



Gene Flow of *Phytophthora infestans* Between Refuse Piles, and Organic and Conventional Potato Fields in Southern Flevoland, The Netherlands

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Abstract

Phytophthora infestans causes late blight, a devastating disease of potato and tomato. Previous work in the Netherlands showed that the population is highly diverse due to sexual reproduction and that oospores can overwinter in the soil. We sequenced archived DNA from multiple nuclear and mitochondrial loci from *P. infestans* sampled in 1994 from 17 conventional and 5 organic potato fields and 7 potato refuse pile sites in the Southern Flevoland. We assessed the genealogical history using sequences from two regions of the single-copy nuclear *ras* gene (intron 1 and exons 3–6) and several mitochondrial loci (P3-*rpl14*, *rpl5* and tRNAs and P4-*cox1*) from 83 isolates. There were 10 heterozygous sites in the *ras* gene and 12 in the mitochondrial genes that were phylogenetically informative. Five nuclear and 5 mitochondrial haplotypes were shared among fields and several unique haplotypes were found. Nucleotide diversity and population mean mutation rates (θ_w) were higher in samples from refuse piles and organic fields than conventional fields for both nuclear and mitochondrial loci. Subdivision was observed between conventional fields and refuse piles. Migration analysis suggested that gene flow may have occurred first from conventional potato fields to organic fields and then to refuse piles. Recent mutations were found in rare lineages from organic fields. Ancestral mutations in mitochondrial loci were derived from samples from refuse piles documenting the importance of refuse piles as a source of inoculum for late blight epidemics in the organic and conventional fields.

Keywords Gene flow · Late blight · Potatoes · *Ras* gene · *Rpl14* · *Rpl5* · tRNAs and *cox1*

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Introduction

Phytophthora infestans (Mont.) de Bary causes late blight, a devastating disease of potato and tomato worldwide. *Phytophthora infestans* is a heterothallic oomycete plant pathogen and infects a wide range of *Solanum* species (Ristaino 2020). Yield losses caused by late blight and the cost of its control measures have been estimated to exceed 6 billion Euros annually (Haverkort et al. 2008). The disease is very severe in the Netherlands where more than half of all fungicide use in the country is directed for control of late blight (Pacilly et al. 2016).

Phytophthora infestans can reproduce asexually by means of sporangia and zoospores and sexually by oospores when both A1 and A2 mating types are present. *Phytophthora infestans* in Western Europe was highly uniform with limited genetic diversity until A2 mating-type strains were found in Switzerland in 1981 (Hohl and Iselin 1984). Since then, A2 mating-type strains have been reported in Eastern Germany, England and Wales, The Netherlands (Drenth et al. 1993a; Fry et al. 1991; Fry 2008), Poland and many other European countries (Drenth et al. 1993b; Yuen and Andersson 2012; Ristaino 2020).

With the introduction of A2 mating-type strains, sexual reproduction became possible and the concomitant increase in phenotypic and genotypic diversity of populations of *P. infestans* in Europe. First introductions of *P. infestans* into Europe were largely confined to a single clonal lineage of the Ia mtDNA haplotype FAM-1 (Martin et al 2013; Ristaino et al. 2001; May and Ristaino 2004; Ristaino 2006, 2020; Ristaino and Hu 2009; Saville and Ristaino 2021). Over time, other clonal lineages of the A1 mating type migrated to Europe (including US-1, Ib mtDNA haplotype) and then these lineages were gradually replaced by more aggressive populations with both A1 and A2 mating-type strains (Day and Shattock 1997; Cooke et al. 2012; Ristaino 2020). In some European countries, low genetic diversity has been detected in some populations with both A1 and A2 mating types. However, higher diversity has been reported from Poland, The Netherlands and Scandinavia where sexual reproduction plays a role in the disease cycle (Drenth et al. 1993a; Yuen and Andersson 2012).

Previous work in The Netherlands showed that populations of *P. infestans* are highly diverse due to sexual reproduction and that oospores can overwinter in the soil (Drenth et al. 1995). Prior to the 1980s, several early studies had shown that refuse piles were more important inoculum sources for late blight epidemics than tuber-borne inoculum from volunteers (Bonde and Schultz 1943; Hirst and Stedman 1960; Boyd 1974). In Southern Flevoland, The Netherlands, Zwankhuizen et al. (1998) analysed the genotypic diversity by characterising isolates of *P. infestans* collected between 1994 and 1996 from conventional and organic potato fields, potato refuse piles and allotment gardens. In that work, mating type and RFLP fingerprints were used to characterise the genetic diversity of populations. Refuse piles were identified as the most important inoculum source for commercial fields in 1994 and 1995 and some organic fields were identified as a source of mid-season infection of conventional fields in 1994 (Zwankhuizen et al. 1998). Moreover, 138 rare genotypes (81%) were documented in organic potato fields

and allotment gardens between 1993 and 1996 (Zwankhuizen et al. 2000). In 1994 and 1995, the common genotypes were found mostly in refuse piles and conventional potato fields. Over time, the number of A2 mating types increased from 4% in 1994 to 56% in 1996 (Zwankhuizen et al. 2000). However, since RFLP were used in the previous work (Zwankhuizen et al. 1998), migration and gene flow analysis among fields and sample sites, population sub structuring and ancestry could not be determined.

To prevent late blight, growers in the North and West of Europe apply fungicides for late blight on a weekly basis. The cost of fungicides' application in excess of 115 million Euros in The Netherlands, as well as environmental regulations on pesticide use, led to an increase in organic potato production (Haverkort et al. 2008, Haverkort et al. 2009). However, late blight is the most limiting disease problem for organic growers and is responsible for the significantly lower yields achieved by organic growers than conventional yields (Lammerts van Bueren et al. 2008). Devastating late blight epidemics in 2007 led to decreased plantings of organic potatoes in some areas (Lammerts van Bueren et al. 2008, Willer 2009).

In this study, we reanalysed a subset of archival DNA from *P. infestans* populations sampled from 29 organic and conventional potato fields and refuse piles collected by Zwankhuizen et al. (1998) in 1994 in Southern Flevoland. We chose to sample from the 1994 populations since more refuse sites were sampled in that year and spread from a putative source population could be evaluated. This is an area in The Netherlands with intensive potato cultivation. The objective was to examine the sources of inoculum and the genetic structure of *P. infestans* populations sampled from refuse piles, organic and conventional potato fields within a region during a single growing season using multilocus genotyping, nucleotide sequence diversity estimates, population subdivision tests and genealogical analyses and to evaluate migration and gene flow among organic, conventional and refuse piles in the sampled fields.

Materials and Methods

“Late blight epidemics differed dramatically from each other with regard to the date of first appearance of late blight, the overall disease level, and the development pattern during the growing season” (Zwankhuizen et al. 1998). During the growing seasons of 1994, late blight development was monitored over an area of 150 km² in Southern Flevoland, a polder in the central part of the Netherlands. Details are described in Zwankhuizen et al. (1998). *Phytophthora infestans* isolates were collected from sporulating tubers in refuse piles in the early growing season (April through June). Due to hot and dry weather in July, disease intensities decreased and almost no newly infested potato sites were found after that time. Isolates were also collected from conventional and organic potato fields (Fig. 1). The sampling strategy was set up to determine whether organic fields could be a source of inoculum for the conventional fields.

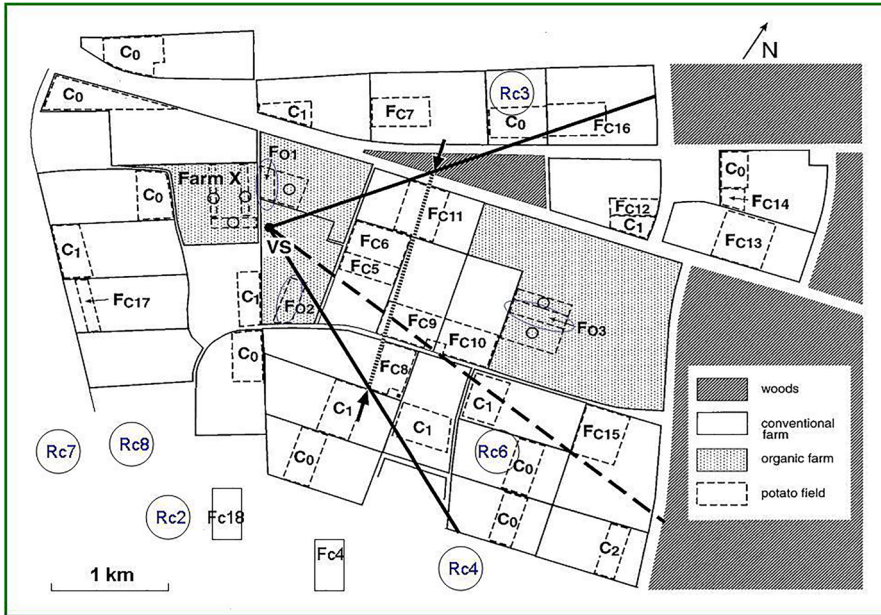


Fig. 1 Conventional and organic potato fields sampled in Southern Flevoland, the Netherlands, between September 1993 and September 1996 (Zwankhuizen et al. 1998; 2000). For this study, only 1994 samples were used. Fc, conventional potato field; Fo, organic potato field; Rc, refuse piles; VS, virtual source of dispersal; C₀, no disease was found in these conventional fields; C₁, conventional fields were not sampled; C₂, conventional field was severely infested but destroyed before sampling. Not shown are organic fields Fo4 and Fo5 that were located 7.25 km northwest of proposed virtual source of dispersal and conventional field Fc1 that was located at 8.5 km northeast of proposed virtual source of dispersal. Refuse piles Rc1 and Rc3 were located at 4.5 and 6.5 km, respectively, northeast of proposed virtual source of dispersal. The main part of the figure is reproduced from Zwankhuizen et al. (1998)

Isolates

We chose to sequence multiple loci from several nuclear and mitochondrial genes in this work. These same loci had proven useful to studying migration, gene flow, ancestry and population substructuring among a larger set of global samples of *P. infestans* in our previous work (Gómez-Alpizar et al. 2007). DNA preparations from isolates sampled in each field and representing diverse genotypes (as determined previously by RG57 fingerprinting) and both A1 and A2 mating types were selected for further DNA sequencing (Zwankhuizen et al. 1998) (Table S1). The RFLP lineage of each isolate is shown in Table S1. A total of 83 isolates from organic and conventional potato fields and potato refuse piles collected in 1994 were sampled (Table 1). Among these isolates, 15 were from 5 organic potato fields (Fo1, Fo2, Fo3, Fo4 and Fo5); 49 isolates were from 17 conventional potato fields (Fc1, Fc2, Fc4-Fc18) and 19 isolates were from 7 potato refuse pile sites (Rc1, Rc2, Rc3, Rc4, Rc6, Rc7 and Rc8) located in close proximity in Southern Flevoland (Fig. 1).

Table 1 Summary of isolates of *Phytophthora infestans* collected from organic, conventional and refuse piles in Southern Flevoland during 1994

Field	Number of isolates	Mating type (number of isolates)	RG-57 genotype (number of isolates)
Organic potato fields			
Fo1	3	A1 (2), A2(1)	NL-41 ^c , 76, 77
Fo2	3	A1 (2), A2(1)	NL-1, 27, 76
Fo3	3	A1 (3)	NL-48 (3)
Fo4 ^a	3	A1 (3)	NL-76 (2), unknown
Fo5 ^a	3	A1 (3)	NL-48, 69, 76
5 fields	15	A1 (13), A2 (2)	NL-41 (1), 76 (5)
Conventional potato fields			
Fc1 ^b	3	A1 (3)	NL-41 (3)
Fc2	3	A1 (3)	NL-75 (3)
Fc4	3	A1 (3)	NL-41 (3)
Fc5	3	A1 (3)	NL-41, 74, 76
Fc6	3	A1 (3)	NL-76 (3)
Fc7	3	A1 (2), A2(1)	NL-41, 55, 76
Fc8	3	A1 (3)	NL-41, 76 (2)
Fc9	3	A1 (3)	NL-36, 75, 76
Fc10	1	A1 (1)	NL-41
Fc11	3	A1 (2), A2(1)	NL-41, 76, 105
Fc12	3	A1 (2), A2(1)	NL-41, 76, 77
Fc13	3	A1 (3)	NL-76 (3)
Fc14	3	A1 (3)	NL-41 (2), 76
Fc15	3	A1 (3)	NL-41, 76 (2)
Fc16	3	A1 (3)	NL-41, 76 (2)
Fc17	3	A1 (3)	NL-41 (2), 76
Fc18	3	A1 (3)	NL-41 (2), 75
17 fields	49	A1 (46), A2 (3)	NL-41 (20), 76 (19)
Refuse piles			
Rc1 ^c (D3)	3	A1 (2), A2(1)	NL-41, 76, 100
Rc2 (D4)	3	A1 (3)	NL-41 (3)
Rc3 ^c (D7)	1	A1 (1)	NL-76
Rc4 (D6)	3	A1 (3)	NL-69 (3)
Rc6 (D5)	3	A1 (3)	NL-75, 76 (2)
Rc7 (D1)	3	A1 (3)	NL-69 (3)
Rc8 (D2)	3	A1 (3)	NL-69 (2), 107
7 fields	19	A1 (18), A2 (1)	NL-41 (4), 76 (4)

^aTwo organic fields were located 7.25 km northwest of the proposed virtual source of dispersal

^bField Fc1 was located at 8.5 km northeast of proposed virtual source of dispersal

^cRefuse piles Rc1 and Rc3 were located at 4.5 and 6.5 km, respectively, northeast of proposed virtual source of dispersal

^dThe two most common RFLP genotypes were NL-41 and 76

DNA amplification and sequencing

DNA samples were housed in cryostorage in the second author's laboratory at Wageningen University, The Netherlands. Two regions of the *ras* gene, a single-copy nuclear gene, were amplified including intron 1 (224 bp) and a 542-bp region including exons 3–6 and introns 3–5 (Chen and Roxby 1996). Two regions of the mitochondrial genome, P3 and P4, were also amplified and sequenced (Avila-Adame et al. 2006; Griffith and Shaw 1998; Paquin et al. 1997). The P3 region includes the genes *rp114*, *rp15* and tRNAs (1180 bp) and the P4 region includes a part of the *cox1* gene (832 bp). The PCR primers and their location on the GenBank accession, U30474 for *ras* (Chen and Roxby 1996) and U17009 for mitochondrial loci (Paquin et al. 1997), can be found in Gómez-Alpizar et al. (2007). Two 50- μ l reactions were carried out per primer set and isolate. Each reaction contained 1 \times PCR buffer (20 mM Tris–HCl; 50 mM KCl), 1.8 mM MgCl₂, 100 μ M of each dNTP, 0.4 μ M of each forward and reverse primer, 0.1 mg/ml BSA, 1 unit of *Taq* polymerase (Invitrogen, Waltham, MA), and ~5–10 ng genomic DNA. Cycling conditions were 96 °C (1 min); then, 35 cycles of 96 °C (1 min), 55 °C (1 min) and 72 °C (2 min); and a final extension of 72 °C (10 min). Two independent PCR reactions were done with appropriate controls. The two PCR products were pooled and purified with QIAquick PCR Purification Kits (QIAGEN, Hilden, Germany). Purified fragments were sequenced directly in both the forward and reverse directions by using the same primers as those used in the initial amplification. Sequencing reactions were prepared using the ABI PRISM® BigDye Terminator Cycle Sequencing Ready Reaction Kit and analysed on an ABI PRISM® 377 automated sequencer (Applied Biosystem, Waltham, MA).

Data statistical analysis

All statistical analyses of the nucleotide sequences were performed in SNAP Workbench version 2.0 (Price and Carbone 2005). Multiple DNA sequences were aligned and edited manually with BioEdit (Hall 1999). Sequence data from nuclear loci (IntronRas+Ras) and mitochondrial loci (P3 and P4) were combined separately into alignment files using SNAP Combine (Aylor et al. 2006). Sequences were collapsed into unique haplotypes using SNAP Map (Aylor et al. 2006) after removing insertions and deletions (indels) from each of the aligned multilocus data sets and excluding infinite-sites violations. Base substitutions were categorised as phylogenetically informative or uninformative, transitions or transversions, and nonsynonymous (replacement) or synonymous amino acid changes in the coding region of each alignment. Resultant haplotype data sets were used to examine the overall support or conflict among the variable sites in the DNA sequence alignment. A site compatibility matrix was generated from each haplotype data set using SNAP Clade and Matrix (Aylor et al. 2006). Compatibility matrices were used to examine compatibility/incompatibility among all variable sites, with any resultant incompatible sites removed from the data set. This was important as subsequent coalescent analyses

assume that all variable sites are fully compatible. Data sets were also evaluated using RecMin (Aylor et al. 2006) for evidence of recombination boundaries and for estimating the minimum number of recombination events. Conflicting data partitions or putative recombinant haplotypes were also excluded from further analyses, except when testing for population subdivision using Hudson's test statistics as recombination increases the power of these tests (Hudson et al. 1992a; 1992b). Non-recombining data sets were collapsed into unique haplotypes excluding infinite-site violations using SNAP Map. Two non-recombining data sets were defined as follows: the nuclear gene region intron 1 of *ras* and *ras* (IntronRas + Ras) and the mitochondrial regions P3 and P4. Nuclear and mitochondrial haplotypes were numbered as mH1 and rasH1, respectively.

Neutrality tests and population subdivision

Nucleotide polymorphism analysis was carried out on non-recombining data sets using the programme DnaSP version 4.50.3 (Rozas et al. 2003). Estimates of the number of segregating sites and sequence diversity statistics, including Watterson's θ (estimated as θ_w) (Watterson 1975) and Tajima's π (Tajima 1983; 1989), were calculated for the entire sample and each locus separately, as well as for the organic, conventional and refuse piles and the pooled data. Sequence variation was tested for deviations from neutrality by using Tajima's D (Tajima 1989), Fu and Li's D^* and F^* (Fu and Li 1993) and Fu's F_s tests (Fu 1997). Neutrality test statistics were calculated and by using 5000 simulations to test the hypothesis that mutations in the genes were selectively neutral was tested (Kimura 1980).

Genetic differentiation among samples from organic and conventional fields and refuse piles were analysed using SNAP Map, Seqtomatrix, Permtest and S_{nn} (Hudson et al. 1992a; 1992b) implemented in SNAP Workbench. Permtest is a non-parametric permutation method based on Monte Carlo simulations that estimates Hudson's test statistics (K_{ST} , K_S and K_T) under the null hypothesis of no genetic differentiation. Significance was evaluated by performing 1000 permutations for each nuclear and mitochondrial data set including incompatible sites and recombinant sequences.

Migration analysis

Where subdivision was observed, we further tested the null hypothesis of isolation between organic, conventional or refuse piles using MDIV and the "isolation with migration" programme (IM) developed by Hey and Nielsen (2004). Under the null hypothesis of isolation, we would expect migration (M) to be near 0. If migration rates are nonzero and populations split a long time ago, then divergence is consistent with a two-island model and equilibrium migration rates. IM implements Markov chain Monte Carlo simulations to estimate the posterior probability distribution of multiple demographic parameters including effective population size, divergence time and migration rates for a pair of closely related populations or species. The IM

model assumes neutrality and no recombination, and therefore, these analyses were performed on non-recombining and neutrally evolving loci.

Genealogical analysis

The ancestral history of the samples from organic and conventional fields and refuse piles was determined for the non-recombining loci (nuclear IntronRas + Ras (combined data shown) and mitochondrial (P3 + P4)) were inferred using Genetree (Version 9.0) and Treepic (Griffiths and Tavaré 1994; Bahlo and Griffiths 2000) in SNAP Workbench (Aylor et al. 2006). The genealogy with the highest root probability, ages of mutations, the TMRCA of the sample and the geographic distribution of the mutations were estimated from coalescent simulations. Coalescent analyses with population subdivision were performed and a backward migration matrix was estimated for each locus using IM. Recombinant haplotypes were identified a priori using SNAP Clade and excluded from the analysis. Coalescent simulations were performed assuming an infinite-sites model, constant population size and population subdivision. Gene genealogies for each locus were inferred using five million simulations of the coalescent. Additionally, we performed five independent runs of five million simulations using a different starting random number seed for each run to ensure convergence.

Results

Nuclear DNA sequence variability

A total of 2778 nucleotides were sequenced corresponding to 766 nucleotides in the nuclear (intron 1 and exon 3–6 regions of the *ras* gene) and 2012 nucleotides in the mitochondrial regions P3 (*rpl14*, *rpl5* and tRNAs) and P4 (part of *cox1*) (Table 2). Allele sequences were used for the analysis due to the heterozygous sites in the *ras* gene. There are two allele sequences for each isolate. In the case of a homozygous sequence, two identical sequences were used for the analysis.

Ten segregating nucleotide sites were identified, including 5 in intron 1 and another 5 in the exons 3–6 of *ras* that were phylogenetically informative (Aylor et al. 2006) (Table 2). Five different haplotypes were identified among isolates from organic and conventional fields and refuse piles (Table 3). *Ras* haplotype 1 (rasH1) was the most abundant haplotype (~51%) and distributed among all fields and refuse piles that were sampled. Two isolates were homozygous and were found in organic and conventional fields and were also haplotype rasH1. Haplotypes rasH2 and rasH3 were found less frequently than haplotype rasH1 and present in all fields (Table 3). Rare haplotype rasH4 (0.6%) was only found in one conventional potato field (Fc17) and haplotype rasH5 (1.3%) was found in both an organic (Fo2) and conventional (Fc2) field.

Sequence diversity in the intron 1 of *ras* was higher (2.23%) than in the exons 3–6 of *ras* (0.92%). Two synonymous substitution sites were found in exon 5

[illegible]

^aNumbering in vertical columns indicates the site number of GenBank accession no. U30474 for *ras* (Chen and Roxby 1996) and U17009 for mitochondrial loci (Paquin et al. 1997; Avila-Adame et al. 2006). Sites 561–701 are located in intron 1, and sites 1064–1351 are located in introns and exons 3–6, respectively, of the *ras* gene. Sites 2988–3974 are located in the P3 region (*rnmM*, *rpl* 1/4, *rpl*5 genes) and site 10,138 is in the P4 (*cox*1 gene) region, respectively

b_i, transitions; *v*, transversions; *i*, phylogenetically informative sites; -, uninformative sites; *r*, nonsynonymous (i.e., replacement) substitutions; *s*, synonymous substitutions

Table 3 Haplotype frequency of *Phytophthora infestans* among organic and conventional fields and potato refuse piles

	Nuclear (IntronRas + Ras) ^a					Mitochondrial (P3 and P4) ^b				
	rasH1	rasH2	rasH3	rasH4	rasH5	mH1	mH2	mH3	mH4	mH5
Organic	16	8	5		1	1		3	10	1
Conventional	46	32	10	1	1				49	
Refuse piles	19	8	11				1		18	

^aGenBank accession U30474 for *ras* gene. The intron 1 and introns and exons 3–5 were sequenced (Chen and Roxby 1996)

^bGenBank accession U17009 for mitochondrial loci. The P3 region (*trnM*, *rpl 14*, *rpl5* genes) and P4 (*cox1* gene) region, respectively, were sequenced (Paquin et al. 1997; Avila-Adame et al. 2006)

of the *ras* gene (Table 2). Nucleotide diversity (π), the average number of nucleotide differences per site between two sequences, for the pooled sample was 6.02×10^{-3} (Table 4). The Watterson's estimate (θ_w) of population mean mutation rate for the pooled sample was 1.774. Samples from b refuse piles and organic fields had higher nucleotide diversity and mean mutation rates (θ_w) compared to the pooled samples or those from conventional fields (Table 4).

Mitochondrial gene sequence variability

A total 2012 nucleotides were sequenced and analysed for the combined P3 and P4 regions (Table 4). There were 11 segregating sites in the P3 region and only 1 site was found in the P4 region (Table 2). Overall, five different haplotypes were found; however, only one mitochondrial haplotype (mH4) occurred in conventional fields (Table 3). mH4 was the most abundant haplotype (~93%) and was also found in organic fields and refuse piles. Other rare haplotypes including mH1, mH3 and mH5 were only found in the organic fields (Table 3). The single isolate identified as haplotype mH2 was an IIa mitochondrial haplotype (Griffith and Shaw 1998), while the mH1, mH3, mH4 and mH5 haplotypes were Ia mitochondrial haplotypes (Table 3, Table S1).

Sequence diversity in the P3 region was higher (0.93%) than the P4 region (0.12%). Three synonymous substitution sites and seven nonsynonymous substitutions were found in the P3 region (3 in *rpl14* and 4 in *rpl5*) that led to amino acid changes (Table 2). Nucleotide diversity (π) estimates for the pooled sample was 0.17×10^{-3} . The Watterson's estimate (θ_w) for the pooled sample was 2.405 (Table 4). Samples from the refuse piles had higher nucleotide diversity and mean mutation rates (θ_w) compared to those from organic fields. Since only one dominant haplotype was found in the conventional fields, estimates of π and θ_w values were null for these loci.

Table 4 Population statistics, diversity estimates and neutrality tests based on variation in nuclear and mitochondrial loci of *Phytophthora infestans*

Locus/population	Sample summaries					Parameter estimates		Test of neutrality			
	<i>l</i>	<i>n</i>	<i>s</i>	<i>h</i>	<i>k</i>	π (SE) ($\times 10^{-3}$)	θ_w	Tajima's D	Fu and Li's D*	Fu and Li's F*	Fu's Fs
Nuclear (IntronRas + Ras)											
Organic	766	30	10	4	4.726	6.17 (0.48)	2.524	2.596***	1.444**	1.995**	6.851**
Conventional	766	90	10	5	4.488	5.86 (0.18)	1.972	3.169***	1.388**	2.318***	8.795***
Refuse piles	766	38	10	3	4.953	6.47 (0.27)	2.380	3.077***	1.434**	2.197***	10.475***
POOLED	766	158	10	5	4.610	6.02 (0.13)	1.774	3.694***	1.349**	2.576***	11.297**
Mitochondrial (P3 + P4)											
Organic	2012	15	3	4	0.610	0.30 (0.09)	0.923	-0.915	-1.177	-1.166	-1.420
Conventional	2012	49	0	1	0	0	0	ND	ND	ND	ND
Refuse piles	2012	19	9	2	0.947	0.47 (0.41)	2.575	-2.045**	-3.546***	-3.312***	2.747
POOLED	2012	83	12	5	0.336	0.17 (0.11)	2.405	-2.246***	-5.334***	-4.811***	-2.736

l, consensus sequence length; *n*, sample size (number of isolates for mitochondrial locus and number of alleles for nuclear locus); *s*, segregating nucleotide sites; *h*, haplotypes; *k*, average number of pairwise nucleotide differences; π , average number of base differences per site; SE, standard error; θ_w , population mean mutation rate of Watson's θ estimator; ND, not determined because there was no polymorphism; *, $0.01 < P < 0.05$; **, $0.001 < P < 0.01$; ***, $P < 0.001$

Parsimony analysis

A parsimony analysis with heuristic searching in PAUP (assumes no recombination) was done for the *ras* gene sequence and resulted in three equally parsimonious trees each with a consistency index of 0.8333. However, for mitochondrial genes, only one tree was found and no homoplasy was detected.

Tests of neutrality

Neutrality tests were run in order to determine whether the data were consistent with the expectations of the neutral model of molecular evolution. These neutrality tests assume a constant population size, no recombination, no migration and that each sample is taken from a single randomly mating population and random sampling. Several of the assumptions were violated in this study since we sampled DNA from different previously characterised genotypes from known fields in equal number. We could also not rule out the possibility of migration or recombination. A significant test result may suggest a departure from neutrality, the presence of subdivision, population growth or background selection. Neutrality tests for both nuclear and mitochondrial genes were significant for the pooled sample, so the neutral model of evolution was rejected for DNA sequence variation in these regions (Table 4).

For the nuclear *ras* gene sequence, all four tests of neutrality were significant and positive for organic and conventional fields and refuse piles indicating an excess of ancient mutations or common alleles (right-side test). However, for the mitochondrial loci, the tests of neutrality were not significant for samples from organic and conventional fields, so the equilibrium model of neutral evolution could not be rejected. Nevertheless, in the samples from refuse piles, the values for Tajima's *D*, Fu and Li's *D** and *F** were significant and negative indicating an excess of recent mutations or rare alleles (left-side test) but not significant for Fu's *F*s which suggests balancing selection (Fu 1997).

Population subdivision

Hudson's tests (K_{ST} , K_S , K_T and S_{nn}) were performed to quantify population subdivision within and among organic and conventional fields and refuse piles. For the

Table 5 The S_{nn} (the nearest neighbour statistic)^a test for population subdivision for the nuclear *ras* gene (upper right matrix) and for mitochondrial genes (lower left matrix)

	Organic	Conventional	Refuse piles
Organic		0.619628	0.497272
Conventional	0.642081		0.592032
Refuse piles	0.528867	0.651697**	

^aGenetic differentiation among populations was analysed using SNAP Map, Seqtomatrix, Permtest and S_{nn} (Hudson et al. 1992a; 1992b) implemented in SNAP Workbench. Permtest estimates Hudson's test statistics (K_{ST} , K_S and K_T) under the null hypothesis of no genetic differentiation

******, $0.001 < P < 0.01$

ras gene, samples from organic fields were not genetically subdivided from those from conventional fields and refuse piles (Table 5–upper right). However, among the 7 refuse piles, samples from Rc1 and Rc8 were genetically subdivided ($S_{nn}=0.7$, $P=0.046$) from each other. For mitochondrial genes, pairwise comparisons between samples from refuse piles and conventional fields were genetically differentiated ($K_{ST}=0.096538$, $K_S=0.130482$, $K_T=0.144425$, $S_{nn}=0.651697$, $P=0.002000$) (Table 5–lower left); however, gene flow occurred since shared haplotypes were observed among refuse piles and conventional fields (Table 3).

Migration analysis

Only mitochondrial genes were analysed for migration since removing incompatible sites in the *ras* sequence resulted in identical sequences. Mean population mutation rates for samples from organic fields were higher ($\theta_1=8.00$) than those from refuse piles ($\theta_2=4.85$) (Table 6). Mean population mutation rates were also higher ($\theta_1=8.94$) for samples from organic than conventional fields ($\theta_2=0.19$). Mean population mutation rates for samples from refuse piles were also higher ($\theta_1=4.94$) than those from conventional fields ($\theta_2=1.06$). Although migration occurred into both organic fields and refuse piles, a higher mean number of migrants into refuse piles were observed ($m_2=6.35$) compared to the mean number of migrants into organic fields ($m_1=4.04$). Similarly when organic field sites and conventional field sites were compared, there were higher mean numbers of migrants into organic fields ($m_1=5.90$) than into conventional fields ($m_2=1.00$) (Table 6). Since there was no subdivision found between organic and conventional fields, we pooled these samples and compared the migration rates to samples in the refuse piles. There were higher mean numbers of migrants into refuse piles ($m_2=6.75$) than pooled conventional and organic potato fields ($m_1=3.61$). MDIV also showed some evidence for gene

Table 6 Isolation model parameter estimates for *Phytophthora infestans* populations from refuse piles and organic and conventional potato fields based on variation in mitochondrial loci

Parameter	Organic fields (1) versus refuse piles (2)	Organic fields (1) versus conventional fields (2)	Conventional fields (1) versus refuse piles (2)	Pooled conventional and organic fields (1) versus refuse piles (2)
θ_1	8.00 (7.87–8.19)	8.94 (8.80–9.12)	1.06 (1.03–1.09)	1.70 (1.61–1.83)
θ_2	4.85 (4.53–5.28)	0.19 (0.17–0.23)	4.94 (4.62–5.36)	4.08 (3.76–4.51)
θ_A	22.10 (21.79–22.53)	7.81 (7.67–7.99)	19.84 (19.53–20.27)	20.70 (20.39–21.13)
t	6.32 (6.21–6.47)	7.25 (7.14–7.40)	7.55 (7.44–7.70)	1.23 (1.12–1.38)
m_1	4.04 (3.93–4.19)	5.90 (5.79–6.05)	6.81 (6.70–6.96)	3.61 (3.50–3.76)
m_2	6.35 (6.24–6.50)	1.00 (0.89–1.15)	5.35 (5.24–5.50)	6.75 (6.64–6.90)

In estimating these parameters, the inheritance scalar was set to 0.25 for the mitochondrial loci. In the table, the parameters are as follows: θ_1 , θ_2 and θ_A are the mean population mutation rates for population 1, population 2 and the ancestral population (A), respectively; t is the mean time of divergence of populations from a common ancestor; m_1 is the mean number of migrants into population 1; m_2 is the mean number of migrants into population 2. The 90% highest posterior density interval is shown in parentheses for those parameters in which the complete posterior distribution was estimated from the data

flow between potato fields and refuse piles using mitochondrial genes ($M=0.28$, $T=0.03$). There was gene flow between fields in both directions since the migration rates were not close to the zero. These results suggest that *P. infestans* migrated more from conventional fields to organic fields and then subsequently to refuse piles.

Genealogical analysis

The ancestral relationships of the mitochondrial lineages were examined using mitochondrial genes with GENETREE. Two ancestral lineages derived from a common ancestor were found in the mitochondrial gene genealogy (Fig. 2). The oldest lineage was mH2 and these isolates were type IIa mtDNA haplotypes. There were three unique haplotypes (mH1, mH3 and mH5) found in organic fields and these haplotypes diverged more recently than haplotype mH2. Both lineages were derived from isolates from refuse piles, hence providing further evidence of the importance of refuse piles as a source of inoculum in the region. The most common haplotype mH4 was derived from refuse piles.

Discussion

We utilised nucleotide sequences from both nuclear and mitochondrial gene regions to examine the genetic diversity of *Phytophthora infestans* from conventional and organic potato fields and potato refuse piles in samples collected from a large region of Southern Flevoland, The Netherlands. The data from this experiment represents one of the largest spatially sampled field experiments conducted to date for examining potato late blight disease foci, gradients and inoculum sources in organic and conventional fields and refuse piles (Zwankhuizen et al. 1998; 2000). Zwankhuizen documented the importance of refuse piles as sources for late blight epidemics in conventional and organic fields. However, in the previous study, migration analysis, direction of gene flow and ancestry of populations was not done.

Zwankhuizen et al. (1998; 2000) reported that the two most frequent RFLP genotypes, NL-41 and NL-76, were found commonly in conventional potato fields (44% and 34%, respectively) and refuse piles (38% and 23%, respectively). Overall, in this prior study, Zwankhuizen et al. (1998, 2000) found many more rare RG-57 genotypes in organic potato fields than in conventional potato fields and refuse piles.

Our results using multi-gene sequencing found similar results and indicated that the most common haplotypes were shared between isolates from refuse piles, organic and conventional fields. Haplotypes found in refuse piles were also found in organic and conventional fields. We documented spread of clonal lineages of the pathogen from refuse piles to conventional and organic fields over a large area (Fig. 1).

Populations in conventional fields were dominated by a few genotypes. We also found a higher frequency of the most common nuclear (rasH1 and rasH2) and mitochondrial haplotypes (mH4) in conventional fields. In fact, there was only one dominant mitochondrial haplotype found in the conventional fields (mH4). This

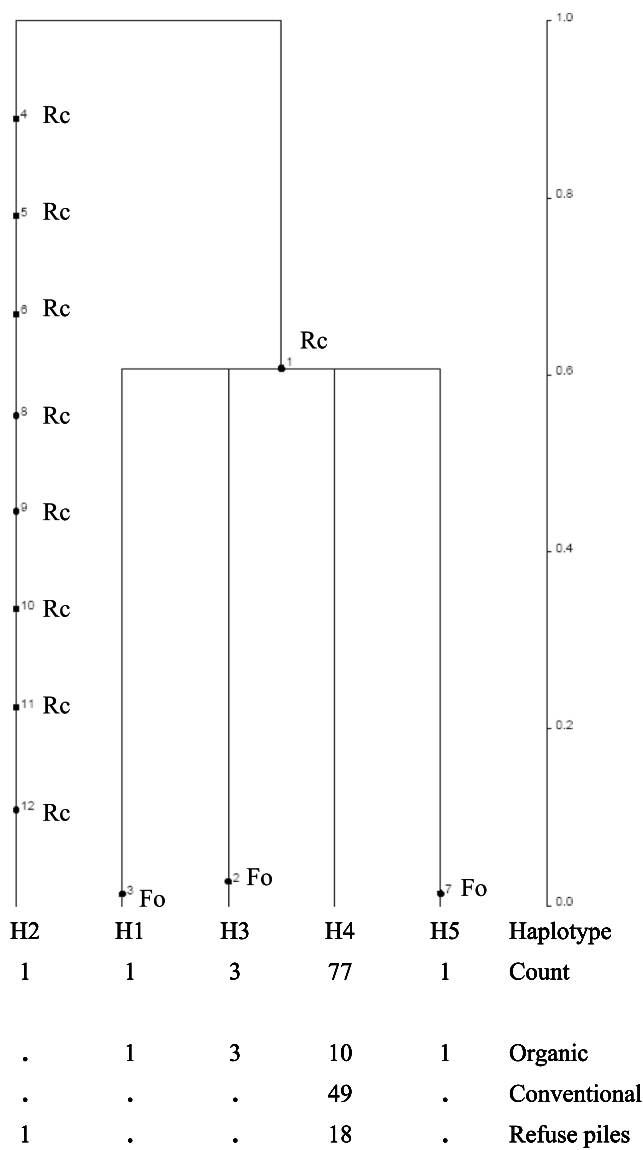


Fig. 2 The rooted coalescent-based gene genealogy showing the distribution of mutations from samples from organic fields (Fo), conventional fields (Fc) and refuse piles (Rc) for combine mitochondrial (P3+P4) loci of *P. infestans* generated using GENETREE (Griffiths and Tavaré 1994). Time scale is in coalescent units of effective population size. The direction of divergence is from the top (past-oldest) to the bottom (present-youngest). The numbers below the tree from top to bottom designate each distinct haplotype and its count (i.e., the number of occurrences of the haplotype in the sample) and the count of each haplotype in organic, conventional and refuse pile sites. Simulations were performed assuming constant population size. Maximum likelihood estimates of the tree with the highest root probability, standard deviation (SD) and Watterson's θ were as follows: likelihood = 1.3276×10^{-9} , SD = 8.7272×10^{-12} , $\theta = 1.605$. Estimates were based on 5 million coalescent simulations and 5 independent runs to ensure convergence

predominance of a few genotypes in the conventional fields (NL-41 and NL-47) was also similar to those noted by Zwankhuizen et al. (2000). Both mating types of the pathogen have been found in the region, but according to Zwankhuizen, the wet weather of 1994 and conditions conducive for disease spread caused the early spread of a few genotypes from refuse piles to the conventional fields. In addition, use of fungicides in conventional fields also likely prevented spread of multiple haplotypes into conventional fields.

Populations of *P. infestans* from organic potato fields and refuse piles were more genetically diverse than those from conventional potato fields. In our study, isolates from both refuse piles and organic potato fields had higher nucleotide diversity (π) and mean population mutation rates (θ_w) (6.47×10^{-3} , 2.38; 6.17×10^{-3} , 2.524, respectively) compared to the pooled sample (6.02×10^{-3} , 1.774) for the nuclear *ras* gene (Table 4). In addition, populations from refuse piles had highest π and θ_w values (0.47×10^{-3} , 2.575) when compared to the pooled sample (0.17×10^{-3} , 2.405) for the mitochondrial loci examined (Table 4). Zwankhuizen found that both mating types were found among isolates from some refuse piles and organic fields (Table 1). Since refuse piles are unsprayed and contain infected tubers from fields, both asexual inoculum and inoculum resulting from sexual reproduction could have survived in the refuse piles and spread to organic fields, accounting for the higher genetic diversity observed between both sites.

In the organic fields, crops are grown once every six or 7 years, which is common practice for organic farming in the Netherlands. The foliage of infested organic potato crops is flamed, which reduces the possibility of contamination of the soil with oospores formed in crop. However, recombination could have occurred in the refuse piles once the infected tubers sporulated. Zwankhuizen reported in 1994 that the level of diversity changed with time and followed the epidemic pattern with higher diversity in the refuse piles early in the season. Towards the end of the season, higher levels of diversity were found in the organic fields, while a lower level of diversity was found in conventional fields.

Although the most common haplotypes were found to be shared between populations from refuse piles, organic fields and conventional fields, overall, rarer haplotypes were found among populations from organic fields. Occurrence of rare haplotypes in the organic fields could have been a result of oospores inoculum. Since conventional fields are sprayed with fungicides to control late blight, clonally reproducing populations are reduced by fungicide sprays and go through severe selection pressure and genetic bottlenecks, whereas more rare haplotypes can survive in populations from unsprayed organic potato fields. Indeed, Zwankhuizen et al. (1998) reported greater diversity of RFLP genotypes over several years in the unsprayed organic fields and suggested the possibility of survival by oospores resulting from sexual reproduction. Our data confirm their results.

For the nuclear *ras* gene, all four tests of neutrality were significant for organic and conventional fields and refuse piles so the equilibrium model of evolution was rejected. The departure from neutrality could be the result of both background selection or population growth. However, for mitochondrial genes, only the tests of neutrality for populations from the refuse piles were significant and negative and could indicate balancing selection (Fu 1997). One example of balancing

selection is called frequency-dependent selection—a situation in which genetic variants are advantageous if they are present at low frequency, but become less beneficial as they reach a higher frequency.

The rasH1 haplotype identified using the *ras* gene was also the most common haplotype (~60%) found in our previous study of the Andean origins of *P. infestans* (Gómez-Alpizar et al. 2007). However, haplotypes rasH3 and rasH4 were unique to this study and different from the 12 haplotypes previously identified among the world populations of *P. infestans* (Gómez-Alpizar et al. 2007). This indicates that these novel haplotypes most likely evolved in the Dutch potato fields rather than via migration from populations outside the country.

We used coalescent analysis to determine ancestry of the populations of *P. infestans* from conventional and organic fields and refuse piles. Two ancestral lineages were found in the mitochondrial gene genealogy (Fig. 2). The oldest and ancestral lineage was mH2 and was clearly derived from populations from refuse piles. This lineage also contained the most mutations. Three unique haplotypes (mH1, mH3 and mH5) found in organic fields diverged more recently in the other lineage. These data document the importance of refuse piles as a source of inoculum from which new haplotypes of the pathogen can arise in organic fields.

We also conducted migration analysis using MDIV. Some evidence for low-level gene flow between potato fields and refuse piles ($M=0.28$, $T=0.03$). Gene flow was observed into both organic fields and refuse piles using IM (Hey and Nielsen 2004). The migration rate was above zero and indicated the gene flow in both directions. However, a higher mean number of migrants into refuse piles occurred than into organic fields. In addition, higher mean numbers of migrants into organic than conventional fields were observed (Table 6). These results suggest that the initial populations migrated from conventional potato fields to organic fields and then to refuse piles. Potentially, greater culled potatoes in organic fields than conventional fields could explain these data. In addition, use of fungicides in the conventional field reduced the number of migrants into the culled potatoes in refuse piles and the conventional fields. Nevertheless, ancestral populations arose from potatoes in refuse piles indicating that populations of the pathogen from refuse piles were the source of inoculum and migrated from refuse piles back into both organic and conventional potato fields in subsequent years.

Refuse piles have been reported as a source of inoculum for late blight for many years (Bonde and Schultz 1943; Bouws and Finckh 2007; Couzens and Evans 1968; Kirk 2003; Zwankhuizen et al. 1998). Local spread of the pathogen from sporangia that formed on potatoes that sprouted from refuse piles occurred in adjacent unsprayed fields hundreds of metres downwind of such sites (Bonde and Schultz 1943). The risk of crop infection from diseased potatoes in refuse piles is dependent on the thermal temperature of the overwintered culls (Kirk 2003). Refuse pile management by spraying culls with fungicides, covering the sites with mulch or plastic, burning culled potatoes, feeding culls to animals or spreading culls over fields to encourage freezing in colder climates has been recommended.

Nearly 15% of all early late blight infections can be traced to refuse pile sites in the Netherlands (Schepers et al. 2009). Potato refuse piles may become an increasingly important source of inoculum for late blight epidemics in future

years in northern countries due to global warming. It is predicted that with global climate change that milder winters in these regions of the world will prevent cull pile freezing thus enable survival of inoculum and thus earlier attacks of late blight (Hansen et al. 2001). The need to be more vigilant in management of refuse piles inspired the Dutch campaign to cover refuse piles by April 15 of each year.

Late blight is still a serious disease problem in organic potato production (Finckh et al. 2006, Hu et al. 2012). Due to the public awareness and health and environmental concerns, organic farming is on the rise around the world. The number of organic farms worldwide has increased with 32.2 million hectares of agricultural land managed organically (Willer 2009). In Europe, organically managed land increased by 0.33 million hectares since 2006. One of the benefits of organic agriculture is to reduce the usage of pesticides and synthetic chemicals hence to enhance biodiversity among beneficials in fields. However, late blight remains a significant disease challenge in organic production systems (Lammerts van Bueren et al. 2008) and has reduced acreage planted in the Netherlands from 1555 in 2002 to 1212 ha in 2007 (Lammerts van Bueren et al. 2008). Some European countries have banned the use of copper, which was approved in organic farming systems for late blight control (Dorn et al. 2007). Our data with mitochondrial and nuclear loci showed that organic fields had more diverse haplotypes of *P. infestans* than conventional fields or refuse piles. Since either fields are left unsprayed or nonsynthetic pesticides are sprayed in organic production, more rare haplotypes of the pathogen are likely to survive within or between seasons. Higher population mutations rates in organic fields could explain the higher diversity of rare haplotypes found there. Our data also indicate that more recent mutations in some lineages (mH1, mH3, mH5) led to the evolution of novel haplotypes in the unsprayed organic fields. Thus, improved management of the pathogen on organic farms is needed to reduce the development of novel genotypes of the pathogen.

Using multilocus gene sequencing, we have documented the importance of refuse piles in the spread of late blight in this region of The Netherlands, but also documented the direction of gene flow. Greater numbers of migrants into organic than conventional fields highlight the importance of maintaining late blight control in organic fields. It is also important to find alternative means to control late blight in organic production systems to avoid development of novel genotypes of the pathogen. The need for resistant varieties developed from traditional breeding programmes, biopesticides or alternative organic pesticides may help to reduce late blight, which is a limiting factor in the growth of the organic potato industry in Europe.

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Declarations

Conflict of interest The authors declare no competing interests.

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