0 scoring for the different alleles as a first step in the calculations. There was a surprisingly high level of nuclear diversity within populations of *P. infestans* in Tunisia combined with a low diversity in mtDNA. Both allelic (gene) and genotypic diversity values were relatively high. Among all 12 markers used, the gene diversity was always higher than 39%. Most alleles were shared between regions and only eight alleles were unique for one of the four regions (North, North-East, North-EastT and North-West). Indeed, the gene diversity in Tunisian population was much higher than that

detected in several populations collected in other countries (Lees *et al.*, 2006, Vargas *et al.*, 2008, Guo *et al.*, 2009, Cardenas *et al.*, 2011).

The clustering based on genetic distances does show a clear regional structure in some cases for instance in China (personal communication), or in other studies no clear substructure is found (Cardenas *et al.*, 2011). In Tunisia a clear substructure is found and a dominant clonal lineage NA-01 (A1, Ia) present in all sampling regions within which some sub-clonal genotypes are predominant. This cluster consists isolates collected from potato and tomato. Indeed, almost all isolates from North-EastT and North-West are grouped in this clonal lineage. In these two regions only A1 isolates are found. The gene and genotypic diversity values are relatively low compared with the overall diversity. Between these two geographic sites we observed the lowest value of Fst and the highest number of migrants between populations (Table 4). Remarkably, all isolates in this clonal lineage that originate from North-EastK (Table 1) and collected from tomato. The isolates originated from potato in this clonal lineage were collected from other sub-regions in North-East area (North-EastS, North-EastH, North-EastB). Furthermore, this cluster displays generally simple race structures (Fig. 4A, 4C). This clonal lineage generates clonal variation by asexual recombination and/or deletions and mutations. The old clonal lineage NA-01 is not similar to the US-1 genotype found to dominate in many parts of the world and still found in Ecuador on tomato (Oyarzun *et al.*, 1998). Nevertheless, the NA-01 clonal lineage found in Tunisia is reminiscent of population analysis described by Goodwin *et al*. (Goodwin *et al.*, 1994b) in that an 'old' clonal population of *P. infestans* appeared less aggressiveness compared with the 'new' population and is being replaced. Although in this case we have found evidence of sexual recombination in the North-East and North, this clonal lineage dispersed effectively throughout Tunisia. Interestingly, the potato population in the North-EastK region seems to be highly changed and the old population was completely replaced by a new, more diverse, population again reminiscent of the displacements previously described in other regions in the world (Drenth *et al.*, 1993a, Goodwin *et al.*, 1998, Spielman, 1991, Zwankhuizen *et al.*, 2000). However, similar to the situation in Ecuador (Oyarzun *et al.*, 1998) the old clonal lineage can still be found on tomato. In the Northern area, *P. infestans* population is a mix of the old NA-01 clonal lineage and the new population. We speculate that the most adaptive genotypes of this recombining population could have increased dramatically by asexual reproduction under the exceptionally conductive environmental conditions in the fall of 2007 and the spring in 2008 (rainy autumn, moderate winter and rainy spring). Indeed, in the population collected

from the midseason crop in 2008 we find a relatively high proportion of A2 mating type isolates (36%). Although few isolates are available from Tunis and the Coastal area (2 from each), we can conclude that the isolates in these areas are no longer strictly clonal as in both cases we found one isolate that belongs to Cluster II. The genetic structure is strongly confirmed by the model-based approach showing the clonal lineage (NA-01) and diverse sub-populations (Cluster II). Structure analysis also shows high membership of genotypes to their geographic sub-populations. Despite the presence of some genotypes (Fig. 2) that were not completely membership for their geographic populations such as isolates TU0645, TU0887 and TU0897 collected from the North-West region, these isolates could be derived from a sexual recombination probably occurred in some sub-regions of the North-West area. In this way, we could speculate that among North-West population showing less genetic changes comparing with other geographic locations, new genetic changes are happening with a probable occurrence of sexual reproduction.

Furthermore, some sub-clonal genotypes undergo more mutations than the stable clonal lineage including 23 isolates collected from all sampling regions. They probably play an important role in the pathogen epidemiology with some genotypes from Cluster II. As A1 genotypes are found everywhere the spread of the A2 isolate from the North reconvenes the two mating types, which could explain the high recombination ratio is identified in 2007 (TU0792 and TU0793...). The presence of many isolates from both North and North-East areas in the same genotype indicates that these isolates may have spread asexually via provincial exchange of potato seeds. Thus, some genotypes from North-EastK region could activate the sexual recombination in the population during the next two sampling years. We speculate that some clonal isolates from 2006 activated the sexual cycle and leaded to new generation in 2007 (TU0776, TU0779).These offspring probably involved in the sexual reproduction during 2008 in which new more complex genotypes (TU0825, TU0821..) are identified. In North-EastT, the genotype detected during 2006 with IIa haplotype did not play an effective role during next years and this could be due to the selection pressure on tomato crop. More detailed marker analysis particularly on mtDNA haplotypes could assist in the parental analysis of these isolates.

Depending on host, collection season and geographic origin, the genetic diversity is either high or low. The clonal lineage is detected all over the year in three seasons and during three sampling years. This could be explained by the overlapping mode of strains between seasons. Also for the new highly diverse population, the highly genetic similarity

is found among isolates collected from different seasons. The most likely explanation is that at least some of unique genotypes generated by sexual reproduction survive and spread over seasons demonstrating their potential to start new epidemics. In addition we note that the genetic diversity is higher in the population collected from potato than that from tomato. The phylogenic clustering proves that potato *P. infestans* population is more diverse than tomato *P. infestans*. We conclude that in Tunisia, the sexual cycle does occur less frequently (if at all) on tomato than on potato, which may affect the selection. The host selection of *P. infestans* isolates in Tunisia, as reported previously by Harbaoui and Hamada (Harbaoui & Hamada, 2008), seems to take place primarily on potato. These results confirm the study of Legard *et al.* (Legard *et al.*, 1995) who reported a host specialization to tomato of isolates from USA, Canada, Mexico and the Netherlands. Also they confirmed the results found by Reis *et al.*(Reis *et al.*, 2003) who found that isolates collected from tomato crops in Brazil belonged to the clonal lineage BR-1, which indicated a strong host specificity of the lineages of *P. infestans* in Brazil. A similar situation seems to occur in Ecuador that the US-1 clonal lineage was found on tomato whereas the EC-1 clonal lineage was found on potato (Oyarzun *et al.*, 1998).

With the identification of 21 races among 31 tested isolates shows a highly diverse virulence spectrum. The isolates virulent on almost all R differentials are collected from North-EastK and North areas. Moreover, the virulence pattern correlates with the genetic diversity. The isolates from the clonal lineage NA-01 consistently show less complex races than isolates from the genetically diverse group. The lack of *Avr* factors in North-EastK and North (only *Avr5* and *Avr9* are detected) and the high number of races in both areas could explain the changes of the race structure of *P. infestans* in recent years. Possibly the occurrence of sexual reproduction and recombination drives the generation of new genotypes and generates more complex race structure. Alternatively the migrants that probably came to Tunisia and including the A2 mating type, have a more complex race structure from the start. Selection for complex virulence patterns could have resulted from the fact that these two geographic sites were recently used as experimental zones for screening resistance levels between subsets of potato varieties against *P. infestans*. However, in both North-EastT and North-West areas, we deduce the presence of six *Avr* factors (*Avr2, Avr5, Avr8, Avr9, Avr10*, and *Avr11*) and seven *Avr* factors (*Avr5, Avr4, Avr6, Avr8, Avr9, Avr10*, and *Avr11*) respectively in all tested isolates. Comparing with the other two regions it concludes that the dominant clonal lineage is less complex and less virulent on plants. Interestingly, tested isolates collected from tomato have less complex

races than those from potato. It demonstrates North-EastT isolates from tomato (TU0765, TU0646, and TU0614) have less complex races, while isolate TU0618 from potato shows a more complex pattern. Indeed, the phenotypic diversity could be due to epigenetic changes that may be different between potato and tomato. These changes could result from the climate conditions conductive to the selection pressure from hosts and the occurrence of new generations with more complex races. This high virulence diversity was reported in many countries in the world (Śliwka *et al.*, 2006, Bouws & Finckh, 2007, Guo *et al.*, 2009a, Runno-Paurson *et al.*, 2009, Van Poppel *et al.*, 2009). On the other hand, some *R* genes from *S. demissum* (*R5, R8, R9, R10,* and *R11*) could be still effective in some regions. The R5, R8 and R9 differentials capable to withstand almost all tested isolates can be highly useful for resistance breeding in Tunisia, in contrast to Toluca Valley in Mexico from where race 5, race 8 and race 9 were detected in more than half of the tested isolates (Rivera-Peña, 1990). Nevertheless, our results confirmed those found by Guo *et al*. (Guo *et al.*, 2009) and Li *et al.* (personal communication) who concluded that these three differentials were the most resistant cultivars against Chinese *P. infestans*. Furthermore, R10 and R11 are still effective for more than 80% of isolates but more aggressive isolates are found that can overcome these resistances.

The import of potato seeds from Europe to Tunisia yearly is the main way for the introduction of European strains especially from the Netherlands from where more than 50% of seeds is imported. Recently some strains that cause late blight epidemics in Europe such as "Blue_13" (Fry *et al.*, 2009) are probably introduced in Algeria via potato imported seeds (Corbiere *et al.*, 2010). Sofar, the aggressive EU genotypes are not detected in Tunisia (data not shown) at least in this survey. It suggests that the environmental conditions may unfavourably inhibit the spread of imported genotypes and the race structure of Tunisian population still be comforting although the apparition of new more complex races.

Altogether, Tunisian *P. infestans* population has a clear structuring and shows the presence of one clonal lineage NA-01 and a highly genetic diverse group with the sexual reproduction in North and North-East areas. The presence of sexual progeny could be the infection source during years of sampling and within regions. Probably, it is evidence of oospore conservation in the soil. This assumption is based on the fact that the oospore could be a soil-borne inoculum as reported elsewhere (Howard S, 1997, Rubin *et al.*, 2001, Fernandez-Pavia *et al.*, 2004). Oospores play an important role in the epidemiology of late blight and sexual progeny could generate genetically new genotypes that are

epidemiologically important (Gavino *et al.*, 2000). Sexual cycle depends on favourable climate conditions for oospore germination that probably occur i) after a long period of incubation in soil during summer during autumn's rain in combination with high temperature and ii) in the midseason when temperature starts to increase in combination with cloudy skies. Therefore, it will be highly important to monitor the dynamics of the *P. infestans* population and apply *R*-genes identified from other species of *Solanum* that can still be useful to control late blight in Tunisia (Park *et al.*, 2005, Vleeshouwers *et al.*, 2008, Vleeshouwers *et al.*, 2011).

MATERIAL AND METHODS

Sampling, isolation and storage

Samples were collected from potato crops and tomato greenhouses and fields from five major crop regions in Tunisia (Fig. 1): North (Bizerte), North-East (Cap-Bon), North-West (Beja and Jendouba), Tunis area and Coastal Zone (Chott Meriem). Samplings were carried out from different host plants, organs, regions and seasons during 2005, 2006, 2007 and 2008 (Table.1). Small pieces from the biotrophic part of blighted samples (leaf, stem, tuber, and fruit) freshly collected from fields or greenhouses, were placed in Petri dishes under desinfected tuber slices and incubated at 18°C and 16h in light, 8h in dark for 6 to 7 days in a climate chamber. When mycelium was growing on the top of the potato slice, the mycelium was transferred to fresh rye agar medium amended with rifampicin (24 mg.L^{-1}). *P. infestans* mycelia were purified by repetitive transfers to rye agar medium amended with rifampicinafter microscopic checks. Pure cultures were maintained at 18°C in the dark until further use. For long-term storage, two plugs of pure and fresh mycelium were placed in cryotube vial (1.8 ml) filled with 15% Dimethylsulfoxid (DMSO) solution. Vials were frozen slowly (in Polystyrene box) at -80°C during 24h and transferred to the liquid nitrogen container. To retransfer strains into fresh medium, vials can be taken from liquid nitrogen, kept at room temperature until thawing and plugs can be placed on medium plates.

Virulence bioassay

A representative set of 31 isolates (Table.1) was selected from the whole population based on genotypic and geographic diversity (see results) and used to inoculate a differential set of potatoes carrying the major resistance *R* genes (*R1-R11*) of *Solanum demissum*(Mastenbroek, 1953). Cultivar Bintje was used as a positive control of infection as it has no known *R* genes. From each potato genotype, two freshly cut leaves were incubated in water-saturated florists foam with abaxial side up on moist filter paper in a tray (60 x 25 cm). Fresh mycelia grown for 1-2 weeks on rye agar medium was flooded by sterile water and incubated for 2h at 4°C to stimulate the release of zoospores from sporangia. The spore suspension was adjusted to a concentration of 10^4 spores/ml. For each compound leaf, five droplets of 10µl zoospore suspension were placed in one side of each of 5 individual leaflets. In the other side of the same leaf, the inoculum of another isolate is placed by the same method. Trays covered by plastic bags were placed at 18°C under a photoperiod of 16h in light and 8h in dark during 7 to 8 days. Scoring was determined by calculating the infection

efficiency (IE) and by macroscopic scoring. Macroscopic scoring was performed by examining the leaves and rating the plant on a scale from 1 to 8 from sensitive to resistant depending on infected area and degree of sporulation in each point of interaction. Also the infection efficiency (IE) was determined by dividing the number of lesions per leaf by the number of inoculation spots (i.e. 10 per experiment). The *Solanum* genotype was classified as resistant (R) or susceptible (S) based on both the macroscopic phenotyping data as well as the IE. The interaction bioassay was repeated twice in the same experiment and twice in an independent experiment.

DNA extraction

To produce mycelia, 1 cm^2 plugs of pure mycelium were placed in Petri dish filled by 20ml liquid pea broth After 5 to 7 days, developed mycelia were rinsed with sterile distilled water and filtered. After drying the biomass in a flow-cabinet for 30 min, the mycelium was used for DNA extraction. The protocol used for the genomic DNA extraction was obtained from the International Potato Centre with slight modification. The biomass sufficiently dried (50-100 mg) is grinded in the liquid nitrogen, transferred in a sterile Eppendorf tube to which 500µl extraction buffer (EDTA 0.05 M, Tris 0.1 M at pH 8.0, NaCl 0.5 M, 0.7% ß-mercaptoethanol, and 0.25% SDS) were added. The suspension was incubated for one hour at 65°C and mixed by inversion each 15 min. The supernatant obtained after centrifugation (10min, 13000 rpm) was transferred to a new tube and 1/3 V of NaOAc 5 M was added and the mix was placed on ice for 20 min. After 10 min of centrifugation at 13000 rpm, the supernatant was transferred to another Eppendorf tube for which 10 µg/µl of RNase solution was added and the mix was incubated at 37°c for 30 min. 1:1 phenol: chloroform was added, mixed by vortex and centrifuged (1min, 13000rpm) and the watery phase was extracted twice with 1volume chloroform to eliminate possible phenol contamination. To precipitate DNA, 1volume of cold isopropanol was added to the supernatant. To increase the efficiency of nucleic acid recovery, tubes were kept at –20°C for 1h. A final centrifugation (10 min, 13000 rpm, 4°C) yielded a pellet of DNA that was washed with 70% of cold ethanol and centrifuged for 5 min at 13000 rpm. The pellet was dried overnight under the flow-cabinet and resuspended in 1X TE buffer (Tris-HCl 10 mM, pH8, EDTA 1 mM, pH8). Genomic DNA extracts were stored at -20°C until use.

Mating type determination

Two techniques were used for the mating type determination: while the first consisted on a molecular analysis using CAPS markers (Cleaved Amplified Polymorphism Sequence) and was mostly adopted since it is fast and reproducible, the second is based on a bioassay through *in vitro* crossing with two reference isolates with opposite mating type for confirmation of some doubted situation. CAPS technique consisted on amplifying genomic DNA with two primers hybridizing to a sequence of a marker linked to gene encoding for the mating type in *P. infestans* genome followed by an enzymatic restriction of the amplified sequence with *Hae*III enzyme that yielded two different patterns A1 and A2. Primer pairs W16-1(5'AACACGCACAAGGCATATAAATGTA3') and W16-2 (5'GCGTAATGTAGCGTAACAGCTCTC 3') used here were designed by Kim and Lee (Kim & Lee, 2002). The bioassay consisted on placing plugs of mycelium taken from fresh culture of unknown isolate in one side of a Petri dish containing pea broth medium in which tester isolates TU0646 for A1 or TU0820 for A2 was placed in the other side. After 7 days, the presence of the oospores was checked with a microscope at 100x

magnification. Chi-square values and significance of mating types frequencies were calculated using GenAlex software 6.4 (Peakall & Smouse, 2006).

Haplotype characterization

Mitochondrial DNA haplotypes were characterized using the PCR-RFLP technique. Specific sequences of mitochondrial genome of each sample were amplified by primer pairs P2 (F2 5'TTCCCTTTGTCCTCTACCGAT3'; R2 5'TTACGGCGGTTTAGCACATACA3') and P4 (F4 5'TGGTCATCCAGAGGTTTATGTT3'; R4 5'CCGATACCGATACCAGCACCAA3') designed by Griffith and Shaw. Amplified fragments were respectively digested by *Msp*I and *Eco*RI enzymes yielding restriction patterns by which the isolates could be classified into four haplotypes: Ia, IIa, Ib and IIb.

SSR markers

Of 165 collected and purified isolates, 157 were analyzed with twelve SSR markers in a multiplex PCR technique. Markers used in this study are: D13, G11, Pi4B, Pi04, Pi63, Pi70, PinfSSR2, PinfSSR3, PinfSSR4, PinfSSR6, PinfSSR8 and PinfSSR11 (Table 2). The forward primers of 11 markers and the reverse primer of SSR6 were labelled with FAM, VIC, NED or PET. These primers were prepared in dark cryotubes. PCR reactions were prepared with multiplexing all the 24 primers in one PCR tube for each DNA sample using the QIAGEN Multiplex PCR Kit (Qiagen, Germany). PCR plates were run in PTC200 thermocycler (MJ Research, Waltham, Massachusetts, USA), with initial denaturation at 94°C for 2 min, followed by 13 touch down cycles of 94°C for 30 seconds, from 66 to 53°C for 30 sec, and 72°C for 30 sec, then by 28 cycles of 94°C for 30 sec, 53°C for 30 sec, and 72°C for 30 sec, and a final extension at 72°C for 7 min. 1-2 μl of PCR product from successful amplifications were added to 1 μl de-ionized formamide loading buffer, denatured at 92°C for 3 min. The resulting amplification products were sized by capillary electrophoresis on an automated ABI 3730 using the molecular standard GeneScan-500 ROX and scored using GeneMapper3.7 software (Applied Biosystems).

Data Analysis

Genetic diversity based on SSR data was estimated according to the number of total alleles. The gene diversity and the genetic distances between populations were computed according to Nei (Nei, 1973) using the POPGENE software, v.1.31 (Yeh *et al.*, 1997). The multilocus microsatellite genotype (MLMG) for each isolate and the number of MLMG in each population were determined using the program MULTILOCUS V.1.3 (Agapow & Burt, 2001). Isolates with the same MLMG were treated as clones. Several indices of clonal diversity were calculated for each population, including i) the number of different multilocus genotypes, ii) the number of the most frequent genotype and iii) the clonal fraction (the proportion of isolates originating from asexual reproduction), calculated as $1 - [(\text{number of different genotypes}) / (\text{total number of isolates})]$ (Zhan *et al.*, 2003). The same program was used to compute the index of association I_A and the correlation coefficient r_d in order to deduce the possible linkage disequilibrium between individuals in each sub-population and in the whole population. The occurrence of specific allele and allele frequencies were computed using CONVERT software v. 1.31 (Glaubitz, 2004). The genetic differentiation (Fst) among *P. infestans* isolates collected from different regions and the gene flow (Nm) were further investigated by the Analysis of Molecular Variance (AMOVA) using ARLEQUIN 3.0 software (Excoffier *et al.*, 2005). Tolerance was set to 5% of missing per locus. The SSR data were analyzed by the phylogenetic software

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package TREECON® for Windows Version 1.3b (Van de Peer & De Wachter, 1994). The tree was rooted using the US-1 isolate VK1.4. Population structure was performed using a model-based software program, STRUCTURE 2.2 (Pritchard *et al.*, 2000). In this model, a number of populations (K=1....7) are assumed to be present, each of which is characterized by a set of allele frequencies at each locus. Individuals in the sample are assigned to populations (clusters), or jointly to more populations if their genotypes indicate that they are admixed. All loci are assumed to be independent, and each K population is assumed to follow Hardy–Weinberg equilibrium. The posterior probabilities were estimated using a Markov Chain Monte Carlo (MCMC) method. The MCMC chains were run at 10^5 burn-in period lengths followed by 10^6 iterations using a model allowing for admixture and correlated allele frequencies. However, the inference of the exact value of K (gene pool) was not straightforward, because the estimated log-likelihood values appeared to be an increasing function of K for all examined values of K. Therefore, it may not be possible to find the true value of K. In this situation, Campana and collaborators (Campana *et al.*, 2011) propose to use CorrSieve software 1.6-3 that can calculate the ΔK todetermine meaningful value of K. This program can summarize the ΔK statistic directly from STRUCTURE outputs. The population K that has the highest value of ΔK is considered as the meaningful population.

ACKNOWLEDGEMENTS

CHAPTER 8

General discussion

CHAPTER 8

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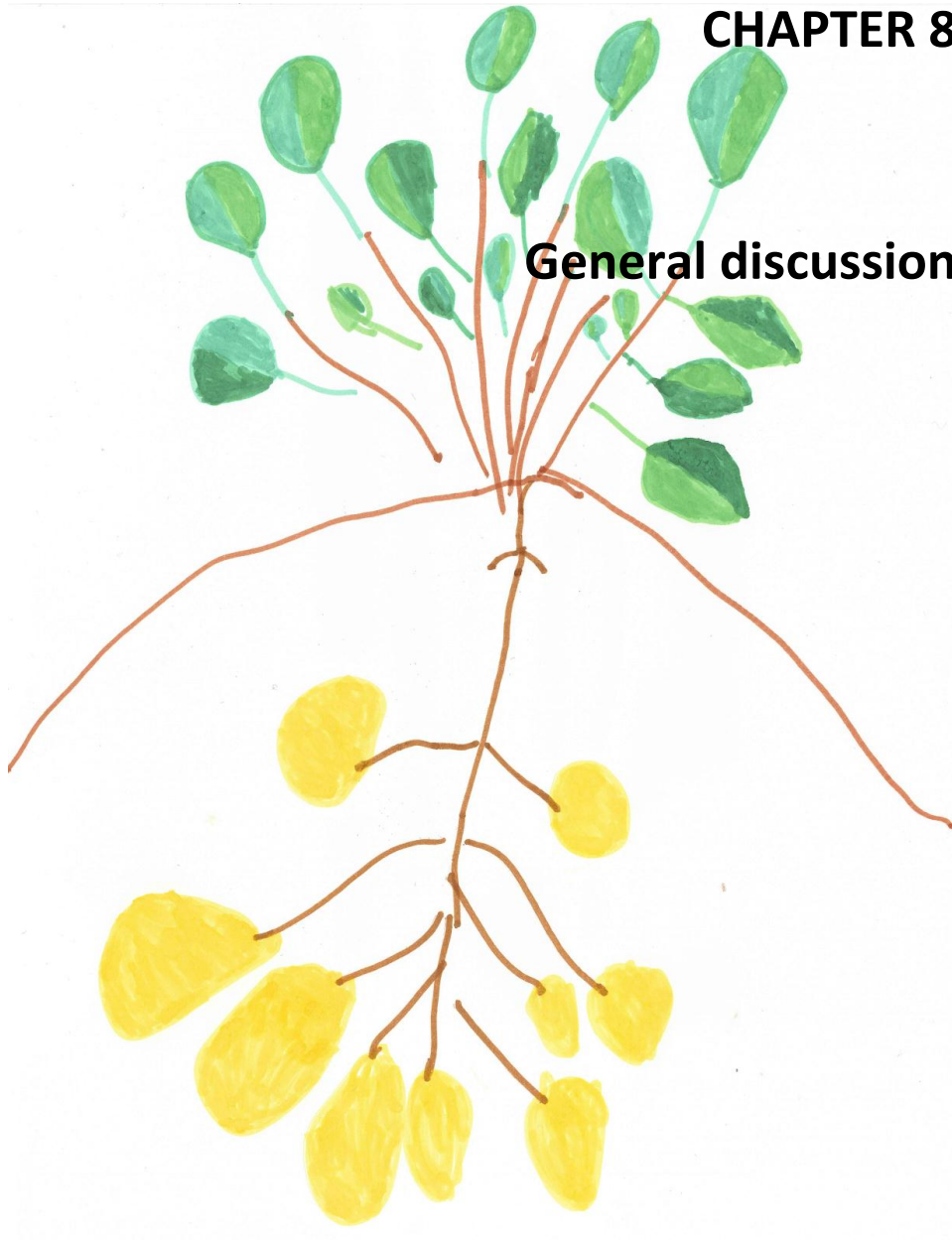
GENERAL DISCUSSION

Few genetic markers, if any, have found such widespread use as microsatellites, or simple/short tandem repeats. Microsatellites have been popular molecular markers ever since their advent in the late eighties (Ellegren, 2004). Despite growing competition from new genotyping and sequencing techniques, the use of these versatile and cost-effective markers continues to increase, boosted by successive technical advances (Guichoux *et al.*, 2011). Next-generation sequencing technologies allow the identification of large numbers of microsatellite loci at reduced cost in non-model species. So far, however, only a limited number of informative microsatellite loci have been identified in the potato late blight pathogen *Phytophthora infestans* and none have been mapped (Knapova & Gisi, 2002, Cooke & Lees, 2004, Lees *et al.*, 2006).The structure of global *P. infestans* populations changed significantly after new genotypes of both A1 and A2 mating type isolates were introduced in the late 1970s (Drenth *et al.*, 1994), which raises the question whether new genotypes in the pathogen population may have evolved through sexual reproduction of isolates and what the real structure of current global population is. So we propose the right *R*-gene management strategy in potato breeding, but also in potato production, should include the direct monitoring of pathogen populations. The selection of broad spectrum *R*-genes and stacking of them is dependent on the population analysis of late blight in the potato growing regions of interest. In future, another important way to manage late blight is an additional screening for the presence or absence of known functional *Avr* genes which is directly related with cross reacting *R*-genes. Based on the results in this thesis, this chapter is devoted to discuss the application of microsatellites in monitoring genetic diversity of late blight and the potential use in the resistance breeding.

Global standard tool for the *P. infestans* genetic diversity

There are several requirements for a standard protocol for genetic analysis of (i) high resolution of genetic variation (ii) it has to be able to handle large population sizes, (iii) should allow the integration of global datasets derived from different research groups generated by different types of equipment, (iv) automatic scoring of raw data and standards, correct identification and analysis of markers.

To achieve these goals, the research community of *P. infestans* was already looking for the most optimal approach for many years. In the past, genotypic characterization of *P. infestans* isolates included allozyme variation patterning at the loci coding for

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glucose-6-phosphate isomerase (*Gpi*) and *peptidase* (*Pep*), mitochondrial DNA haplotyping, RFLP fingerprints with RG57, and AFLP fingerprinting (Fry *et al.*, 1992, Drenth *et al.*, 1994, Van der Lee *et al.*, 1997, Van der Lee *et al.*, 2001, Wangsomboondee *et al.*, 2002, Van der Lee *et al.*, 2004, Gotoh *et al.*, 2007). Due to the limited genomic knowledge of *P. infestans*, few molecular markers were used in population studies. This is not allowing to "fine" genotype different populations with high resolution and to learn the sub-variances within dominant clonal lineages. The traditional genotyping assays mentioned above are very time-consuming and labor intensive. Moreover, researchers use different assays and protocols which make difficult to integrate all raw data to a global database. Earlier studies described populations where clonal lineages dominated with mainly one mating type (Drenth *et al.*, 1994, Goodwin *et al.*, 1998, Cohen, 2002), while in recent twenty years with the occurrence of both mating types more complicated and diverse populations were reported (Sujkowski *et al.*, 1994, Goodwin *et al.*, 1992a, Goodwin *et al.*, 1998, Hermansen *et al.*, 2000, Cooke *et al.*, 2011). A better molecular tool was needed to distinguish the more complex population structure in *P. infestans* efficiently.

With slowly unveiling the genome sequence of *P. infestans*, the traditional markers have been fading out. Meanwhile, a new generation of molecular markers was getting more popular for studies on population genetics and genetic identity due to its higher informative value, multi-allele detection and integration of datasets. Recent advances in genomics are revolutionizing the field of *Phytophthora* research. They also form a crucial resource from which valuable DNA-based markers which can be coupled with advances in fingerprinting technology and laboratory automation which are facilitating affordable, high-throughput analysis of multiple DNA-based markers. Co-dominant markers such as microsatellites, also known as simple sequence repeats (SSRs), have improved the ability to detect cryptic outcrossing in fungi (Vandenkoornhuyse *et al.*, 2001), and may provide a better understanding of the overall genetic structure of clonal species. SSRs have been used to investigate the genetic structure and reproductive biology of numerous plant pathogens (Tenzer *et al.*, 1999, Sirjusingh & Kohn, 2001, Gramacho *et al.*, 2007, Njambere *et al.*, 2010, Jänsch *et al.*, 2011), such as different fungi but also *P. capsici* (Wang *et al.*, 2009). SSRs are currently the preferred type of marker due to their ease of handling, high level of polymorphism, co-dominant scoring and the ease in which scorings can be automated. Therefore, it is very promising that SSRs can be applied in key fields such as population biology and epidemiology of *P. infestans* populations,

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Recent years, several SSRs were developed for *P. infestans* and used widely (Cooke & Lees, 2004, Knapova & Gisi, 2002, Li *et al.*, 2010, Lees *et al.*, 2006). However, facing the problem of a big amount of isolates sampled in the field, the accomplishment of a high-throughput detection method was next question to be answered. Fluorescence-based SSR detection on the automated sequencer is one of the fast and accurate ways for SSR genotyping. This approach allows fast, cheap and robust genotyping of individual samples on large-scale or *in planta* fingerprinting. Technically, one advantage of fluorescence-based SSR genotyping is that several SSRs can be simultaneously separated applying different colors and/or size ranges for the different SSR loci. Simultaneously several SSRs are amplified in a single PCR reaction (Hayden *et al.*, 2008). Multiplex PCR now forms the basis for many studies, on both diploid and polyploidy species (Jewell *et al.*, 2010, Raabová *et al.*, 2010), reducing very significantly the costs and time of genetic analyses. Automated analysis after data production can be standardized with guidelines for quality control and downstream analysis. Afterwards, the integrated databases should be easily imported to international standards/consensus. The first multiplex SSRs for examining *P. infestans* populations were attempted by Li, *et al*, 2010 (Li *et al.*, 2010) (**Chapter 2**). From a screening of 300 non-redundant SSR loci that were identified in ESTs and genomic sequences from *P. infestans*, based on their positions on the genetic linkage map and on the information content in populations eight SSR markers were selected. These eight highly informative SSR markers were assembled into **two multiplex PCR sets** using fluorescent labeled primers that allow all markers to be scored after one single capillary electrophoresis run.

In light of the threats from changing *P. infestans* populations worldwide, particular emphasis has to be placed on the utility of existing SSRs and set up a global standard "ruler" of genotyping analysis. The one should be able to be maximized by the rapid public release of protocols and applications, ideally collated into a global database to link the populations from different countries. To accommodate the needs, the one-step multiplex PCR method was developed by us to facilitate worldwide high-throughput screening of *P. infestans* populations (**Chapter 3**). The SSR markers reported in the earlier studies were re-evaluated and integrated, of whom the twelve most informative SSRs were selected. This **one-step multiplex PCR method** significantly increases the resolution of genotyping compared to the previous SSR set in **Chapter 2**.

Moreover, how many markers should be used in population studies? This type of question has been discussed for a long time, especially for SNPs in general (Smouse, 2010) and for human beings (Jorde & Wooding, 2004, Edwards, 2003). For sure, the detection of

subtle genetic population differences is only possible if enough genetic markers are used(Rosenberg *et al.*, 2005). We have been enamored with the idea that if we just have enough polymorphic Mendelian segregating loci with a sufficiently large worldwide sample pool, the genetic misclassification probability becomes close to zero. With increasing availability of polymorphic molecular markers across genomes, examining population structure using a large number of loci has become a common practice in evolutionary biology and human genetics (Steinmetz *et al.*, 2000). To dissect sub-clonal lineages, it suggests to integrate SSR markers with genomic SNP screening when the whole genome sequence is available. For *P. infestans*, there are not many robust markers published or used, therefore, we made the best of what was available, taking advantage of published or well-known SSRs and selecting the most informative ones to set up a standard set for the population analysis.

In assigning individual membership and inferences, investigators have found that some markers (or variants) are more informative than others (Kalinowski, 2004). In such cases, many loci are typed on samples from these populations, and subsets of these loci (typically those that appear most divergent between the populations) are chosen for analysis. Selecting and using only the most informative markers for population assignment can reduce both time and genotyping costs while retaining most of the power of the complete set of markers. Our study represents different SSR markers which are more or less informative for different regions. Within different populations, G11 and PinfSSR4 always show highest PIC values (e.g. mean PIC value of G11 is 0.78 and 0.76 for PinfSSR4), while G11 is less informative in Ecuadorian *P. infestans* (0.34). Pi70 is always less informative in the populations, e.g. in China Pi70 shows no diversity, but has 0.53 for PIC value within Tunisian *P. infestans*.

The 12-plex SSRs are distributed over different chromosomes (**Chapter 3**), which is more than in other related studies to detect subtle genetic population differences. Our new SSR fingerprint protocol facilitates world-wide genotyping of *P. infestans* isolates and the generation of an integrated high resolution database under the EUCABLIGHT project (www.eucablight.org). We tested the assay in two different laboratories (Wageningen University, the Netherlands and the James Hutton Institute, UK) and standardized the allele binning and nomenclature across all 12 multiplexed loci. This assay should serve as a single means of scoring *P. infestans* diversity that is applicable for international use allowing high-throughput screening on large *P. infestans* populations. In previous studies, higher levels of ploidy or aneuploidy of *P. infestans* (Tooley & Therrien, 1991, van der Lee *et al.*, 2004) were reported. Such a mixture of isolates of different levels of ploidy makes the analysis of the resultant SSR data more complex (Bruvo *et al.*, 2004, Clark & Jasieniuk, 2011).**Chapter**

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3 is not to explore the specific details of the populations in question, butpresents means of analyzing such data.

SSR markers provide the backbone of the population analysis

Early studies described populations that were clonal or dominated by a few discrete lineages (Drenth *et al.*, 1994a, Goodwin *et al.*, 1998, Cohen, 2002), whereas more recent analyses highlight the appearance of many new genotypes via migration and/or sexual recombination (Goodwin *et al.*, 1992, Sujkowski *et al.*, 1994b, Goodwin *et al.*, 1998, Hermansen *et al.*, 2000, Cooke *et al.*, 2003). The one-step multiplex SSR detection, as discussed above, plays a key role to dissect the more complex *P. infestans* populations (**Chapter 3**). The first populations to try the "ruler" were the analysis of Chinese (**Chapter 4**), Dutch (**Chapter 5**), Ecuadorian (**Chapter 6**) and Tunisian (**Chapter 7**) populations, showing very interesting population diversities and remarkable differences.

China is still growing as the biggest potato cultivation and seed potato multiplication country with an important and frequent interprovincial trade of consumption and seed potatoes. The A2 mating type was first found in northern China in 1996 (Zhang *et al.*, 1996) and later detected in other regions, including Yunnan in Southern China close to the Vietnamese border (Zhao & Zhang, 1999). Although both mating types are found in China, to this date, no evidence of an actively sexual cycle based on changes in allele frequency was found (Yang *et al.*, 2008). With the 12-plex SSRs, a total of 229 *P. infestans* isolates, collected from seven main potato growing regions in China between 2002 and 2009 and a few from a previous study (Guo *et al.*, 2009), were genotypically characterized (**Chapter 4**). One of the three dominant clonal lineages in this Chinese population was genetically similar to "Blue_13", a dominant genotype found in Europe since 2004. It is the first report of "Blue_13" outside Europe. Within the Chinese P. *infestans* population, the genotypes strongly clustered according to their sampling provinces, which seem not to be influenced by the frequent interprovincial trading activities. The same phenomenon was found in the *P. infestans* population study of Tunisia (chapter 6). The genetic diversity based on the new SSR approach and reported here is not consistent with the nation-wide admixed population reported previously (Andrivon *et al.*, 2011). Nevertheless, the number of genotypes identified in this study exceeded those previously observed (Guo *et al.*, 2009) and it was clearly shown that different genotypes can be found in all regions including Northern China. The reason for these differences compared with the previous study most likely is due to the use of robust

genetic markers and anextended number of isolates.

The Netherlands has a long history of population studies on local *P. infestans* isolates (Spielman *et al.*, 1991, Drenth *et al.*, 1993b). During the 1980's, following a renewed global migration of both mating types Dutch population studies were the first to report that a new *P. infestans*population rapidly displaced the US1 clonal lineage (Spielman *et al.*, 1991, Drenth *et al.*, 1994a). In this thesis we describe a survey of the structure and dynamics of Dutch *P. infestans* population including 652 isolates, collected during a ten year period, 2000 – 2009 (**Chapter 5**). The results emphasize the importance of the sexual reproductionfor genetic recombination and the importance of the asexual clonal lineage together with spontaneous mutations as the driver for the selection of successful genotypes to disperse over larger areas in the Netherlands.

In Ecuador, potato landraces are cultivated on small acreages, with low input of pesticides and often several landraces are grown together. They constitute a potential source of genetic variation for breeding purposes, like quality, earliness and resistance to biotic and abiotic stresses. Studies on *P. infestans* in Ecuador, showed the presence of two closely related species, *P. infestans* on potato or tomato and *P. andina* on non-tuber bearing host*,* but they wereclearly distinguished as two genetic groups based on their SSR profile (**Chapter 6**). The *P. infestans* group is an ancient clonal lineage in Ecuador, but with the 12-plex genotyping a large sub-clonal variationwas detected in isolates collected from potato and it suggests that polyploidization and loss of alleles play a key role in the sub-clonal variation.

In Tunisia where potato is cultivated in three to four partly overlapping seasons, while on tomato *P. infestans* can damage either greenhouse or field grown tomatoes in most of the producing regions. However, little is known about genetic diversity and epidemiologic aspects of *P. infestans*in Tunisia. To genetically characterize the Tunisian population of *P. infestans*, 165 isolates were collected from North, North-East, North-West, Tunis area and Coastal zone of Tunisia between 2006 and 2008 (**Chapter 7**). Although mitochondrial genotyping showed little genetic diversity, the 12-plex SSRs revealed high genetic diversity in the population. It revealed the presence of a major clonal lineage (NA-01_A1), but sexual reproduction helped to overlap periods of this pathogen between two growing seasons, potentially playing a role for the epidemics in coming planting season. Despite the massive import of potato seeds from Europe, Tunisian *P. infestans* population is still clearly distinct. Strict management on late blight control may avoid the spread of aggressive *P. infestans* genotypes such as "Blue_13" from Europe or neighbouring countries to this country. It is also

possible that genotypes like "Blue_13" are not yet sufficiently adapted to this region and that it is a matter of time to come so far.

With the advantage of the standard 12-plex SSRs, it becomes possible to compare the *P. infestans* from different countries or regions. We performed an extensive population structure analysis using the SSR profiling of the four populations (Chinese, Dutch, Ecuadorian and Tunisian, data not shown). It is clear that the population structure is strongly related to the geographic origin except of "Blue_13" detected in China and the Netherlands grouped together. The local clonal lineage plays a dominant role in all four countries, while it reveals in the Netherlands and Tunisia that the sexual reproduction potentially facilitates the epidemics within the season and/or between seasons.

Besides longitudinal comparison among countries, the "ruler" could also help to track and trace the dynamics of a clonal lineage through the years. In the past it was possible to include a known isolate to detect certain dominant clonal lineages among the putative genetic structure (Flier *et al.*, 2003, Cardenas *et al.*, 2011). However, within one simple clonal lineage the sub-clonal variation only can be perceptible by the availability of the high-resolution SSR set, as shown in the Chinese (**Chapter 4**) and Dutch (**Chapter 5**) "Blue_13" clonal lineage and Ecuadorian ancient clonal lineage (**Chapter 6**).

Clonal lineages are a useful concept for organisms with asexual reproduction and with very simple homogeneous population structures so that the individual lineages can be identified. Clonal lineages have been discussed by Anderson and Kohn (Anderson & Kohn, 1995), and a paraphrase of their definition of a clonal lineage is useful: '*the asexual descendants of a given genotype differing from the originator only via mutation and mitotic recombination*'. As mentioned before, the world is suffering the big change of the *P. infestans* population structure during the past 30 years. Several clonal lineages of *P. infestans* have been defined in North America (Goodwin *et al.*, 1994a), Europe (Lees *et al.*, 2009) and other parts of world (Gotoh *et al.*, 2007, Guo *et al.*, 2009). These lineages are based on diverse genetic markers: mating type, the *Gpi* allozyme locus, the *Pep* allozyme locus and RFLP probe RG57. The definition of "clonal lineage" is challenged due to the high-resolution SSRs applied and the large sub-clonal variations observed. We found large sub-clonal variations and clear genetic recombination that occurs only during the sexual cycle with the 12-plex SSRs. To the situation in Ecuador where we found only a clonal population of *P. infestans* (**Chapter 6**), the loss of an allele causes the sub-variation within the clonal lineage. Within the "Blue_13" clonal lineage of Dutch *P. infestans*, a few rare alleles presented for the sub-variance (**Chapter 5**). In Dutch *P. infestans* isolates, the sexual

propagation plays an important role for the genetic recombination. The sexual cluster was illustrated by the absence of clonal lineages and the presence of both mating types, multiple haplotypes and the most aggressive isolates (data not shown). The isolates lacking the *Avrblb1/ipiO* class I gene were indeed found to be virulent on potato plants carrying the *Rpi-blb1* resistance gene, only found in this cluster. Sexual reproduction could be a method by which novel genotypes (or more aggressive isolates) are created (Halkett *et al.*, 2005). The occurrence of sexual recombination dictates major *R*-genes would not be durable, as genetic recombination would yield more frequently virulent strains (**Chapter 5**). However, within the clonal population of *P. infestans* in Ecuador, still some of the more virulent isolates were identified.

Potential of monitoring *P. infestans* genetic diversity for effector variation and breeding

New possibilities offered by marker assisted breeding and GMO breeding have sparked renewed international efforts to breed for durable potato late blight resistance using broad spectrum major *R*-genes. *P. infestans*is, however, known for its adaptability to resistance genes. This potential has been better understood by recent discoveries on the structure and gene order of the whole *P. infestans* genome (Haas *et al.*, 2009). In the past *R*-genes derived from *Solanum demissum*, were used for breeding late blight resistant potato cultivars. However, resistance with these major *R*-genes proved not to be durable since *P. infestans* was able to adapt and break these *R*-genes in rapid succession (Cassells *et al.*, 1991, Van Der Plank, 1971, Turkensteen, 1993). One of the new possibilities to enhance the durability of newly introduced host resistance genes is to monitor the pathogen population for virulence prior to and after their introduction. Monitoring of the local *P. infestans* populations for new virulent genotypes and/or screening for effector variation (**Chapter 5**), allows early detection of adaptation within the *P. infestans* population in a certain region. This gives the possibilities to determine which *R*-genes are broad spectrum and to adapt the control strategy to the new situation. One way of doing that is to replace the existing varieties by other varieties with new *R*-genes by classical breeding or to add (additional *R*-genes) to existing susceptible varieties. In a transgenic or cisgenic approach, *R*-genes could be added by normal transformation and later, if needed, improved by re-transformation with additional broad spectrum *R*-genes.

The Dutch *P. infestans* population was monitored during the growing seasons of recent decade. These *P. infestans* isolates were collected from blighted production fields and from

trap fields in which *R*-gene containing potato clones were grown without fungicide protection. When we focused on the presence of the *Avrblb1/Ipio* class I gene in the isolates and the resistance reaction of an *Rpi-blb1* containing transgenic Desiree plant on these isolates (**Chapter 5**), it was clear that the isolates lacking the *Avrblb1/Ipio* class I gene were virulent. Superimposing the virulence screening on the SSR genetic backbone indicated that loss of the *Avrblb1/Ipio* class I gene was unique to the genetically highly diverse sexual isolates and no variation was found among members of the clonal lineages. So far the asexual spread of such identified virulent isolates has been limited. It is also suggested that such kind of loss of presence mutations of the *Avrblb1/Ipio* class I gene are less frequently found in existing clonal lineages. This indicates that although the majority of the late blight infections are caused by clonal spread of dominant lineages for the exploitation of resistance genes such as *Rpi-blb1*, the main risk may come from yet marginal genotypes that arise from the sexual cycle in which some offspring are missing the *Avrblb1/Ipio* class I gene because of unequal crossing over. The necessity of monitoring such minor virulent genotypes in local populations cannot be ignored. It means that the application of the *Rpi-blb1* resistance gene could be more durable in regions with a *P. infestans* population only or mainly consisting of one mating type.

From these findings, it is highly recommended that a monitoring system should be part of future potato late blight control strategy. The monitoring system should include not only monitoring the population diversity, but also screening of the mating type and screening of (a)virulence by the presence and absence of individual *Avr*-genes in *P. infestans*. The known backbone of SSR genotyping can locate the virulent isolates in the population structure, either within the dominant clonal lineage or the sexual progeny. Genomics of *P. infestans* brought a lot of information on effectors, potentially containing *Avr*-genes directly interacting with specific *R*-genes leading to HR (hypersensitivity reaction). Resistance and genomics research in potato and crossable species provided a number of candidate *R*-genes. At the moment a number of them have been cloned belonging to 7 different classes (*R1, R3a, Rpi-blb1, Rpi-blb2, Rpi-blb3, Rpi-oka1, Rpi-chc1* (Ballvora *et al.*, 2002, Huang *et al.*, 2005, Van Der Vossen *et al.*, 2003, van der Vossen *et al.*, 2005, Park *et al.*, 2005) in the wild species *S. demissum, S. bulbocastanum, S venturii* and *S. chacoence*, respectively . It is important that not only *R*-genes are isolated but also the interacting *Avr*-gene(s) in the pathogen. This is the case with all above indicated *R*-genes (Vleeshouwers pers. comm). After isolation of as many *Avr*-genes as possible it is possible to screen all kind of isolates of *Phytophthora*on the presence of functional *Avr*-genes. Such a screening of isolates shows in this way the potential

spectrum of the *R*-geneand which *R*-genes can be used where and in which combination in order to come to sustainable resistance. The resulting information on the dynamics of virulence within the local *P. infestans* population can then be used to enhance the durability of newly introduced host resistance and the further strategies of the resistance breeding, e.g. *R*-gene stacking for cisgenic potato.

Generally, *R*-gene cloning from donor wild species, followed by one-step transformation into cultivated plants can be considered as an efficient method of domesticating cisgenic *R*-genes into the agricultural environment. Stacking of several *R*-genes is expected to provide more durable and broadened resistance as compared to the introduction of a single *R*-gene, which was proven to be ineffective in the past. Zhu *et al.* (2012) performed to explore the strategy of functional stacking of 3 broad spectrum *R*-genes (Zhu *et al.*, 2012), *Rpi-sto1* (Vleeshouwers *et al.*, 2008a), *Rpi-vnt1.1* (Pel *et al.*, 2009) and *Rpi-blb3* (Lokossou *et al.*, 2009). This approach provides a new foundation to distinguish the functionality of each stack component and the expected broadened resistance spectrum based on the functional combination of the three individual *R*-genes, which indeed was achieved in the triple *R*-gene transformants. These three *R*-genes were specifically selected with help of monitoring Chinese *P. infestans*isolates from six provinces. It was observed that *Rpi-sto1* and *Rpi-vnt1.1* were potentially useful in all six sampled Chinese provinces and *Rpi-blb3* in three provinces and to a lesser extent in Yunnan Province of China (data not shown). Another observation was that none of *P. infestans* isolates from China was avirulent to *R3a* (Li *et al.*, 2009), indicating the general breakage of *R3a*. In trap field trials, individual *Rpi-vnt1.1* and *Rpi-sto1* were still applicable in the Netherlands, but *Rpi-blb3* and *R3a* were widely broken (Kessel *et al.* unpublished). A first field trial in the Netherlands and Belgium showed complete resistance of such triple *R*-gene transformants (Zhu and Jacobsen, personal communication). It is expected that the same observation could be made in the 6 Chinese provinces. The durability enhancing measures such as stacking *R*-genes combined with low input chemical control strategies specifically designed for resistant cultivars are explored by e.g. the DurPh project (www.DurPh.wur.nl) in the Netherlands.So, the further step is to launch these triple *R*-gene plants into field experiments in China and other countries to determine their resistance to the local *P. infestans* populations. On another hand, with advantage of the standard genotyping tool of local *Phytophthora* isolates more potato countries could benefit from the application of *R*-gene stacking by cisgenesis. Cisgenesis is marker-free transformation of potato cultivars with stacked *R*-genes which is more acceptable for consumers in Europe as the Eurobarometer (2010) clearly indicated.

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This thesis adds the one-step multiplex SSR detection method to the existing haplotyping, mating typing, (a)virulence and *Avr* gene determination methods in order to unravel late blight populations in potato growing areas in more detail. This knowledge can be used during potato cultivation but also for breeding of late blight resistant potato varieties with more durable resistance obtained by *R*-gene stacking.

SUMMARY

Potato is the most important non-cereal crop in the world. Late blight, caused by the oomycete pathogen *Phytophthora infestans*, is the most devastating disease of potato. In the mid-19th century, *P. infestans* attacked the European potato fields and this resulted in a widespread famine in Ireland and other parts of Europe. Late blight remains the most important pathogen to potato and causes a yearly multi-billion US dollar loss globally. In Europe and North America, late blight control heavily relies on the use of chemicals, which is hardly affordable to farmers in developing countries and also raises considerable environmental concerns in the developed countries.

The structure of *P. infestans* populations can change quickly by migration, sexual recombination and sub-clonal variation. Migration and the reconvening of the two mating types considerably raised the level of genetic diversity in the global *P. infestans* population, leading to a more variable population with a presumed higher level of adaptability as compared to the previously, purely asexually, reproducing population. How canthe *P. infestans* population efficiently be monitored with such diverse genotypes? A high-throughput, high-resolution and easy-handled set of markers would be favorable for this purpose. Few genetic markers, if any, have found such widespread use as SSRs. Sequencing allows the identification of large numbers of microsatellites by bioinformatics. So far, however, only a limited number of informative microsatellite loci had been described for *P. infestans* and none have been mapped.This thesis first describes the development and mapping of SSR markers in *P. infestans* and integration with other SSRs to generate a multiplex SSR set and its application in the population analysis of *P. infestans* from four countries are described with the developed multiplex SSRs. Finally, the use of this knowledge in resistance breeding of potato is shortly indicated and discussed.

Chapter 1 describes the historic population changes of *P. infestans* at the global level and the current population trends.It summarizes microsatellite as favorite molecular markers for studying pathogen population diversity and assesses monitoring of population dynamics in more detail for resistance breeding in potato.

The selection and identification ofnew SSR markersis presented in **Chapter 2.** From EST and genomic sequences from *P. infestans* we identified 300 non-redundant SSR loci by a bioinformatic screening pipeline. Based on the robustness, level of polymorphism and map position eight SSR markers were selected, which were assembled in two multiplex PCR sets

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and labeled with two different fluorescent dyes to allow scoring after single capillary electrophoresis.

This successful multiplex SSR approach encouraged the development of fast, accurate and high-throughput genotyping, in an one-step multiplex PCR method to facilitate worldwide screening of *P. infestans* populations. Published SSRs and the 8new SSRs were integrated.All these SSR markers were re-evaluated and the 12 most informative SSRs were selected to set up a standard set for global application (**Chapter 3**). The 12-plex SSRs are distributed over different chromosomes, significantly increasing the resolution of genotyping compared to the previous set of 8 SSRs. The 12-plex SSRs were integrated to one-step fluorescence-based multiplex reaction, which plays a key role to facilitate highly paralleled genotyping and efficient dissection of the more complex *P. infestans* populations. This multiplex PCR for *P. infestans* populations is (i) simple, as only one PCR is needed to perform multi-locus typing with twelve markers; (ii) rapid, as the genotyping results can be available in 1 day; and (iii) reproducible and adapted to different laboratories. The genotyping data from different geographic populations were submitted to the Euroblight database. With the same SSR set and the bin set, a comparable global database can easily be achieved.

As indicated earlier, more recent analyses of *P. infestans* populations highlight the appearance of many new genotypes via migration and/or sexual recombination. To practice the newly developed 12-plex SSR set and dissect the current population structure, several *P. infestans* populations from 4 different continents were selected for analysis. These include Chinese (**Chapter 4**), Dutch (**Chapter 5**), Ecuadorian (**Chapter 6**) and Tunisian (**Chapter 7**) populations.

China has become the largest potato producing country not only for potato cultivation area but also in Megaton potato production. Interprovincial trade of consumption and seed potatoes is very important and frequent in China. Although both, the A1 and A2 mating types are found in China, to this date, no evidence of an active sexual cycle based on changes in allele frequency was found. With the ten SSRs, a largegenotypic survey of in nation-wide collection of 228 *P. infestans*isolates was performed (**Chapter 4**). One of the three dominant clonal lineages CN-04 (A2) in this Chinese population was genetically similar to a major clonal lineage identified in Europe, called "Blue_13" with A2 mating type. It was not possible to critically assess the origin of this clonal lineage. This study is the first report of "Blue_13" outside Europe. The virulence spectrum of selected Chinese *P. infestans* isolates showed seven different virulence spectra varying from 3 to 10 differentials. The CN04

genotypes were identified as more aggressive and more virulent genotypes, one of whom had the full virulence pattern after using the potato differential set. Within the Chinese *P. infestans* population, the genotypes strongly clustered according to their six sampling provinces, which seem not to be influenced by the frequent interprovincial trading activities of seed potatoes. The mating type ratio and the SSR allele frequencies indicate that in China the contribution of the sexual cycle to *P. infestans* onpopulation dynamics is minimal. It was concluded that the migration through asexual propagules and the generation of sub-clonal variation are the dominant driving factors behind the Chinese *P. infestans* population structure.

The Netherlands has a long history of population studies on local *P. infestans* isolates and a substantial amount of commercial potato varieties growing in the field. One decade (2000-2009) of isolate sampling in 5 different regions provided the basis for a good understanding of the population dynamics in the Netherlands (**Chapter 5**). The surveyed population revealed the presence of several clonal lineages and a group of sexual progenies. The major clonal lineage with A2 mating type is known as "Blue_13", but also two distinct clonal lineages with A1 mating type in this study have been identified.This survey witnesses that the Dutch population was undergoing dramatic changes in the ten years under study. The most notable change was the emergence and spread of A2 mating type strain "Blue_13". The results emphasize the importance of the sexual cycle in generating genetic diversity and the importance of the asexual cycle as the propagation- and dispersal mechanism for successful genotypes. In addition to the neutral SSR markers a molecular marker for the virulence of isolates on potato lines that contain the *Rpi-blb1R*-gene has been developed. Using this *Avr-blb1* marker and the corresponding virulence assay we report, for the first time, the presence of*Rpi-blb1* breaker isolates in the Netherlands even before a *Rpi-blb1* containing resistant variety was introduced. The 12 breaker isolates only occurred in sexual progeny. So far the asexual spread of such virulent isolates has been limited because of the absence of *Rpi-blb1* containing varieties in the field.

Remarkably, on the other end of the world in the Andes, the region of potato origin, the situation is far less complex as far as *P. infestans* is concerned. There are more than 400 potato landraces in Ecuador and the planting habit by local farmers by traditional cultivation at small scale in the highlands is different from potato cultivation in other potato countries in North America or Europe (**Chapter 6**). *Phytophthora* isolates in Ecuador belong to two closely related species, *P. infestans* (on potato and tomato) and *P. andina* (on non-tuber bearing host), but SSR analysis of 66 isolates indicated that the two species are separated in two clearly distinguished genetic groups. Two ancient clonal lineages of *P. infestans*

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appeared to be dominant in Ecuador one is found only on tomato the other one only on potato. Within the potato isolates, but not in the tomato isolates, there is a large sub-clonal variation caused by(partial) polyploidization and loss of alleles.

In Tunisia, potato is cultivated in three to four partly overlapping seasons while tomato is grown either in greenhouses or as aerial crop in most potato producing regions. **Chapter 7**revealed, among 165 isolates of five regions, the presence of a major clonal lineage (NA-01, A1 mating type, Ia mtDNA haplotype) that seems to consist of races that are relatively simple. Another highly genetic diverse group of isolates was found containing more complex races and isolates with both mating types. Season clustering indicated that at least some of the new genotypes generated by sexual reproduction overlapped between seasons and such a   sexual progeny may play an important role in the next season epidemics. On tomato, mostly asexual progeny was identified with twomtDNA haplotypes but less nuclear genotypes, compared to potato. This study shows that the *P. infestans* population is currently changing, and the old clonal lineage is being replaced by a more complex, genetically diverse and sexually propagating population in two sub-regions in Tunisia. Despite the massive import of potato seeds from Europe, the *P. infestans* population in Tunisia is still clearly distinct from the European population.

Chapter 8 discusses the applicationof microsatellites in monitoring genetic diversity of late blight and the potential use in resistance breeding. Monitoring of the local *P. infestans*population for new virulent genotypes with the differential potato set in combination with screening for effector variation, allows early detection of adaptation of certain genotypes within the *P. infestans* population to particular resistance genes in a specific region. This provides the possibilities to determine which broad spectrum *R*-genes are still useful in order to adapt the control strategy by resistance breeding to the new situation. One way of doing that is to replace the existing varieties by other varieties with stacked non-broken*R*-genes obtained by marker assisted selection or to add additional *R*-genes to existing (*R*-gene containing) varieties by transformation. In a transgenic or cisgenic approach, additional broad spectrum *R*-genes could be added by re-transformation. As we have shown, the right *R*-gene management strategy in potato breeding, but also in potato production, should include the direct monitoring of local pathogen populations by using the differential set and the 12-plex SSR set.

Samenvatting

Naast de graangewassen is aardappel het meest belangrijke voedselgewas wereldwijd. De aardappelziekte, veroorzaakt door de oömyceet *Phytophthora infestans*, is de meest verwoestende ziekte. Tijdens het midden van de 19^e eeuw, tastte P. infestans de Europese aardappelpercelen aan wat in Ierland en andere delen van Europa een grote hongersnood tot gevolg had. De aardappelziekte is bij aardappel sindsdien de belangrijkste ziekte gebleven en veroorzaakt wereldwijd elk jaar nog steeds miljarden dollars schade. In Europa en N. Amerika berust de beheersing van deze ziekte voornamelijk op het frequent gebruik van bestrijdingsmiddelen wat milieu- en gezondheidsrisico's met zich meebrengt. In ontwikkelingslanden zijn deze middelen daarnaast nauwelijks beschikbaar..

De samenstelling van de *P. infestans* populatie kan door migratie, sexuale recombinatie en sub-klonale variatie snel veranderen. Migratie en het weer bij elkaar brengen van de beide paringstypen verhoogde het niveau van de genetische diversiteit van de wereldwijde *P. infestans* populatie. Dit heeft geresulteerd in een meer variabele populatie met, in vergelijking met de vroegere klonale populatie, een beter adaptatie vermogen. Hoe kan de *P. infestans* populatie met zulke verschillende genotypen efficiënt worden beheerst?

Er zijn maar weinig genetische merkers, die op zo'n grote schaal toegepast worden als SSR's. Sequencing en bioinformatica maakt de identificatie van grote aantallen van microsatellieten mogelijk. Tot nu toe echter zijn er slechts een beperkt aantal informatieve microsatellieten van *P. infestans* beschreven en geen enkele is gelokaliseerd. Dit proefschrift beschrijft eerst de ontwikkeling en het in kaart brengen van SSR merkers in *P. infestans* en integratie met andere SSR's om tot een multiplex SSR set te komen en daarna de toepassing van de ontwikkelde multiplex SSR set in populatie-analyses van *P. infestans* in vier verschillende landen. Tenslotte wordt het gebruik van deze kennis voor de resistentieveredeling van aardappel kort aangegeven en besproken

Hoofdstuk 1 beschrijft de historische veranderingen in de populaties van P. infestans op mondiaal niveau en de huidige demografische ontwikkelingen. Het geeft een overzicht van de microsatelliet als favoriete moleculaire merker voor het bestuderen van pathogeen diversiteit en geeft het belang weer van monitoring van de populatiedynamiek voor de resistentieveredeling in aardappel.

De selectie en de identificatie van nieuwe SSR merkers wordt in **hoofdstuk 2** beschreven. Van EST's en genomische sequenties van *P. infestans* werden in een bio-informatica-screening pijplijn 300 niet-redundante SSR loci geïdentificeerd. Op basis van de robuustheid, de mate

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van polymorfie en kaart positie werden acht SSR merkers geselecteerd, die in twee multiplex-PCR-sets werden geassembleerd en voorzien werden van twee verschillende fluorescerende kleurstoffen die, na een eenmalige capillaire elektroforesestap, het scoren van de merkers mogelijk maakten.

Deze succesvolle multiplex SSR aanpak stimuleerde de ontwikkeling van snelle, nauwkeurige en high-throughput genotypering, in een één-staps-multiplex-PCR-methode om wereldwijde screening van *P. infestans* populaties te vergemakkelijken. Eerder gepubliceerde SSR's en de 8 nieuwe SSR's werden geïntegreerd. Al deze SSR merkers werden opnieuw geëvalueerd en de 12 meest informatieve SSR's werden geselecteerd om een standaard set voor wereldwijde toepassing op te zetten (**hoofdstuk 3**). De 12-plex SSR's zijn verdeeld over verschillende chromosomen, wat een aanzienlijke verhoging van de resolutie van genotypering van isolaten ten opzichte van de vorige set van 8 SSR's mogelijk maakte. De 12-plex SSR's werden geïntegreerd in een één-staps fluorescentie gebaseerde, multiplex reactie die een belangrijke rol speelt bij het vergemakkelijken van parallelle genotypering en efficiënte ontleding van de meer complexe *P. infestans* populaties. Deze multiplex PCR methode voor *P.infestans* populaties is (i) eenvoudig, omdat slechts één PCR stap nodig is om multi-locus typeringen te kunnen uitvoeren met twaalf merkers, (ii) snel, omdat de genotypering resultaten binnen 1 dagbeschikbaar zijn, en (iii) reproduceerbaar en toepasbaar in verschillende laboratoria. De genotypering gegevens van verschillende geografische populaties werden ondergebracht in de Euroblight database. Met dezelfde SSR set kan ook een vergelijkbare wereldwijde database gemakkelijk worden opgezet.

Zoals eerder aangegeven ,wijzen meer recente analyses van *P. infestans* populaties op de verschijning van vele nieuwe genotypen die via migratie en / of seksuele recombinatie verkregen of ontstaan zijn. Om de nieuw ontwikkelde 12-plex SSR set toe te passen en de huidige populatieopbouw te ontleden, werden *P. infestans* populaties uit 4 verschillende continenten en landen geselecteerd: China (hoofdstuk 4), Nederland (hoofdstuk 5), Ecuador (hoofdstuk 6) en Tunesië e (hoofdstuk 7).

China is uitgegroeid tot de grootste aardappel producent van de wereld, niet alleen met betrekking tot de oppervlakte, maar voor de totale aardappelproductie in kilo's. Interprovinciale handel in consumptie- en pootaardappelen is erg belangrijk en komt vaak voor in China. Hoewel beide, A1 en A2 paringstypen, in China voorkomen, is er tot op heden geen bewijs voor een actieve seksuele cyclus gevonden gebaseerd op veranderingen in allel frequenties. Met 10 ipv 12 SSR's werd een analyse op 228 landelijk verzamelde *P. infestans* isolaten uitgevoerd (**hoofdstuk 4**). Een van de drie dominante klonale lijnen CN-04

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(A2) in de Chinese populatie was genotypisch gelijk aan een grote klonale lijn die in Europaals "Blue_13" met paringstype A2 aangeduid wordt. Het bleek niet mogelijk om de oorsprong van deze klonale lijn vast te stellen. Wel werd in dit onderzoek de klonale lijn "Blue_13" voor het eerst buiten Europa gevonden. Het virulentie spectrum van deze klonale lijn liet zeven verschillende virulentie spectra zien, variërend met virulentie voor 3-10 verschillende "differentials"van de *Solanum demissum* differential set. De CN04 genotypen werden gekarakteriseerd als agressief en zeer virulent. Eén isolaat was zelfs virulent op de gehele *S. demissum* differential set. Clustering van isolaten gebaseerd op analyse van SSR data kwam sterk overeen met de herkomst van de isolaten uit 6 provincies. Deze clustering lijkt niet te worden beïnvloed door de frequente interprovinciale handelsactiviteiten m.b.t. pootaardappelen. De paringstypenverhoudingen en de SSR-allel frequenties geven aan dat in China de bijdrage van de seksuele cyclus aan de populatiedynamiek van *P. infestans* minimaal is. Er wordt geconcludeerd dat migratie middels sporangia en natuurlijke sub-klonale variatie de dominante drijvende krachten achter de Chinese *P. infestans* populatie- structuur zijn.

Nederland heeft een lange geschiedenis van populatieonderzoek naar *P. infestans*. Daarnaast wordt een aanzienlijk aantal verschillende commerciële aardappelrassen geteeld. Data afkomstig van één decennium (2000-2009) isolaatbemonstering in 5 verschillende teeltregio's vormden de basis voor een studie naar de populatiedynamiek van *P. infestans* in Nederland **(hoofdstuk 5)**. Gedurende het decennium bestond de onderzochte populatie uit een dynamisch geheel van klonale lijnen en seksuele nakomelingen. De belangrijkste klonale lijn met A2 paringstype staat bekend als "Blue_13", maar daarnaast werden ook twee andere klonale lijnen met A1 paringstype in dit onderzoek geïdentificeerd. Dit onderzoek laat duidelijk zien dat de Nederlandse populatie in deze tien jaar dramatische veranderingen onderging. De meest opvallende verandering was de opkomst en verspreiding van de klonale lijn "Blue_13" metparingstype A2. De resultaten benadrukken het belang van de seksuele cyclus bij het genereren van genetische diversiteit en het belang van de aseksuele cyclus als het voortplantings- en verspreidingsmechanisme voor succesvolle genotypen. Naast de neutrale SSR markers is er een moleculaire merker ontwikkeld die virulente isolatenvoor het *Rpi-blb1* resistentiegen herkent. Resultaten van deze toets in combinatie met de bijbehorende virulentie test laten de aanwezigheid zien van isolaten die *Rpi-blb1* doorbreken zelfs voordat een ras met deze resistentie in Nederland is geïntroduceerd. Twaalf *Rpi-blb1* virulente isolaten werden gevonden, allemaal afkomstig uit de seksuele cyclus.. Tot nu toe is de

aseksuele verspreiding van deze virulente isolaten beperkt omdat *Rpi-blb1* bevattende aardappelvariëteiten in het veld nog afwezig zijn.

Aan het andere uiteinde van de wereld in de Andes, het oorsprongsgebied van de cultuuraardappel, is de situatie mbt *P. infestans* veel minder complex. Er zijn meer dan 400 aardappel landrassen in Ecuador, de teelt gebeurt door lokale boeren op kleine schaal in de hooglanden en is erg traditioneel. Dit is een duidelijk verschil met de aardappelteelt in Noord-Amerika of Europa (**hoofdstuk 6**). *Phytophthora*-isolaten uit Ecuador behoren tot twee nauw verwante soorten, *P. infestans* (op aardappel en tomaat) en *P. andina* (op niet-knoldragende *Solanum* soorten), maar SSR-analyse van 66 isolaten geeft aan dat deze twee soorten duidelijk te onderscheiden genetische groepen zijn. Twee oude klonale lijnen van *P. infestans* bleken in Ecuador dominant: één was uitsluitend te vinden op tomaat, de ander uitsluitend op aardappel. Binnen de aardappel isolaten, maar niet binnen de tomaat isolaten, was een grote sub-klonale variatie te vinden die mede veroorzaakt werd door (gedeeltelijke) polyploidisatie en verlies van allelen.

In Tunesië wordt aardappel geteeld in drie tot vier, deels overlappende, seizoenen, terwijl in de meeste aardappel producerende regio's daarnaast tomaat, hetzij in kassen of op het veld, geteeld wordt (**Hoofdstuk7**). Onder 165 isolaten afkomstig uit vijf regio's was één grote klonale lijn (NA-01, A1 mating type, Ia mtDNA haplotype) te vinden die uit relatief eenvoudige varianten leek te bestaan. Een andere, genetisch zeer diverse, groep van isolaten omvatte meer complexe varianten en beide paringstypen. Ten minste enkele nieuwe genotypen, ontstaan uitgeslachtelijke voortplanting, bleken een rol te spelen in de overleving van seizoen naar seizoen. Oosporen als seksueel nageslacht kunnen een belangrijke rol spelen als primair inoculum en epidemieën op gang brengen in volgende seizoenen. Op tomaat werden, in vergelijking met aardappel, voornamelijk aseksuele nakomelingen geïdentificeerd met twee mtDNA haplotypen, maar ook duidelijk minder verschillende nucleaire genotypen,. Dit onderzoek toont aan dat de *P. infestans* populatie in Tunesië op dit moment aan het veranderen is, de oude klonale lijn wordt in tenminste twee sub-regio's van Tunesië vervangen door een meer complexe, genetisch diverse en vaker seksueel voortplantende populatie. Ondanks de massale import van pootaardappelen uit Europa, verschilt de *P. infestans* populatie in Tunesië nog steeds duidelijk van de Europese populatie.

Hoofdstuk 8 gaat in op de toepassing van microsatellieten bij het onderzoek naar genetische diversiteit van *P. infestans* en het mogelijke gebruik ervan in de resistentieveredeling. Monitoring van de lokale *P. infestans* populatie op nieuwe virulente genotypen met een *R*-gen differential set in combinatie met screening op effector variatie,

maakt vroegtijdige detectie van doorbraak van specifieke resistentiegenen in teeltregio's mogelijk. Dit biedt, op zijn beurt, de veredelaar de mogelijkheid vast te stellen welke individuele resistentiegenen nuttig zijn in de nieuwe situatie. Een manier om dat te doen is om bestaande rassen met een doorbroken resistentie te vervangen door andere rassen met een gestapelde, niet-doorbroken resistentie verkregen via merker gestuurde selectie of door toevoeging van extra *R*-genen aan bestaande (*R*-gen bevattende) rassen via genetische transformatie. In een transgene of cisgene benadering kunnen additioneel breedspectrum *R*-genen   door re-transformatie worden toegevoegd.

Dit proefschrift laat het belang van een effectieve *R*-gen managementstrategie zien, zowel tijdens deveredeling, als tijdens de teelt van aardappelrassen.De resultaten van directe monitoring van (adaptatie in) lokale pathogeen populaties met behulp van een geschikte *R*-gen differential set en de 12-plex SSR set zouden leidend moeten zijn voor keuzes binnen deze strategie.

170 x 240 mm

SAMENVATTING

博士论文

英文题目

Multiplex SSR analysis of *Phytophthora infestans* in different

countries and the importance for potato breeding

中文题目

晚疫病多重微卫星群体分析及其对马铃薯育种的意义

李颖

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中文摘要

　　由卵菌 *Phytophthora infestans* 引起的马铃薯晚疫病是马铃薯生产上最严重的病害。十九世纪中叶，该病害曾在欧洲马铃薯生产区广泛流行，导致了举世闻名的爱尔兰大饥荒。至今，马铃薯晚疫病仍是马铃薯生产的第一限制因素，每年在全球造成数十亿美元的损失。在欧洲和北美，晚疫病的防治严重依赖于化学杀菌剂，这已造成了严重的环境污染。在发展中国家，农民无力购买杀菌剂，而无法防治该病。

　　晚疫病菌群体结构变异速度加快，主要由于病原菌迁徙、有性重组和亚群变异等诸多因素造成的。晚疫病菌两个交配型之间可进行迁徙和重新组合，这使得全球范围内的晚疫病菌群体遗传多样性明显提高，因而与无性繁殖群体相比，其适应能力不断增强。因此，高效监控晚疫病菌群体变化能够提高对该病的防控预警。高通量、高分辨率且操作简单的分子标记常用于种群变异研究，其中应用最为广泛的是微卫星标记(SSR)。新一代测序技术可以通过生物信息学手段得到大量微卫星标记，大大地提高了分子标记的精确度。但是目前晚疫病菌遗传分析的微卫星标记数量很少，而且未在遗传图谱上定位。因此，开发马铃薯晚疫病菌的微卫星标记，有利于监控晚疫病菌的群体变异，在马铃薯抗病育种工作中具有重要的理论和实践意义。本文针对晚疫病菌的 SSR 标记进行了系统研究，首次开发微卫星标记并将其定位在遗传图谱上。通过建立微卫星多重 PCR 检测方法对 4 个不同国家的晚疫病群体进行多样性分析。获得如下结果：

　　第一章回顾了全球晚疫病群体结构变化的历史和目前群体变化的趋势。本章总结了微卫星标记在研究病原菌群体多样性中的鉴定作用，并且详细描述了群体监测在对马铃薯抗病育种发挥的重要作用。

　　第二章的研究内容主要是开发鉴定微卫星标记。通过生物信息学分析从已知的晚疫病菌 EST 和基因组序列中获得了 300 个微卫星标记。利用筛选标记的稳定性、多态性水平和图谱定位，最终选择 8 个标记。这 8 个标记被整合到两个荧光标记的多重 PCR 反应中。以上微卫星多重 PCR 方法的成功建立为下一步开发高效率、高通量的一步法多重 PCR 技术奠定了基础。

　　第三章的研究内容是通过对已发表的微卫星标记和 8 个新开发标记进行重新评估和整合，成功建立了包含 12 个标记的多重 PCR 一步法技术。这 12 个标记均匀分布在不同的染色体上。该技术将可以作为一套通用的群体基因型分析工具，对分析晚疫病病原菌群体多样性分布和揭示病原菌移动迁徙的历史经历起到关键作用。通过该技术分析的不同群体数据将作为公益资源被上传到国际马铃薯晚疫病联盟网站上，供全球各地研究人员及学者比较分析。

现今很多晚疫病群体的研究都集中在新基因型的产生和变异上。为了利用此多重 PCR 监测技术，分析当前晚疫病群体结构变化，该工具在本论文研究中被应用到四个国家的马铃薯晚疫病群体监测上，包括中国(第四章)、荷兰(第五章)、厄瓜多尔(第六章)、突尼斯(第七章)。

中国无论在马铃薯生产还是在马铃薯种植面积上都是世界第一。省际间的马铃薯鲜薯和种薯运输越来越频繁省际间的马铃薯鲜薯和种薯运输日益频繁。现阶段虽然晚疫病菌的两个有性交配型都都已在中国发现了，但是仍没有直接证据证明中国马铃薯田间存在有性繁殖。利用 10 个微卫星标记对 228 个分离菌株进行了基因型分析(第四章)，结果。分析表明中国存在 3 个优势无性系，其中的一个 CN-04(A2) 与在欧洲发现的优势无性系"Blue_13"相同。但是根据这些数据还不能断定该无性系的起源。本论文是第一个报道在欧洲以外的地域发现优势无性系"Blue_13"的研究。在被用于生理小种鉴定的中国菌株中，共鉴定出 7 个不同的生理小种组合，其中最少包括 3 个毒性因子，最多包括所有 10 个毒性因子。CN04 无性系包含致病性最强的基因型。对菌株基因型的聚类结果表明，基因型分布与样品来源省份分布几乎相同，因此可以说明马铃薯的省际运输不是影响群体变化的主要原因。交配型和等位基因频率分析结果说明有性繁殖在中国晚疫病群体多样性变异中的作用很小，其实无性繁殖后代的迁徙和亚群内变异是中国晚疫病群体变化的主要原因。

荷兰在马铃薯晚疫病群体研究上有着悠久的历史，而且田间有很多马铃薯栽培品种。通过 10 年(2000-2009)的病原菌收集工作，为系统地了解荷兰晚疫病群体变化提供了扎实的基础(第五章)。这次系统群体调查结果显示荷兰晚疫病群体主要包括几个无性系和有性群体，其中发现无性系包括一个 A2 交配型的优势无性系"Blue_13"和两个截然不同的 A1 交配型无性系。同时发现最重要的优势无性系"Blue_13"的出现和快速扩散。微卫星标记显示有性繁殖在荷兰影响着群体遗传多样性，和优势无性基因型在病原菌繁殖和扩散中的决定作用。本研究通过开发毒性基因标记(*Avr-blb1*)鉴定可以克服广谱抗病基因 *Rpi-blb1* 的菌株，结果表明荷兰其实并没有引进含抗病基因 *Rpi-blb1* 马铃薯品种。在调查群体中一共发现了 12 个毒性菌株，这些菌株都分布在有性群体中。由于荷兰没有含抗病基因 *Rpi-blb1* 马铃薯品种，所以目前这些毒性菌株无性扩散繁殖的可能性不是很大。

第六章的研究结果表明在马铃薯起源地印第安山脉地区，晚疫病群体复杂程度很低。在厄瓜多尔，有超过 400 多个不同的马铃薯农家品种。当地农民在高地上使用传统的栽培方式种植马铃薯，这些小面积的种植模式和欧洲或北美国家的方式截然不

同。在厄瓜多尔，疫霉包括两个非常相近的病原种，*P. infestans* 和 *P. andina*。通过对 66 个晚疫病菌株的微卫星标记分析，这两个病原种被划分成两个明显不同的遗传群体。这两个古老的无性系在当地晚疫病群体中占有主要地位，一个无性系的菌株都是从番茄上分离到的，另外一个都是从马铃薯上收集的。分析结果表明在马铃薯寄主上分离的晚疫病无性系亚群内的多样性差异主要是由多倍性和等位基因丢失造成的。

在突尼斯马铃薯全年种植，全年总共有 3-4 个种植季交叉种植；而番茄主要种植在温室内或者马铃薯田间空闲地上。第七章研究分析了从突尼斯 5 个地域采集的 165 个菌株，结果表明突尼斯晚疫病群体中有一个生理小种简单的优势无性系 NA-01，该无性系为 A1 交配型、Ia 线粒体单倍型。另外一个遗传多样性高度变异的群体表现出复杂的生理小种结构，并且发现存在两个交配型。根据菌株采集季节对群体进行聚类发现有些有性繁殖产生的新基因型存在于种植季交替期内，由此推测该有性后代可能影响下一种植季的晚疫病传播。在番茄上的晚疫病群体存在两个线粒体单倍型，而且与马铃薯上的晚疫病群体相比，番茄上的晚疫病菌存在较小的基因型差异。本研究结果说明目前突尼斯的晚疫病群体正处于动态变化阶段，在调查的两个区域中老的优势无性系正被多样化程度高的有性群体更替。虽然每年突尼斯从欧洲进口大量的马铃薯种薯，但是突尼斯当地的晚疫病群体与欧洲大陆的群体在遗传结构上有着明显不同。

第八章讨论了微卫星标记在监测晚疫病群体上的应用及其在抗病育种中的作用。结合毒性因子变异检测技术，实时监测当地最新毒性菌株的变化可以预警当地不同马铃薯抗病品种的可持续性。通过群体结构和毒性因子的监测可以确定当地应用广谱抗病基因的种类，提高当地的防治效果。针对抗病育种现状，提出了两种同源转基因手段，一种是将未被克服的抗病基因叠加转化到当地主栽品种中，另外一种是将未被克服的抗病基因转入已有抗性的栽培品种中以提高抗病效率。本研究应用生理小种鉴定和微卫星多重检测技术，高效准确的监控晚疫病群体结构，预警马铃薯晚疫病的发生，为马铃薯的抗病育种提供有效的理论基础。

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In the end, I would like to give a sentence to the friends who are being through the difficulties, "*The fade is flowers, but not Spring*", 那凋零的是花，不是春天.

Curriculum Vitae

Curriculum Vitae

Ying Li was born on Jan 22, 1980 in Beijing, China. After completing her academic high school in 1998, she entered China Agricultural University (CAU) and obtained her BSc as Plant Pathology in 2002. Subsequently, Ying studied for MSc degree in Wageningen University (WUR), the Netherlands, following in Plant Biotechnology with specialization in Plant Virology.

After MSc graduation, Ying started work in Biotechnology Department at Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences (IVF-CAAS) to now. During this period, she works as a joint PhD student between IVF-CAAS and WUR under the supervisions of Dr. Theo van der Lee and Prof. dr. Evert Jacobsen. This thesis summarizes the research results of her PhD program in exploring multiplex SSR analysis of *Phytophthora infestans* in different countries and the importance for potato breeding.

After her defence for the doctorate, she will return to IVF-CAAS to continue her scientific career in potato resistance breeding and late blight research while keeping strong connections to WUR and encouraging the cooperation between IVF-CAAS and WUR in various fields.

Publications

1. Guangcun Li, Sanwen Huang, Xiao Guo**, Ying Li**, Yu Yang, Zhen Guo, Hanhui Kuang, Hendrik Rietman, Marjan Bergervoet, Vivianne Vleeshouwers, Edwin A G van der Vossen, Dongyu Qu, Richard Visser, Evert Jacobsen, Jack Vossen. 2011. Cloning and characterization of *R3b*; Members of the *R3* superfamily of late blight resistance genes show sequence and functional divergence. Mol Plant Microbe Interact. 24(10), 1132 – 1142.
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