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The population structure of *Phytophthora infestans* in the Netherlands during the years 2000 - 2009

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SUMMARY
652 *Phytophthora infestans* isolates were collected from commercial potato fields in the Netherlands during a ten year period, 2000 – 2009. Population diversity was assessed using twelve highly informative microsatellite markers. The results describe a population structured in three major groups but also reveal the complexity of the Dutch *P. infestans* population with over 322 unique genotypes as well as the increasing importance of clonal lineages, their dynamics during the potato growing season and their ongoing stepwise displacement over the years. The results also emphasize the importance of the sexual cycle in generating genetic diversity as well as the importance of the asexual cycle as the booster and dispersal mechanism for successful genotypes.

KEYWORDS
Phytophthora infestans, late blight, microsatellites, SSR, population genetics

INTRODUCTION

In the Netherlands the total area under potato cultivation amounts to 165.000 ha and annually yields 7.9 million Mg of potato representing a 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 price (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 under selection pressure exerted by e.g. cultivation of resistant cultivars. One of the prerequisites for durable management of late blight therefore is a thorough up to date knowledge on local *P. infestans* characteristics and high level understanding of population dynamics in order to avoid unnecessary erosion of resistance and development of fungicide resistance.
P. infestans is heterothallic and both, A1 and A2, mating types are required for completion of the

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sexual cycle. Sexual reproduction results in large genetic variation in the offspring and may lead to increased and more rapid evolution of the pathogen. Before the 1980's, both mating types were only found in the highland of central Mexico which is the presumed center of origin of *P. infestans* (Flier *et al.* 2003, Fry *et al.* 1993). At that time, outside central Mexico only the Al mating type was detected. In the early 1980's, A2 mating type isolates appeared in Europe (Frinking *et al.* 1987, Hohl and Iselin 1984, Tantius *et al.* 1986). Nowadays, the A2 mating type is present all over Europe, South- and North American and Asia (Ann *et al.* 1998, Cooke *et al.* 1995, Ghimire *et al.* 2001, Wiem *et al.* 2006). As a results the population structure of *P. infestans* around the globe has undergone major changes over the past 20 years. The predominant 'old' populations of *P. infestans* in Europe, the America's and Asia were displaced by "new" populations (Drenth *et al.* 1994, Fry *et al.* 1993, Spielman *et al.* 1991).

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 (Drenth *et al.* 1993, Spielman *et al.* 1991). Members of the old US1 clonal lineage were not detected in the Netherlands ever since. 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 and Turkensteen 1999).

More than two decades into this displacement process, 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 and Shaw 2009).

On the premise that understanding of the population genetics of plant pathogens will contribute to the development of more durable future 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 between 2000 to 2009. For this purpose, individual *P. infestans* isolates were characterized using a recently developed and standardized set of twelve highly informative microsatellite markers.

MATERIALS AND METHODS

Sampling areas 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 and North West (NW), as well as the Central (C) area is dominated by ware and seed potatoes grown on clay soils. The South West (SW) is also characterized by ware and seed potatoes grown mainly on clay soils. The South East (SE) is characterized by ware potato crops on sandy soils.

Infected leaves were predominantly sampled from production fields but also from allotment gardens, potato dumps and volunteer potato plants. The samples were generally collected between the 1st of April and the end of September. Location, sampling date and cultivar were recorded.

Infected potato leaflets containing a single lesion were hand-picked and positioned upside down in a Petri dish containing 1.5% water agar. A small tissue sample from the edge of the lesion was then placed under a potato disc inside an otherwise empty Petri dish and incubated for one week at 15°C. Mycelium emerging from the top of the tuber slice was transferred to ampicillin containing pea agar (PA). All isolates were maintained in liquid nitrogen as a part of the *P. infestans* collection at Plant Research International, the Netherlands. A total of 652 isolates were obtained.

Mating types were determined by confronting the individual isolates with an A1 (isolate VK98014) or A2 (isolate EC3425) tester isolate on PA. 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. 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.
Isolates were grown in liquid pea broth. After 3-4 days of incubation at 20ºC in dark, mycelium was collected for freeze drying and subsequent DNA extraction. Genomic DNA was isolated from 20mg of lyophilized mycelium using the DNeasy 96 Plant Kit (Qiagen, Hilden, Germany). Elution was done with 200µl ultra-pure water. DNA extracts were stored at -20ºC until further use.
Mitochondrial haplotypes were determined using the PCR-RFLP method of Griffith & Shaw (23). Restriction digestions of the amplified regions P2 (MspI) and P4 (EcoRI) allowed for differentiation of the four mitochondrial (mtDNA) haplotypes Ia, Ib, IIa and IIb.
The Euroblight set of twelve microsatellite markers was used. 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 seconds, 58°C for 90 seconds, and 72°C for 60 seconds, and a final extension at 72°C for 20 min. Electrophoresis and visualization of SSRs was performed on a Herolab type RH-5 geldoc system and ABI3730 DNA analyser (Applied Biosystems). 5 ul PCR product was mixed with 1/6 volume of gel loading buffer (Orange G loading buffer) and seperated on 3% agarose gel. For ABI3730 analysis, the PCR products were diluted 1000 times and one µl of diluted SSR product was added to 9µl of deionized formamide containing 0.045µl of GeneScan-500LIZ standard (Applied Biosystems). Capillary electrophoreses of the mixture was done on an automated ABI3730 according to the manufacturer's instructions. SSR allele sizing was performed and scored using GeneMapper v3.7 software (Applied Biosystems).

GENETIC DATA ANALYSIS

Basic measures of genetic diversity - to analyze the variation in microsatellite loci, the observed number of alleles (na), effective number of alleles (ne) and Shannon's Information index (I) per locus in all populations were estimated using POPGENE. The significance of deviations from the Hardy-Weinberg equilibrium (HWE), using Bonferroni corrections, was determined using exact P values estimated using GENEPOP version 4.0 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 linkage disequilibrium (LD) 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 was employed. 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 frequencies deviates significantly from a probability distribution for allele frequencies under the infinite-alleles model in a neutrally evolving population. Bottleneck software version 1.2.02 was employed to test the bottleneck hypothesis under a two-phased model of mutation (TPM).
The spatial genetic structure was analyzed using the Bayesian clustering program STRUCTURE 2.2. 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.* (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 5 Structure runs from each K into one of 5 groups, each containing a single likelihood from each K. Within each of these 5 groups, we then calculated the necessary differences from Ln'(K) and |Ln''(K)|. |Ln''(K)| was averaged over the 5 groups, and divided by the standard deviation of the likelihood for the ultimate

calculation of ΔK. To validate the genetic substructure, principle component analysis (PCA) using NTSYS software was conducted to construct plots of the most significant axes for grouping pattern verification.

RESULTS

Isolates were collected from 207 different locations comprising the five Dutch potato-growing areas. A total of 652 *P. infestans* isolates were analyzed. The twelve microsatellite loci revealed very high PIC value with an average of 0.534 and different allele frequencies in the total collection. A total of 75 alleles were detected over 12 loci and the average number of alleles per locus was 6.25. SSR markers revealed 28 rare alleles with a frequency lower than 0.05.

The mean expected (He) and observed (Ho) heterozygosity were 0.524 and 0.577. 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. G11 is the most informative locus of the 12 SSRs, with the highest value for the Shannon's Information index (I=2.006). Six loci were not in HWE. After clone-correction (elimination of fully identical genotypes) all of the loci displaying heterozygote excess were still significantly different from HWE (Table 2).

The complete isolate collection shows a high genetic diversity with 311 unique genotypes among 652 isolates. To examine the distribution of genetic variation, among and within populations as defined by the five geographical areas, the data were analyzed by AMOVA. As a result, 95% of the variance was attributed to the regional stratum (within regions) whereas the remaining 5% was attributed to the national stratum (between regions) strongly indicating the absence of separate regional populations.

For the structure analysis, clone corrected data were used. Thus, the genetic structure behind 358 isolates was analysed using the correction for Structure 2.0 outputs as described by Evanno *et al.* (2005). For all K, memberships were consistent between all runs. The first ΔK peak for K=3 corresponds to the presence of three main groups.

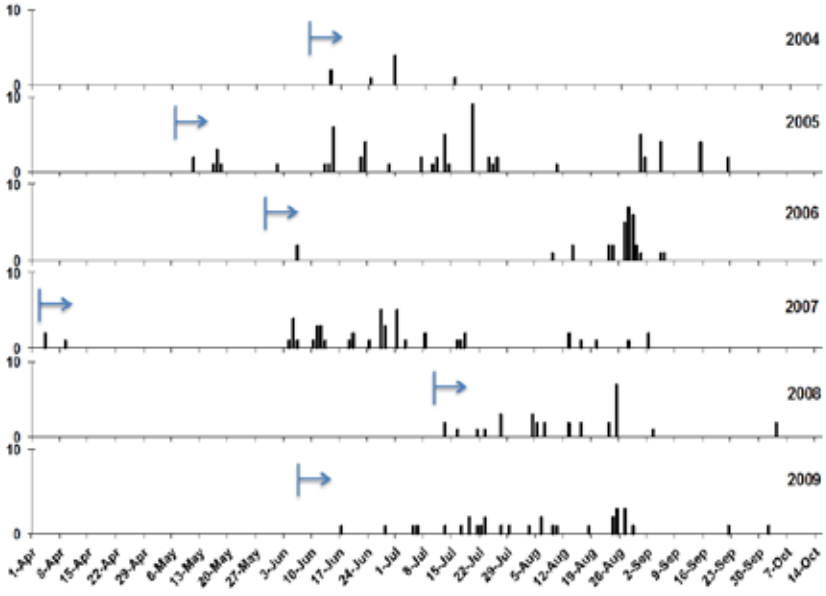

Figure 1. Isolation dates and numbers for genotype GS-001_A2 or EU13-A2 during the period 2004 – 2009. Arrows indicate the first date *P. infestans* was retrieved in the season.

The AMOVA for K=3 indicated that 25% of the variance was attributed to variation between the three groups, 75% of the variance was due to variation within groups. Pair-wise estimates of FST indicated a high degree of differentiation between the three groups with values ranging from 0.18 between group 2 and 3 to 0.42 between group 1 and 2. Based on a plot against the first two dimensions from the principle coordinate analysis, the three groups detected by the structure analysis also separate from each other.

Within the three large groups identified above, three genotypes are dominant on the various temporal and spatial scales. One dominant genotype, called GS-001_A2 (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. GS-001_A2 has the same SSR genotype profile as the EU13-A2 (or Blue-13) clonal lineage previously reported by Lees *et al.* (2009). A second dominant genotype called GS-008_A1 (A1 and Ia) was retrieved 43 times between 2000 and 2009 whereas genotype GS-005_A1 (identical to a the previously reported SSR genotype EU6_A1 or pink 6 (Lees *et al.* 2009)) was found 15 times at low frequency during this survey in 2002, 2005, 2006, 2007 and 2008. Apart from these three dominant genotypes, other much smaller clonal lineages with less than 15 isolates were found in multiple years and multiple regions. The vast majority of genotypes was however only found once. Figure 1 illustrates the dynamics of the GS-001_A2 or EU13-A2 during the years 2004 - 2009.

Both A1 and A2 mating types were found in all years and all regions. Three haplotypes (Ia, IIa and Ib) were found in all years and all regions (Fig.3). Isolates with identical SSR genotypes also shared the same mitochondrial haplotype and the same mating type, further establishing clonality of these isolates.

The genetic structure of Dutch isolates was found to be of intermediate complexity, including multiple, closely related genotypes.

DISCUSSION

This paper aimed to describe and analyze 10 years of *P. infestans* population dynamics in the Netherlands. On the premise that a better understanding of *P. infestans* population dynamics contributes to more durable forms of disease management, the overall population structure, occurrence, dynamics and displacement of clonal lineages were investigated. The Dutch *P. infestans* population was found to be structured in three major groups, each containing one or more clonal lineages in a well (groups 2 and 3) or less (group 1) developed sub-structure. Clonal lineages emerging from an ongoing displacement process were found to be an important population feature. AMOVA revealed that the Dutch *P. infestans* population should be considered a national population rather than a meta-population consisting of several regional populations.

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