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Microsatellite markers reveal two admixed genetic groups and an ongoing displacement within the French population of the invasive plant pathogen *Phytophthora infestans*

JOSSELIN MONTARRY,*† DIDIER ANDRIVON,* ISABELLE GLAIS,* ROSELYNE CORBIERE,* GLADYS MIALDEA* and FRANCOIS DELMOTTE†

*INRA, UMR1099 BiO3P (Biology of Organisms and Populations applied to Plant Protection), F-35653 Le Rheu, France, †INRA, UMR1065 Santé Végétale, F-33883 Villenave d'Ornon, France

Abstract

Potato late blight is an example of a re-emerging disease of plants. *Phytophthora infestans* was first introduced into Europe during the 19th century, where it caused the Irish potato famine. During the 20th century several additional introduction events have been suspected, especially in the mid-70s due to the import of large quantities of potato needed after the shortage caused by drought in 1976. Here, we investigate the genetic population structure of *Phytophthora infestans*, at the first stages of a recent invasion process in France. A total of 220 isolates was collected from 20 commercial fields of the potato susceptible cultivar Bintje, during two consecutive years (2004 and 2005). Clustering analyses based on eight recently developed microsatellite markers reveal that French *P. infestans* populations are made of two differentiated genetic clusters of isolates (F_{ST} = 0.19). This result suggests multiple introductions of *P. infestans* into France, either through the introduction of a composite population of isolates or through the successive introduction of isolates having differentiated genetic backgrounds. Both clusters identified have a strong clonal structure and are similar regarding genetic diversity and mating type composition. The maintenance of differentiation between the two genetic clusters should result from the low or non-existent contribution of sexual reproduction in French *P. infestans* populations.

Keywords: clonality, mating type, microsatellite markers, oomycete, population genetic structure, *Solanum tuberosum*

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Introduction

Human activities are efficient at dispersing parasites on a word-wide scale thereby promoting bio-invasions that pose major threats to public health, to biodiversity and ecosystem functioning, and to agriculture and fisheries (Vitousek *et al.* 1997). While much of the attention focuses on human (and animal) diseases, outbreaks due to invasive pathogens are also increasing among plant diseases. Invasions by plant pathogenic fungi are the most remarkable of fungal invasions, since exotic plant pathogens often cause destructive plant diseases. A striking example is chestnut blight, caused by *Cryphonectria parasitica*, which devastated American chestnut forests and orchards (Jarosz & Davelos 1995). It has been estimated that 65–85% of plant pathogens worldwide (including pathogens of introduced crops) are alien in the location where they were noted to cause epidemics (Pimentel *et al.* 2001). The fact that many agricultural plant species are grown worldwide, together with the globalization of trade of plant products greatly facilitate transportation of pathogenic species out of their native range, and thus provide recurrent opportunities for invasions by

Correspondence: Josselin Montarry, INRA, UR407 Pathologie Végétale, F-84143 Montfavet, France, Fax: 33 (0)4 32 72 28 42; E-mail: josselin.montarry@avignon.inra.fr

exotic fungal plant pathogens (Brown & Hovmøller 2002).

Introduction events confer novel evolutionary opportunities for an exotic plant-pathogen (Desprez-Lousteau *et al.* 2007), and several scenarios of invasion can be distinguished. A first scenario is that the migration of the plant pathogen to the new geographic area does not lead to a successful introduction due to lack of suitable hosts; this is probably a frequent case for introduced species. The second scenario occurs when the introduction of the parasite results in rebuilding a plant–pathogen system, already established elsewhere, in a different geographical area and/or environment; this happens in agricultural crops, most horticultural plants and many forest plantations that are considered as exotic plants in most areas where they are grown. In a third scenario, the introduction results in the formation of a new pathosystem, by putting into contact a plant pathogen species that did not co-evolve with potential hosts absent from its native area, but present in the place into which the introduction takes place. In this case, the success of the introduction is conditioned by the adaptation of the pathogen to its new environmental conditions.

Introductions are events that greatly modify the genetic structure of exotic plant pathogen populations in their new area (compared to source populations), which in turn condition their evolutionary potential (Dlugosch & Parker 2007). On one hand, populations may be subjected to loss of genetic variability because of demographic bottlenecks (introduction of small populations containing little genetic variation), random genetic drift and selection exerted by the novel environment (Sakai *et al.* 2001; Parker & Gilbert 2004). On the other hand, multiple introduction events may increase the level of genetic variance available for selection in the new environment by bringing together different genetic combinations, and thus increase the level of adaptive evolution of the invasive population (Dlugosch & Parker 2007; Delmotte *et al.* 2008). Finally, the establishment of a plant pathogen into a novel environment can modify the biology of the species, in particular the balance between sexual and asexual reproduction during its life cycle (Taylor *et al.* 1999; Smart & Fry 2001). It is therefore useful to assess the genetic characteristics of introduced populations of invasive plant pathogens to get more insights into the genetic changes that accompany bio-invasions, and to better understand the epidemiology of the diseases these pathogens cause to promote more effective control strategies.

The genetic structure of exotic fungal diseases in their area of introduction has been highlighted in several agricultural or natural plant-pathogen systems: it has been shown that the emergence of the exotic pathogen *Phytophthora ramorum*, causing sudden oak death in North America and Europe, was the result of three independent migration events. Three distinct clonal lineages have been identified in *P. ramorum* populations, while genetic analyses brought evidence for sexual reproduction in the ancestral populations (Ivors *et al.* 2006; Mascheretti *et al.* 2008; Goss *et al.* 2009). Similar results were obtained for another oomycete plant pathogen, *Phytophthora cinnamomi*, which has a devastating impact on native ecosystems in Australia. This parasite was most likely introduced into Australia early in the 19th century, following European settlement. Only three clonal lineages were identified in Australian populations, and these same clonal lineages were present in worldwide populations. No evidence for sexual reproduction between the three clonal lineages has been observed (Dobrowolski *et al.* 2003). At the same time, a growing number of genetic studies addressed the migration rates of agricultural fungal species among continents, such as for *Rhynchosporium secalis* (Linde *et al.* 2009; Zaffarano *et al.* 2009), *Mycosphaerella graminicola* (Stukenbrock & McDonald 2008), *Ustilago maydis* (Munkacsi *et al.* 2008), *Venturia inaequalis* (Gladieux *et al.* 2008), *Sclerotinia sclerotiorum* (Kohli *et al.* 1995), or *Puccinia striiformis* (Hovmøller *et al.* 2008). Phylogeographical and population genetics approaches are indeed useful to bring insights about the movements of plant pathogens at a worldwide scale. These studies have enabled investigators to identify source populations and to disentangle migration due to human activities (mainly transport of infected plants) from natural dispersion of fungal plant-pathogen spores.

Late blight is the most destructive disease of the cultivated potato (*Solanum tuberosum* subsp. *tuberosum* L.) worldwide. The European epidemics of *P. infestans*, that led to the Irish potato famine in the 1840s (Bourke 1964; Fry & Goodwin 1997), are illustrative of the ecological, economic and social consequences of plant pathogen invasions. *P. infestans* has indeed repeatedly emerged as major potato disease as the pathogen has migrated into new countries and reformed the plant–pathogen system with the cultivated potato cultivars. It has been postulated that *P. infestans* originates from Central Mexico (Niederhauser 1991; Fry *et al.* 1992, 1993; Goodwin *et al.* 1994), but a recent study has challenged this hypothesis by suggesting – as for the host plants – an Andean origin of the pathogen (Gomes-Alpizar *et al.* 2007). The first introduction of *P. infestans* occurred around 1842 to North-Eastern USA, where it caused a new disease of the cultivated potato *Solanum tuberosum*. In 1844, it was introduced into Europe, decimating potato production in the next few years and causing the Irish potato famine (Bourke 1964). The pathogen was subsequently transported throughout the world, probably within exported seed tubers (Fry *et al.* 1993). All *P. infestans*

isolates found outside Mexico between the mid-1840s and the mid-1970s were thought to be direct descendants of the original introduction, represented by a genotype called US-1 (Goodwin *et al.* 1994), but recent phylogenetic data based on mitochondrial and nuclear polymorphism suggest that US-1 was probably a member of a second wave of invasion in the 1940–1950s (Ristaino *et al.* 2001; Gomes-Alpizar *et al.* 2007). US-1 was itself quickly displaced in Europe during the mid-1970s (Spielman *et al.* 1991), following the introduction of new *P. infestans* populations, presumably with the import from Mexico and North America of large quantities of potato needed after the shortage caused by drought in 1976. This migration strongly increased the genetic diversity of European *P. infestans* populations, as revealed by allozymes, mitochondrial DNA haplotypes and RFLP fingerprints, and introduced the A2 mating type in Europe (Spielman *et al.* 1991; Fry *et al.* 1992, 1993). The presence of the two mating types now potentially enables the pathogen to reproduce sexually and allows the recombination of traits, generating new possibilities for quick adaptation in the current European population. In general, the aggressiveness of *P. infestans* isolates coming from this modern introduction event seems to be higher and more variable than that of US-1 (Day & Shattock 1997; Flier & Turkensteen 1999). These new genotypes spread all over Europe and further into other parts of the world (Smart & Fry 2001); however, the frequencies of A1 and A2 isolates vary largely between sites, from close to parity in Nordic European countries (e.g. Drenth *et al.* 1994; Andersson *et al.* 1998; Brurberg *et al.* 1999; Turkensteen *et al.* 2000) to an overwhelming or exclusive dominance of A1 isolates in the British Isles or France, at least until recently (Day & Shattock 1997; Lebreton *et al.* 1998; Carlisle *et al.* 2001; Day *et al.* 2004; Cooke *et al.* 2006; Montarry *et al.* 2006a). In France, while genotypes were different from the original clone US-1 (Andrivon *et al.* 1994; Lebreton *et al.* 1998; Flier *et al.* 2007), the A2 mating type has remained rare before 2003 (Lebreton *et al.* 1998; Montarry *et al.* 2006a). However, the frequency of A2 mating type isolates increased rapidly in the North of the country from 2003 on, up to a complete reversal of mating type frequencies in the most recent years (Dubois & Duvauchelle 2005; Montarry *et al.* 2008). This rapid and drastic change in mating type frequencies can be regarded as the sign of another invasion in French *P. infestans* populations, the origin and number of invading genotypes being unknown. So far, there is no evidence that sexual recombination is active in French *P. infestans* populations, which show a strictly clonal structure (Lebreton *et al.* 1998; Montarry *et al.* 2006a, 2008).

In this study, we took advantage of recently developed microsatellite markers (Knapova & Gisi 2002; Lees *et al.* 2006) to investigate the genetic structure of *Phytophthora infestans* populations in France during the first stages of this last invasion. The underlying hypothesis of this work was a priori the most parsimonious, i.e. that the invasion was due to a single A2 clone displacing previously established A1 lineages.

Materials and methods

Phytophthora infestans *biology*

Phytophthora infestans belongs to the Oomycetes, a group of diploid filamentous protists closely related to the brown algae (Kroon *et al.* 2004). *Phytophthora infestans* can infect all parts of the potato plant (i.e. shoots, stems, leaves, berries and tubers), leading to serious yield losses. It is characterized by a primarily aerial life cycle (Alexopoulos *et al.* 1996) with large multiplication rates (Harrison 1992) and polycyclic epidemics favouring rapid response to selection, which may however be counterbalanced by the large dispersion capacity of the asexual sporangia containing the infective zoospores (Bourke 1964). *Phytophthora infestans* is a heterothallic species: sexual reproduction requires the simultaneous presence of hyphae of the two opposite mating types, designated A1 and A2 (Smoot *et al.* 1958). While infected tubers are the most common source of inoculum at the beginning of the season in temperate climates (Zwankhuizen *et al.* 1998), infections can also start from oospores that result from the sexual cycle.

Phytophthora infestans *collection*

A total of 220 isolates was collected during two consecutive years (2004 and 2005) from 20 commercial fields of the susceptible potato cultivar Bintje, cultivated to a very large extent in France. Fields were located in the two most important French potato production areas, Northern France and Brittany, which are distant by approximately 500 km, and where late blight is often a problem. Infected leaves were collected each year from independent plants during the early stages of the epidemic, and the location of each field was recorded using GPS (Global Positioning System; Table 1). Within a field, we sample only one leaf per plant and the plants were randomly sampled across the field. Single-lesion isolates were established and maintained as axenic cultures on pea agar as previously described (Montarry *et al.* 2006a).

Mating type determination

We determined the mating type of each isolate by pairing it on pea agar with known A1 and A2 testers,

Table 1 Locations and names of potato fields sampled in the two most important French potato production areas (North and Brittany). Numbers of isolates collected are indicated for each field and each year (2004 and 2005)

	GPS position				
	Lat	Long	2004	2005	Total
North					
P01	N50 10 04.4	E2 36 46.2	10	–	10
P02	N50 30 47.9	E2 46 14.0	10	–	10
P03	N50 30 27.8	E2 46 13.4	1	–	1
P04	N50 29 47.7	E2 46 54.2	2	–	2
P05	N50 15 13.8	E2 16 29.2	3	–	3
P06	N50 16 04.9	E2 15 08.4	8	–	8
P07	N50 35 07.1	E2 57 18.9	7	10	17
P08	N49 49 33.6	E2 23 04.1	10	1	11
P12	N50 15 22.2	E3 21 10.1	–	16	16
P13	N50 15 09.6	E3 23 30.3	–	16	16
P14	N50 33 38.5	E2 55 46.8	–	7	7
P15	N50 40 11.4	E2 35 59.4	–	9	9
P16	N50 39 53.5	E2 36 16.8	–	4	4
P17	N50 20 20.3	E2 52 40.8	–	11	11
P18	N50 18 53.7	E2 52 30.3	–	4	4
P19	N50 18 57.2	E2 53 20.8	–	5	5
Total North			51	83	134
Brittany					
P09	N48 22 27.7	W4 44 48.7	11	9	20
P10	N48 30 00.6	W4 19 14.7	14	17	31
P11	N48 29 59.9	W4 19 19.1	11	8	19
P20	N48 06 29.1	W1 47 35.3	–	16	16
Total Brittany			36	50	86
Total			87	133	220

incubating them in the dark at 18 °C for 10 to 14 days, and observing cultures for oospore formation under a microscope (Shaw *et al.* 1985). Isolates forming oospores with the A1 tester were rated as A2 mating type, while those that formed oospores with the A2 tester were rated as A1 mating type.

DNA extraction and microsatellite amplification

Isolates were grown in pea broth, previously sterilized by autoclaving for 20 min at 120 °C. After 10 to 15 days of incubation at 18 °C, mycelium was washed three times in sterile water and lyophilized. DNA was extracted as described by Lebreton *et al.* (1998) and stored in TE buffer containing 10 M Tris-HCl and 0.1 M EDTA (pH 8.0). DNA concentration and purity were estimated using a spectrofluorimeter (SpectraMax M2, Molecular Devices).

Eight polymorphic microsatellite loci were chosen, although a total of 10 were tested. Markers used were Pi4B, Pi4G and PiG11 (Knapova & Gisi 2002) and Pi04, Pi16, Pi33, Pi56 and Pi70 (Lees *et al.* 2006); Pi02 and Pi63 were also amplified for allele detection but not analysed because a number of isolates showed multiple banding pattern at those microsatellite loci.

Microsatellite polymerase chain reactions (PCR) were performed in a 12.5 μL volume containing between 20 and 200 ng of DNA of *P. infestans*, 2.5 μL of 5X PCR Buffer (Promega), 0.3 mM of each dNTP, 2.5 mM of $MgCl_2$ (Promega), 0.3 μM each of forward and reverse primers, and 1.25 U of Taq DNA polymerase (GoTaq® flexi DNA polymerase, Promega). PCR was performed in a thermocycler (PTC200, MJ Research) under the following conditions: the PCR started with a cycle of 2 min at 95 °C, followed by 30 cycles of 20 s at 95 °C, 25 s at 56 °C (for PiG11), 58 °C (for Pi02, Pi04, Pi16, Pi33, Pi56, Pi63, Pi70 and Pi4B) or 60 °C (for Pi4G) and 60 s at 72 °C, and finished with an elongation cycle of 5 min at 72 °C. In order to detect simultaneously the alleles at several loci, primers were labelled with three fluorescent dyes: FAM (PiG11, Pi33, Pi 63, Pi70, Pi02, and Pi4B), NED (Pi56, Pi04, Pi4G) and HEX (Pi16). Amplification products were pooled into three groups, based on expected allele sizes: PiG11, Pi56 and Pi33; Pi63, Pi04, and Pi70; and Pi02, Pi4G, Pi16 and Pi4B, respectively. Ten microlitre samples, comprising 9.84 μL of deionized formamide Hi-Di™ (Applied Biosystems), 0.06 μL of 400 HD ROX™ Size standard (Applied Biosystems), and 0.1 μL of PCR multiplexed product, were loaded into an ABI Prism 3130*xl* DNA sequencer run according to manufacturer's instructions (Applied Biosystems). DNA fragments were automatically sized with the GeneMapper™ 3.5 software. Allele sizes were calibrated to the allele sizes of reference isolates kindly provided by Drs A.K. Lees and D.E.L. Cooke of the Scottish Crop Research Institute, UK (Lees *et al.* 2006).

Data analyses

The genotyping of *P. infestans* isolates with eight independent microsatellite markers allows their assignment to several groups of multilocus genotypes (MLGs), sharing the same alleles at all loci. Because an MLG may result from distinct sexual reproduction events or clonal reproduction, we estimated the probability (P_{sex} *n* re-encounter) of observing at least *n* times a MLG resulting from sexual reproduction, given the observed allele frequencies and assuming Hardy–Weinberg equilibrium (here *n* equals the number of copies of the MLG). The calculations were performed for the overall data set using the software GENCLONE 2.0 (Arnaud-Haond & Belkhir 2007; Arnaud-Haond *et al.* 2007).

A fundamental prerequisite of any inference on the genetic structure of populations is the definition of populations themselves. Because the genetic structure of populations is not always matching the geographical

proximity of individuals, isolates were clustered on the basis of their genetic relatedness (rather than their geographic origin) using two different individual-based clustering methods: a Bayesian algorithm and multivariate analyses.

The Bayesian clustering approach to genetic mixture analysis was performed using the software Structure 2.2 (Pritchard *et al.* 2000; Falush *et al.* 2003a). This method can be used to estimate parameters independently of the posterior probability distribution of allele frequencies. Parameters are estimated under the null model of panmixia, where each locus is at Hardy–Weinberg equilibrium and independent of the others. Nonetheless, this Bayesian algorithm is robust to some deviations from these assumptions (Falush *et al.* 2003a; Halkett *et al.* 2005a), and only physical linkage of loci can lead to spurious results (Kaeuffer *et al.* 2007). Therefore, it has been successfully used to assess genetic admixture in partially asexual organisms such as bacteria (Falush *et al.* 2003b), aphids (Halkett *et al.* 2005a), and fungal and oomycete plant pathogens (Delmotte *et al.* 2008; Fournier & Giraud 2008; Gladieux *et al.* 2008). Simulations were performed using data set without multicopies. Using the admixture model, we estimated the number K of genetic clusters (here between $K = 1$ and $K = 6$) to which the isolates should be assigned. For all simulations, we did not force the model with predefined allele frequencies for source clusters. Five independent runs were conducted to assess the consistency of the results across runs, and all runs were based on 500 000 iterations after a burn-in period of 100 000 iterations. We followed the method developed by Evanno *et al.* (2005) to identify the number of genetically homogeneous clusters (K).

A principal component analysis (PCA) was also performed using the procedure available in the package adegenet (Jombart 2008) for the statistical freeware R version 2.7.2 (The R foundation for Statistical Computing 2008). PCA has the important advantage over other methods such as the Bayesian clustering algorithm implemented in Structure 2.2 (Pritchard *et al.* 2000; Falush *et al.* 2003a) that it does not require strong assumptions about an underlying genetic model, such as the Hardy–Weinberg equilibrium or the absence of linkage disequilibrium between loci (Jombart *et al.* 2009). PCA was followed by a clustering analysis using the classical Ward's method available in R, which is a hierarchical method designed to optimize minimum variance within clusters.

Clonal diversity within each cluster was described, using GENCLONE, as the genetic richness (R), the Simpson evenness index (V), and the complement of the slope of the Pareto distribution of clonal membership, which were recommended by Arnaud-Haond *et al.* (2007) as the most parsimonious set of non-redundant indices of clonal diversity. In order to describe the intermingling of repeated MLGs, we estimated the spatial aggregation index Ac as described by Arnaud-Haond *et al.* (2007): this index ranges from 0, when the probability of identity between nearest neighbours does not differ from the average one, to 1 when all nearest neighbours preferentially share the same MLG. The statistical significance of the aggregation index was tested against the null hypothesis of spatially random distribution of isolates using a re-sampling approach based on 100 permutations.

Because the inclusion of clonal multicopies can strongly distort linkage disequilibrium between loci, estimates of heterozygosity and other F-statistics, standard population genetic tests were performed without multicopies using the software Genepop 4.0 (Raymond & Rousset 1995a): unbiased estimates of F_{IS} and F_{ST} across loci were calculated according to Weir & Cockerham (1984), allele frequencies and unbiased expected heterozygosity were calculated according to Nei (1978), and the number of pairs of loci showing significant linkage disequilibrium was computed with the exact test using the Markov chain algorithm of Raymond & Rousset (1995b).

We also assessed whether random sexual reproduction occurred by estimating the index of multilocus linkage disequilibrium (r_d) using the Multilocus software version 1.3 (Agapow & Burt 2001). This index is based on the index of association (I_A), allowing one to test for random recombination between pairs of loci by comparing the observed and expected variance of genetic distance between all pairs of individuals (Maynard Smith *et al.* 1993). The r_d statistic is much less dependent on the number of loci than the index of association (Maynard Smith *et al.* 1993). Departure from the null hypothesis (no linkage disequilibrium, i.e. $r_d = 0$) was assessed by permuting alleles between individuals independently for each locus (1000 permutations).

To assess the possible evolutionary relationships among the *P. infestans* MLG, a matrix of genotype distances based on the number of different alleles was calculated. A minimum spanning network was calculated from the matrix using MINSPNET (Excoffier & Smouse 1994) and the network was visualized using GRAPHVIZ (http://www.graphviz.org).

Results

Multilocus genotype analysis

A total of 25 alleles were detected over the eight microsatellite loci, with two to five alleles per locus. The 220 *P. infestans* isolates genotyped split into 70 unique

MLGs ($R = 0.32$), 48 of which being represented by a single isolate. Half of the *P. infestans* isolates belonged to six multilocus genotypes (MLGs 03, 09, 17, 30, 55 and 63; Table 2). The high P_{sex} values (Table 2) indicated that the over-representation of these MLGs might result from clonal amplification. Ten of the 22 repeated MLGs were sampled during the two consecutive years, and six were found in both French potato production areas (Table 2). However, two MLG (MLG 17 and 30) included isolates that present different mating types (Table 2). The most likely explanation for the fact that the MLG 17 and 30 included isolates of different mating types is that, in spite of the good resolving power of the combination of SSR markers used, these two MLGs are actually a mix of different genotypes that we are not able to discriminate. It is also possible that the presence of both mating types within an MLG reveals mutations or sex reversion at the mating type locus. Finally, this result is an indication that the determinant of mating type, which is a single locus in *P. infestans* (Judelson *et al.* 1995), may not be linked to any microsatellite markers used in this study.

Clustering analyses

Clustering performed using Structure on the 70 MLGs discriminated in the data set clearly indicated that the posterior distribution of the allele frequencies among clusters was best explained with a grouping into two genetic clusters. Assuming a threshold of $q > 0.8$ for assignment to cluster A (and $q < 0.2$ for cluster B), about 89% of the MLGs clearly belonged to only one cluster, indicating that the two clusters were highly differentiated. This was confirmed by the highly significant genetic differentiation between the two clusters ($F_{ST} = 0.19$). The distribution of allele frequencies between clusters also differed significantly at all loci, except Pi56 (Table 3). Six of the eight loci gave F_{ST} estimates higher than 0.1. Five of the 25 alleles were private for (i.e. specific to) one cluster (Table 3): three were restricted to cluster A (allele 206 at locus Pi33, allele 198 at locus Pi70 and allele 165 at locus Pi4G), and the other two were restricted to cluster B (allele 168 at locus Pi04 and allele 163 at locus Pi4G).

Principal component analysis (PCA) followed by clustering based on Ward's method also revealed two clusters of *P. infestans* isolates. Axes 1 and 2 of the PCA accounted respectively for 32% and 16.4% of total genetic variability (Fig. 1). The clusters discriminated in the multivariate analysis were in perfect agreement with the clusters inferred using the Bayesian clustering algorithm (Fig. 1). Since the Structure clustering output is supported by results from multivariate analyses, this indicates that the assignment obtained with Structure is reliable despite the deviations from the assumptions of the model. Further genetic analyses were thus conducted by grouping isolates into two clusters as obtained with these two clustering methods.

Table 2 Characteristics of the 22 repeated multilocus genotypes (MLG) that were discriminated using eight microsatellite loci on the 220 *P. infestans* isolates. Cluster assignment, number of isolates (n), mating type, P_{sex} n re-encounter, clone range (i.e. the maximum distance between two identical isolates) and sampling year(s) are indicated for each repeated MLG (that are represented by at least two isolates)

Repeated MLG	Cluster $K = 2$	n	Mating type	P_{sex} n re-encounter	Clone range (km)	Sampling year(s)
MLG 09	a	33	A1	1.14E-75	572	2004–2005
MLG 03	a	19	A1	6.07E-38	572	2004–2005
MLG 17	a	11	9A2 + 2A1	1.07E-33	46	2005
MLG 65	a	8	A1	1.62E-16	47	2004–2005
MLG 38	a	7	A1	4.36E-20	34	2004
MLG 37	a	4	A1	5.06E-07	34	2004–2005
MLG 69	a	4	A1	7.56E-09	34	2004–2005
MLG 45	a	4	A1	5.15E-15	511	2004
MLG 15	a	3	A1	2.19E-03	34	2004–2005
MLG 13	a	3	A1	2.96E-05	562	2004–2005
MLG 18	a	3	A2	1.73E-05	<1	2005
MLG 19	a	3	A2	1.34E-07	3	2005
MLG 31	a	2	A1	4.99E-02	<1	2004
MLG 35	a	2	A1	6.21 E-03	34	2005
MLG 62	a	2	A1	3.31 E-03	<1	2004
MLG 55	b	20	A2	1.43E-77	73	2004–2005
MLG 63	b	19	A1	1.32E-43	628	2004–2005
MLG 30	b	10	8A1 + 2A2	6.68E-16	576	2004–2005
MLG 04	b	9	A1	3.03E-15	94	2005
MLG 66	b	2	A1	2.09E-02	46	2004
MLG 48	b	2	A1	1.82E-02	48	2005
MLG 51	b	2	A1	2.96E-03	<1	2004

Table 3 Allelic frequency at eight loci of the two clusters of *P. infestans* MLG revealed by Bayesian clustering analysis. F_{ST} (A/B) values indicate genetic differentiation between the two clusters

Locus alleles	Allelic fr. cluster A	Allelic fr. cluster B	F_{ST} (A/B)
PiG11			
154	0.372	0.194	0.1168***
156	0.269	0.032	
158	0.051	0.032	
160	0.115	0.468	
162	0.192	0.274	
Pi56			
174	0.308	0.274	−0.0070
176	0.692	0.726	
Pi33			
203	0.859	1.000	0.1176**
206	0.141	0.000	
Pi04			
166	0.474	0.371	0.0287***
168	0.000	0.161	
170	0.526	0.468	
Pi70			
192	0.679	0.710	−0.0058*
195	0.244	0.290	
198	0.077	0.000	
Pi4G			
157	0.154	0.742	0.5208***
159	0.795	0.097	
161	0.026	0.032	
163	0.000	0.129	
165	0.026	0.000	
PiM6			
176	0.474	0.081	0.3075***
178	0.526	0.919	
Pi4B			
205	0.205	0.016	0.2291***
213	0.090	0.532	
217	0.705	0.452	
All locus			0.1937***

The level of differentiation significance is indicated by stars (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$).

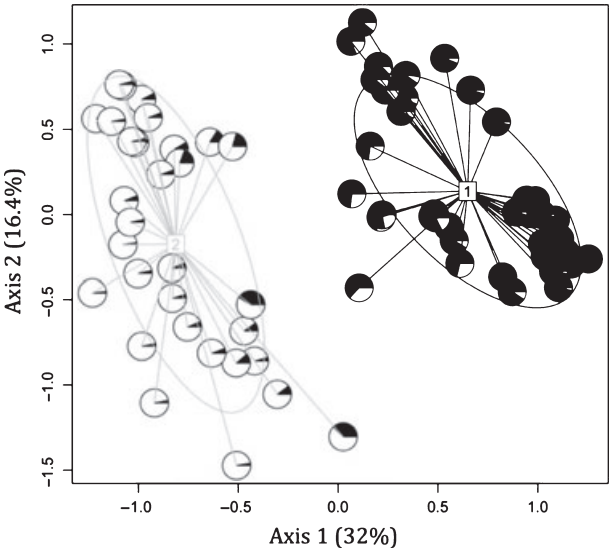

Fig. 1 Principal component analysis (PCA) performed on the 70 multilocus haplotypes of *P. infestans* based on eight microsatellite markers followed by a non-hierarchical classification with clustering based on Ward's method. Each point represents an isolate, and is connected to the mean point of its group by a black or grey segment. The ellipses are used to point out the dispersion of isolates within each group. In addition, for each isolate the probability of assignment to each of the two clusters obtained with the Structure software is reported on the PCA analysis using a pie-chart legend: cluster A in black and cluster B in white.

Genetic properties of the two clusters

Both clusters had a clonal structure, as shown by their low genotypic diversity (or richness) and the presence of isolates with the same multilocus genotype (66 to 71% of repeated genotypes; Table 4). Three of the six most repeated MLGs were assigned to cluster A, the other three belonging to cluster B (Table 2). Both clusters had identical Simpson evenness index values, and similar slopes of the Pareto distribution, reflecting equitable distributions of clonal membership among clusters. However, clonal MLGs from cluster A were much more aggregated than those from cluster B (Table 4).

The mean number of alleles per locus and the genetic distance between MLGs (DAS intra-cluster) were very similar for both clusters. F_{IS} multilocus estimates were highly variable among loci, ranging from −0.9 to 0.35 for cluster A and from −0.810 to 0.628 for cluster B (Table 4). A significant deficit in heterozygotes was detected in cluster A, but not in cluster B. Association between pairs of loci indicated significant linkage disequilibrium in 6 of 28 tests for cluster A and in 1 of 21 tests for cluster B.

The r_d tests on all individuals (i.e. with multicopies) rejected the null hypothesis of recombination ($P < 0.001$), but the r_d tests after clone correction could not do so for cluster B ($P = 0.470$). Taken as a whole, these analyses were consistent with a clonal reproduction regime, but suggest that *P. infestans* group B also reproduces sexually. However recombination has not fully disrupted the associations between alleles caused by clonal reproduction, and the signal of sexual reproduction was apparent only when the data are clone-corrected (Table 5).

Occurrence of mating types within clusters

Each cluster included both mating types A1 and A2 (Table 2 and Fig. 2). However, the frequency of mating

Table 4 Genetic features of the two clusters of *P. infestans* isolates revealed by the Bayesian clustering analysis. The set of non-redundant indices of clonal diversity recommended by Arnaud-Haond *et al.* (2007) was calculated for each cluster on the global data set (including multicopy genotypes): *N*, sample size; G, number of distinct multilocus genotypes discriminated; R, the genotypic richness; V, the Simpson eveness index; c(pareto), the slope of the Pareto distribution of clonal membership; Ac, Aggregation index. Within each cluster, the population genetic statistics and tests were performed without clonal multicopies: mean number of alleles per locus, H_O, observed heterozygosity; H_E, expected heterozygosity; DAS, genetic distance intra-cluster; L.D., number of pairs of significant linkage disequilibrium

Statistics	Cluster A (N = 132)	Cluster B (N = 88)
No. of distinct genotypes (G)	39	31
Genetic richness (R)	0.29	0.34
Simpson evenness (V)	0.839	0.768
c(pareto)	1.372	1.337
Aggregation index Ac	0.304†	0.177†
Mean no. of alleles per locus	2.875	2.750
H_E	0.465	0.404
H_O	0.561	0.415
F_{IS} per locus		
PiG11	0.350*	0.433*
Pi56	−0.312	−0.366*
Pi33	−0.152	nc
Pi04	−0.900*	−0.186
Pi70	0.091*	0.077
Pi4G	−0.183	0.628*
Pi16	−0.795*	−0.071
Pi4B	−0.007	−0.820*
F_{IS} multilocus	−0.210*	−0.029
DAS intra cluster	0.147	0.141
L.D.	6/28	1/21

*Statistically significant F_{IS} ($P < 0.05$).
†Statistically significant Ac ($P < 0.0001$).
nc, can not be calculated.

Table 5 Multilocus linkage disequilibrium (r_d) within the total sample and each cluster. Tests were done on data with and without multicopies. Values that differ significantly from 0 indicate a departure from linkage equilibrium

	With multicopies		Without multicopies	
	r_d	P value	r_d	P value
Cluster A	0.234373	$P < 0.001$	0.0696	$P < 0.001$
Cluster B	0.101036	$P < 0.001$	−0.0009	$P = 0.470$
Total sample	0.175224	$P < 0.001$	0.0488	$P < 0.001$

types within each cluster varied strongly with the sampling area. A2 isolates were restricted to samples from Northern France, where their frequency increased between 2004 and 2005. All A2 isolates collected in 2004 belonged to cluster B, whereas they were split between clusters in 2005 (Fig. 2).

Spatio-temporal distribution of genetic clusters

The frequencies of clusters A and B over years and locations were calculated using all isolates. In 2004, the *P. infestans* population from Brittany belonged exclusively to cluster A, while the population from northern France included both clusters (43% of isolates assigned to cluster A and 57% to cluster B). In 2005, populations from both potato production areas included both clusters (86% A–14% B, and 37% A–63% B, in Brittany and northern France, respectively). In northern France, some potato fields (P03, P04, P08 and P15) were attacked by *P. infestans* populations including only isolates from cluster B. All other populations included both clusters. By contrast, in Brittany, five of the seven populations sampled included only isolates from cluster A, isolates from cluster B being present (along with cluster A) in the remaining two crops sampled (Fig. 3).

Discussion

The genetic structure deduced from the microsatellite genotyping is not consistent with our initial hypothesis of a single A2 invading clone, but instead reveals that this invasion results from the admixture of two genetically differentiated clusters of predominantly clonal lineages (Fig. S1). This original, and unexpected, population structure raises three major questions about the evolutionary dynamics of the invasive and resident (i.e. present before the invasion) populations.

The first of these questions is obviously 'do the two genetic clusters originate from distinct introductions, and where do they come from'? The geographic and temporal distribution of the two clusters lead us to suppose that one of them (cluster A) was the resident population in France before 2003, and that cluster B is the invasive one. This hypothesis is consistent with the fact that isolates belonging to cluster A were much more frequent in Brittany (where invasion started later, at least according to the date of detection of A2 isolates at a significant frequency) than in Northern France. The fact that frequencies of cluster B increased between the 2 years of the study for both potato production areas could indicate differential reproduction likely due to increased relative fitness of cluster B or that cluster B was introduced later into French *P. infestans* populations. However, this last hypothesis does not fit entirely with the fact that both clusters contain A1 and A2 isolates. Aggregation indices could suggest that colonization was more recent for the cluster A (Ac = 0.304) than

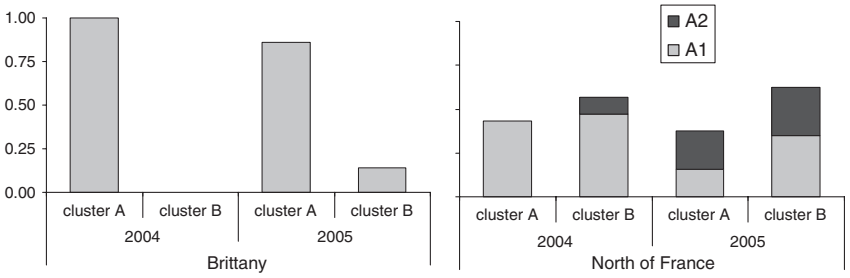

Fig. 2 Frequencies of the two mating types (A1 and A2) and of the two clusters of isolates, revealed by the assignment tests, for each year (2004 and 2005) in the two most important French potato production areas, Bretagne (left part of the graph) and Nord (right part of the graph).

for the cluster B (Ac = 0.177). Indeed, assuming a similar migration rate between clusters, a high aggregation of MLG in space may arise from either recent colonization or from competitive exclusion. Conversely, an absence of aggregation suggests either a full occupation of space by a large number of MLG due to long history or relatively weak competitive interactions among clones (Arnaud-Haond *et al.* 2007). An alternative hypothesis is that both clusters are invasive, following multiple introduction events, either through the introduction of a composite population of isolates or through the separate introduction of lineages with differentiated genetic backgrounds. Such multiple introduction events have been described in several plant pathogen species which recently spread into a novel geographic area, such as the oomycetes *P. ramorum* and *P. cinnamomi* causing the sudden oak death and dieback diseases on a wide range of woody plants, respectively (Dobrowolski *et al.* 2003; Mascheretti *et al.* 2008; Goss *et al.* 2009), the basidiomycete *Puccinia striiformis* f. sp. *tritici* causing yellow rust on wheat (Hovmøller *et al.* 2008) or the agent of sunflower downy mildew, *Plasmopara halstedii* (Delmotte *et al.* 2008). Neither hypothesis can be validly tested with only the set of data used here; proving or disproving them will require the genotyping, with the same set of microsatellite

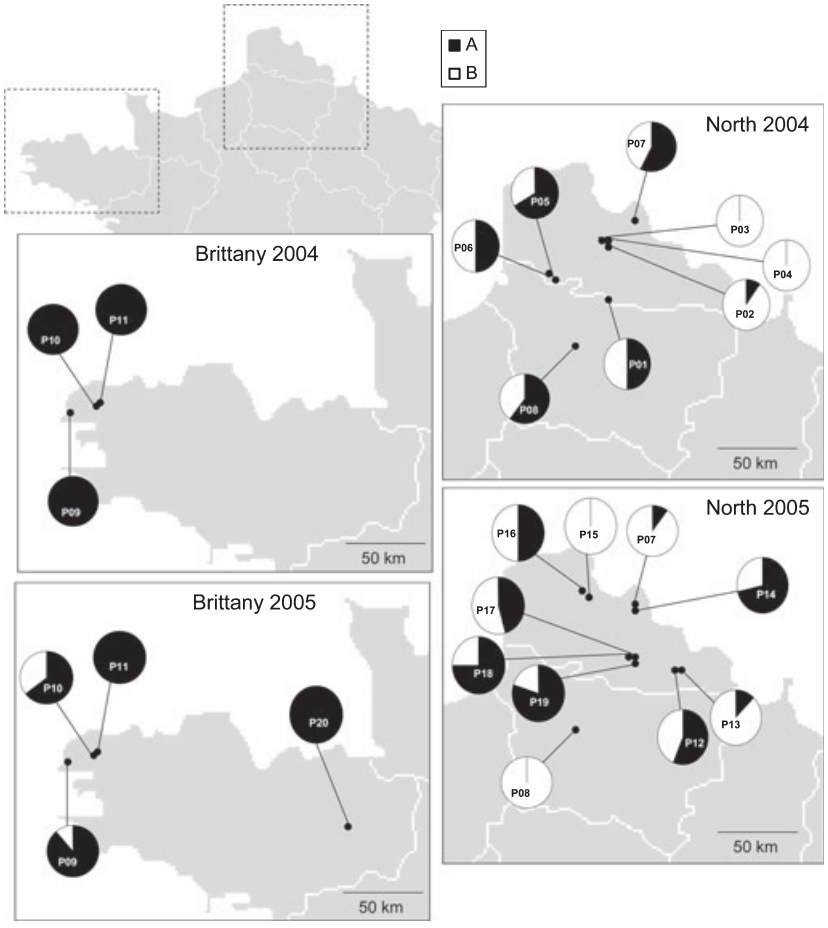

Fig. 3 Spatial distribution of the 11 and 14 fields sampled in 2004 and 2005, respectively, and frequency of *Phytophthora infestans* isolates belonging to cluster A (black) and cluster B (white) for each field. The global proportions of cluster A and cluster B isolates are indicated for each potato production area × year combination.

markers, of *P. infestans* collections sampled before and after that last invasion event. The analysis of invasion routes also requires additional data, to determine whether the genetic clusters present in France are found in other European countries, and compare their genotype profiles with those in potential source populations. Fortunately, adequate data sets allowing these comparisons can now be produced at a pan-European scale, thanks to the design of polymorphic marker sets (Knapova & Gisi 2002; Lees *et al.* 2006) and the setup of a shared database developed by the European Concerted Action on Blight that groups 24 European partners from 14 countries (http://www.eucablight.org).

The second major question is 'why do French *P. infestans* populations show no (or few) signs of sexual reproduction, when both clusters contain both mating types and do co-exist locally'? The high proportion of repeated multilocus genotypes and the P_{sex} values associated to these genotypes brings strong evidence for clonal reproduction in these populations. The negative F_{IS} values indicating an excess of heterozygotes relative to random mating could result either from recent migration events or from asexual reproduction (Balloux *et al.* 2003; Halkett *et al.* 2005a,b; Goyeau *et al.* 2007). Negative F_{IS} are indeed expected in clonal organisms as a result of independent accumulation of mutations on the two sets of chromosomes that never recombine under strict clonal reproduction (Mark Welch & Meselson 2000). The significant multilocus F_{IS} and the higher level of linkage disequilibrium within cluster A might be an indication that genotypes of this cluster are more strictly clonal than those of cluster B. Moreover, because the test of multilocus linkage disequilibrium (r_d) performed without multicopies does not allow one to reject the null hypothesis of recombination within cluster B, it is possible that, in addition to asexual reproduction, *P. infestans* isolates within this cluster also undergo sexual reproduction. The present study and the fact that a pairing on pea agar between an A1 isolate from cluster A and an A2 isolate from cluster B produced oospores (data not shown) are strong indications showing that the maintenance of the differentiation between clusters mainly results from the low rate of recombination events in French *P. infestans* populations. It is worth noting that the population structure of *P. infestans* in France contrasts with results obtained using a comparable set of microsatellite markers in South-West Sweden (Widmark *et al.* 2007). These authors indeed found that soilborne oospore inoculum resulting from sexual reproduction contributed significantly to initiate the late-blight epidemic in the potato field studied. Furthermore, based on the five microsatellite loci (Pi4B, PiG11, Pi16, Pi56 and Pi70) used in both the French and the Swedish studies, the comparison of the genotypic structure revealed no shared genotype between these two countries (data not showed). Two non exclusive hypotheses may account for this result: recombination that mixes alleles into genotypes might have blurred the genotypic similarities, but it is also very likely that a barrier to gene flow exits between French and Swedish populations.

The third main issue is 'why do the A2 components within clusters A and B increase in frequency relative to their A1 counterparts'? Consistent with the clonal reproductive mode of *P. infestans* in France, we found that more than 45% of the multilocus genotypes identified in 2004 could be sampled the following year, and therefore persisted in local populations. The increase of A2 genotypes could thus result from either increased aggressiveness (i.e. quantitative pathogenicity), a better persistence between seasons (higher survival rates – see Montarry *et al.* 2007), and/or more efficient dispersal and recolonisation processes. Limited pathogenicity comparisons showed no significant difference between A1 and A2 isolates, and even a slightly higher aggressiveness of A1 vs. A2 (Montarry *et al.* 2006b; Corbière *et al.* 2009), suggesting that higher epidemic fitness is not the cause for A2 extension. The mean geographic range of clones as determined in the present study was 186 km (median = 46 km), but six clonal genotypes were found to occur more than 500 km apart. Although the largest clone ranges correspond here to A1 genotypes, this is not sufficient to rule out differences in dispersal efficiency, because we focused on the earliest steps of invasion in an A1 population background. Furthermore, it is difficult to disentangle aerial dispersion of asexual sporangia (Bourke 1964) from migrations due the transportation of infected tubers. The present data are therefore not sufficient to conclude about the extent and speed of migration events. Nevertheless, the spread between 2004 and 2005 of cluster B, both within Northern France and between regions, confirms that *P. infestans* is highly mobile throughout France (Montarry *et al.* 2008).

Our finding that French *P. infestans* populations cluster into two distinct genetic groups sheds new light on the population genetic structure and evolution of *P. infestans*. The admixture pattern detected may provide the raw material for more complex evolutionary processes than the straightforward directional selection leading to the classical 'boom and bust' cycles (Vanderplank 1968). Uncovering – and predicting – which of these processes actually shape population changes over large spatial and temporal scales should be the foundation for host cultivar construction and deployment schemes, as well as for the design of matching cropping systems. Long out of reach, it becomes more feasible now that adequate markers are available, used in a

coordinated way across a number of laboratories in Europe (Eucablight) and suitable for both biovigilance and for evolutionary analysis of invasive processes.

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This research is part of the PhD research of J.M. on the population biology and genetics of *Phytophthora infestans*. J.M.'s main research interests are in evolutionary biology, ecology and population genetics of plant-pathogens. D.A.'s research is focused on the epidemiology and population biology of *P. infestans*. I.G., R.C. and G.M. are scientific assistants working in the research group led by D.A. F.D. is a population geneticist studying the adaptation of plant pathogenic oomycetes (grapevine and sunflower downy mildews) to agricultural selective pressures by combining disease ecology, population genetics and genomics approaches. All authors share a strong interest in (agro)biodiversity, the relationship between crops and their wild relatives and the genome organization of disease resistance and reproduction-related genes in different species.

Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Minimum-spanning network showing the relationships among the *Phytophthora infestans* multilocus genotypes (MLGs 1 to 70) of both clusters (A and B). Branch sizes are proportional to genetic distance (i.e. the number of different alleles) and surface of the circles to the numbers of isolates detected.

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