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Emerging virulence arising from hybridisation facilitated by multiple introductions of the sunflower downy mildew pathogen *Plasmopara halstedii*

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ABSTRACT

The sunflower downy mildew pathogen *Plasmopara halstedii* is an invasive plant pathogen in Europe of American origin. Despite efforts to produce resistant host varieties, nationwide monitoring in France has revealed the rapid emergence of new virulent races increasing the number from one founder identified in 1966 to as many as 14 today. We have genotyped 146 samples (including all 14 races) using 13 nuclear and one mtDNA marker. Samples of the same race were found to share alleles/mtDNA haplotype and the two most common races had individuals with multiple matching genotypes. Cluster analyses confirmed that the samples form three groups to which races strongly adhere. Clusters were highly differentiated (F_{ST} 0.65) and characterised by high inbreeding coefficients. Despite this, samples of recently emergent races, including six that are unique to France had mixed ancestry between the groups suggesting they have arisen *in situ* due to hybridisation. Five such samples also had conflicting mtDNA and nuclear DNA profiles. This demonstrates that multiple introductions have aided the establishment of this pathogen in France, and suggests recombination facilitated by these introductions is driving the emergence of new and endemic races in response to host resistance.

1. Introduction

The introduction of plant pathogens into new environments, many of which are unintentional, is a phenomenon that has been well documented. Notorious examples such as the introduction of *Phytophthora infestans* on infected potato tubers from South America to Europe (Gomez-Alpizar et al., 2007) that led to the Irish famine from 1845 to 1849, and the Dutch Elm disease causing parasite *Ophiostoma ulmi* that was inadvertently spread into North America from Europe via infected timber (Brasier and Buck, 2001) emphasise the impact of such events. Introduced populations are expected to exhibit particular genetic signatures such as decreased genetic diversity and heterozygosity (Crow and Kimura, 1970) although this does not eliminate all variation (Nei et al., 1975). On the other hand, multiple and successive introduction events increase the level of genetic variation (Dlugosch and Parker, 2007) and then subsequent recombination further increases the genetic pool thereby facilitating the emergence and spread of new genotypes and races that are better adapted to their new habitat (Facon, 2006; Dutech et al., 2010).

Invasive plant pathogens in agro-ecosystems can provide useful models for understanding the evolutionary processes that lead to the emergence of new (virulent) pathogens (Stukenbrock and McDonald, 2008, Montarry et al., 2010). An important selective driver of pathogen evolution in agro-ecosystems is the repeated deployment of resistant host cultivars. Usually a single resistant crop cultivar is widely used until its resistance is overcome by the pathogen and it is then replaced by another. This cycle of pathogen evolution under strong host selection is often referred to as the 'boom and bust cycle' (Thompson and Burdon, 1992) and has been described for many powdery mildews and cereal rusts (McDonald and Linde, 2002).

Several factors affect the emergence of novel virulence in crop pathogen systems. The short generation time of pathogens (compared to their hosts) means they can evolve at a relatively fast rate which can in turn enhance local adaptation (Brasier, 1995; Kaltz and Shykoff, 1998). The high density of uniform hosts at large geographic scales further provides greater opportunity for pathogen populations to increase their density and spread (Stukenbrock and McDonald, 2008). However, the evolution of new pathogenicity does not rely wholly upon recombination and there exists striking examples of evolution by accumulation of mutations. For example, in highly clonal populations of wheat rusts evolution by mutation was sufficient to allow rapid adaptation to resistant host cultivars (Enjalbert et al., 2005, Goyeau et al., 2007). Therefore an

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improved understanding of genetic structure and diversity, as well as rates of clonal versus sexual reproduction in populations of plant pathogenic fungi will help inform changes in pathogenicity that accompany bio-invasions (McDonald and Linde, 2002).

Sunflower downy mildew is a plant pathogen that is rapidly changing in France (Tourvieille de Labrouhe et al., 2000). It is native to North America where it was first identified in 1922 (Young and Morris, 1927) and later reported in France in 1966 (Delanoë, 1971), where it was introduced probably via infected seeds in agricultural trade (Ioos et al., 2007). Since 1992 it has been under quarantine regulation in Europe. It is caused by *Plasmopara halstedii* (Farlow) Berlese and De Tony, a member of the Oomycota, which comprise some of the most important plant diseases known today. It is diploid, homothallic, and can reproduce sexually and asexually. The sexual phase is required to produce over wintering propagules, but during the sunflower growing season from spring to autumn there can be several asexual generations. Like other downy mildews it is an obligate biotroph and can be dispersed by wind via infected seeds but is mostly soil-borne. Mobile zoospores in the soil infect seedlings through the roots and systemic infection follows. Normally this leads to death of the host but surviving plants have to severe dwarfism, yellowing of the leaves and symptomatic accumulation of mycelium on the undersides of the leaves appearing as a white mass. Putative effector genes RXLR and CRN (for crinkling and necrosis) have recently been found to be expressed during *H. annuus* and *P. halstedii* interactions with SNP's being identified in CRN sequences between different races of the pathogen (As-sadi et al., 2011).

In this study pathogen race is defined by its ability to infect genetically resistant sunflower cultivars (susceptibility or resistance). Currently there are 36 identified races of sunflower downy mildew worldwide each named by an international system for race nomenclature in use since 1998 (Gulya et al., 1998). Following the first identification of *P. halstedii* in France in 1966, a dramatic increase in the number of races was observed in the following years and 14 are found in France today (Fig. 1a).

The disease is controlled to some extent by the chemical treatments of seeds, but the most effective method is the introgression of dominant major resistance genes denoted *Pl* into new host cultivars. There are currently 18 resistance genes in use, sought from wild *Helianthus*, some of which are believed to be tightly linked in at least two different linkage groups (Vear et al., 2003, 1997). Dominance in the pathogen avirulence genes is not known, but by analogy with other plant-pathogen species they are probably recessive (Stukenbrock and McDonald, 2008). The only race known to occur in France was 100 until 1988 when race 710 was discovered in central France, closely followed in 1989 by race 703 in south-western France. For a full description of race phenotype nomenclature see Section 2.4. A further 11 races able to overcome the *Pl* loci were discovered over the following years (Fig. 1a) in varying numbers (Fig. 1b). The different races were found in distinct geographical regions with 710, 704, 714 and 314 predominantly in central France and 703, 307, 300 and 304 found predominantly in south-west France. Race 100 was ubiquitous albeit rarely found due to the use of resistant host cultivars. Fig. 2 shows the regions where the samples were collected and races are denoted by different colours. Six of them (304, 307, 314, 334, 704 and 714) have never been documented outside of France even though sunflower hybrids using the *Pl6–Pl7* genes are grown in other European countries (Gulya, 2007). The development of sunflower resistant cultivars that retain the characteristics required for the oil crop is a lengthy process and the rate of new *P. halstedii* virulence emergence is more rapid than the rate that newly resistant hosts can be bred. Host and race evolution in downy mildew is therefore an issue of key agricultural and commercial importance.

Early studies of *P. halstedii* typically found low levels of genetic diversity using RAPD markers (Komjati et al., 2004), ISSR (Intelmann and Spring, 2002) and ITS sequences (Spring and Zipper, 2006; Thines et al., 2005). A study of 77 samples from twelve different countries of six virulence races using 21 RAPD primers found low levels of differentiation within and between samples grouped by race and country (Roeckel-Drevet et al., 2003). By contrast, a more recent study found that 24 samples of *P halstedii* strains had divergent genotypes according to their race. These samples were made up of between three and five samples of the three more commonly found virulence races in France, and just one sample of the 11 more recently emerged races. Cluster based analysis revealed that the samples formed three groups and suggested that the three clusters represented at least three separate introductions of the pathogen into France (Delmotte et al., 2008). However, the limited number of samples in that study provided little information about the relationships among the different races. The same three groups were also found among 22 SNPs found within putative CRN effector sequences. As with the neutral markers these SNPs separated races 100 and 304 from 703 and 710 (As-sadi et al., 2011).

Thus, the aims of this study were to determine the level of genetic diversity of *P. halstedii* in France and to investigate the link between genotypic and phenotypic (pathotypes) characteristics. We have combined data from one mitochondrial and 13 nuclear markers in order to advance our understanding of what is driving the rapid emergence of new virulent races. Specifically we ask if

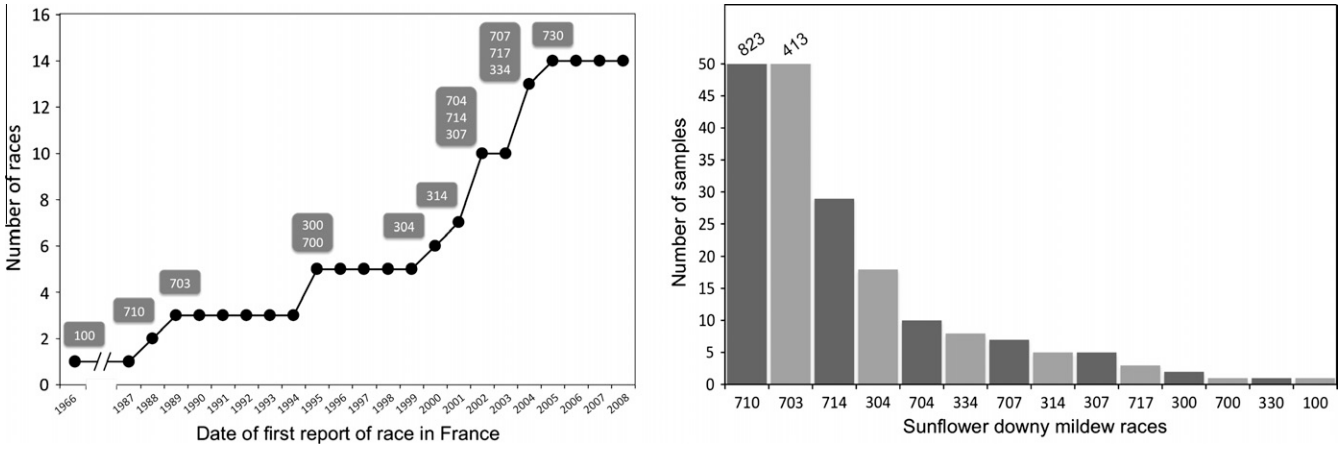

Fig. 1. Graphical representations of *P. halstedii* collections. (A) Graph showing the number of races found against time. (B) Histogram of the number of samples collected for each race during the 25 years of monitoring of *P. halstedii* in France.

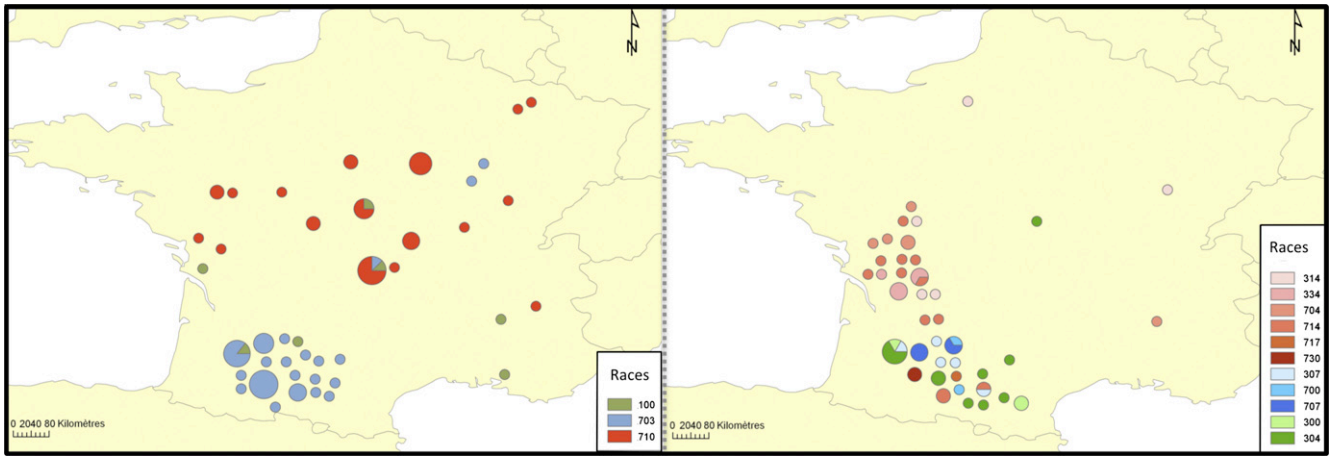

Fig. 2. Geographic distribution of 147 sunflower downy mildew samples in this study. (A) Distribution races 100, 703 and 710 in France. (B) Distribution of virulence races that have emerged since 1994. Nuclear cluster is indicated by the colours: Cluster 1 = green, Cluster 2 = blue, Cluster 3 = red). The size of the circle relates to the number of samples found in each location. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

are our samples from structured populations and are the newly found races a result of hybridisation or have they arisen due to mutations in standing genetic variation.

2. Methods

2.1. Background

Disease prevalence was monitored nationally by Service Régional de la Protection des Végétaux (SRVP) pathologists in sunflower crop fields during the 21 years period from 1988 to 2008 (Moinard et al., 2006; Tourvieille de Labrouhe et al., 2000). During these surveys 1200 *P. halstedii* isolates were collected and were later characterised for their virulence race profile (also known as race identification) (Fig. 1b).

2.2. Sampling

All of the samples were collected from infected sunflower leaves taken from one or two plants in each crop field. Spores from these infected leaves were then propagated onto nine different host lines in order to determine their virulence profile (also sometimes referred to as race). In order to ensure that samples did not contain a mixture of races, a single sunflower cotyledon was then taken from each infected plant and the resulting spores were tested again on the nine differentially resistant lines. This procedure was repeated for all samples. In total 147 samples were used for DNA extraction and genetic analysis. These isolates were chosen to include multiple samples from each of the main sunflower growing regions (i.e. West, Central, and south-western France) and where possible to represent samples collected from different years. For races that had numerous samples, they were randomly selected. We also included pathogen races that are rarely found in order to asses their genetic similarity with more frequently found.

2.3. Race phenotyping

Virulence profile identification was replicated twice in independent experiments by inoculating each isolate on a differential set of sunflower cultivars: race virulence tests were determined according to the ability of sporangia to infect differential lines of resistant sunflowers. Resistance tests were performed as described by Cohen and Sackston, 1974, with recommended modifications (Mouzeyar et al., 1993): 15 days after inoculation, plants were incubated for 48 h in a saturated atmosphere. Plants were scored as susceptible if sporulation was observed in cotyledons and leaves, and as resistant if no sporulation or only light sporulation was seen on cotyledons. For a subset of 15 samples (h56 to h70 in Table S1) monozoosporangium isolation was performed before the second round of infection and multiplication on the host-plant. This subset of samples was used as a reference data base for all subsequent phenotyping of sunflower downy mildew races in France.

2.4. Race nomenclature

Races were named according to the international nomenclature of *P. halstedii* (Gulya et al., 1998) (Table 1). The resistant sunflower cultivars are organised in triplets across the table. Row one of the table shows the number given for a race with resistance to each sunflower cultivar in that triplet. When a host cultivar becomes infected by *P. hasltedii* sporangia, the binary numbers in each triplet are transformed to decimal to give the race name. For example an isolate that was able to infect D1 and none of the others would have the virulence profile 100000000 against the nine lines. Using the scores in row two of Table 1, interpretation of the binary code

Table 1
Virulence profiles of 14 *P. halstedii* races on nine sunflower hosts with differential genetic resistance. S indicates host susceptible to race in column one and R indicates resistance. Races underlined are endemic in France.

Host	Virulence profile on sunflower differential hosts								
	D1	D2	D3	D4	D5	D6	D7	D8	D9
Score	1	2	4	1	2	4	1	2	4
Races									
100	S	R	R	R	R	R	R	R	R
300	S	S	R	R	R	R	R	R	R
304	S	S	R	R	R	R	R	R	S
307	S	S	R	R	R	R	S	S	S
314	S	S	R	S	R	R	R	R	S
334	S	S	R	S	S	R	R	R	S
700	S	S	S	R	R	R	R	R	R
703	S	S	S	R	R	R	S	S	R
704	S	S	S	R	R	R	R	R	S
707	S	S	S	R	R	R	S	S	S
710	S	S	S	S	R	R	R	R	R
714	S	S	S	S	R	R	R	R	S
717	S	S	S	S	R	R	S	S	S
730	S	S	S	S	S	R	R	R	R

in triplets would give one for the first triplet and zero for the other two triplets which would name that isolate as 100. If an isolate was able to overcome resistance in sunflower lines D1, D2, D3, D7, and D8 it would have a resistance profile of 111000110 named 704. For each sample a virulence profile was obtained enabling the race of each sample to be determined (Table S1).

2.5. DNA extraction and Polymerase Chain Reaction (PCR)

Infected leaves were removed from individual storage tubes containing ethanol and a 1 cm^2 section was cut from the leaf and freeze dried over night. DNA was extracted from the dried leaf tissue leaf tissue following a standard cetyltrimethylammonium bromide (CTAB) protocol (Zolan and Pukklia, 1986) but phenol-chloroform was replaced with is isoamyl alcohol and chloroform (ratio 24:1). After extraction the DNA was precipitated with isopropanol and the pellet was washed with 75% ethanol and resuspended in 50 μl of water. For PCR amplification of the 12 EST derived markers, 10 ng of DNA was used in a single PCR reaction and carried out in a final volume of 25 μl with 2 mM $MgCl_2$, 150 μM dNTPs, 5 pmol each primer and 0.2 U *Taq* polymerase (Silverstar, Eurogentec) in the manufacturers buffer. Thermal cycling conditions were as follows: initial denaturation at 96 °C for 5 min followed by 38 cycles of denaturation at 96 °C for 40 s, annealing at 57 °C for 50 s, elongation at 72 °C for 90 s, with a final elongation step of 72 °C for 10 min. Five of these markers were then screened by cleaved amplified polymorphism sequence (CAPS) markers, five by single strand conformation polymorphism (PCR-SSCP) and the remaining marker was resolved by agarose gel electrophoresis. A table of PCR primers, EMBL accession numbers, allele sizes and restriction enzyme used in CAPs analysis is given in (Giresse et al., 2007). Samples were also genotyped at one di-nucleotide microsatellite marker (Genbank accession number JF914932). SSCP and microsatellite PCR fragments were visualised by ABI 3130 genetic analyser and peaks scored using the Genemapper v3.2 software. Positive controls included repeated genotyping of the same samples to ensure repeatability. Repeated samples were included in all SSCP runs in order to standardise allele scoring.

Mitochondrial DNA for each sample was sequenced using taxon-specific primers (Giresse et al., 2010) designed to amplify a 900 base pair region of the cytochrome *b* and nad9 genes and their intergenic space. PCR amplification of the mtDNA was as follows: 25 μl final pcr reaction was made up of 10 ng genomic DNA, 5 μM of each primer, 1.2 mM MgCl and 200 μM of each dNTP with 0.1 units of *taq* polymerase (Silverstar, Eurogentec, Liège, Belgium) in the manufacturers buffer. Cycling conditions were: initial denaturation at 94 °C for 4 min, followed by 32 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 45 s, extension at 72 °C for 30 s followed by a single final extension step of 72 °C for 10 min. PCR products were visualised on 1.5% agarose gels and products were sequenced in the forward and reverse direction. Sequence data were edited and aligned using Codon Code sequence aligner and haplotype variation analysed using DnaSp v5 (Librado and Rozas, 2009).

2.6. Analyses of genetic data

Multilocus genotypes were generated for 147 samples and were analysed in GenClone 2.0 (Arnaud-Haond and Belkhir, 2007) to confirm the number of multiple matching genotypes. Estimates of F_{IS} (Weir and Cockerham, 1984), allelic richness and genetic diversity were calculated in Fstat (Goudet, 1995). Using only a single representative of each multi-locus genotype we performed a Principal Components Analysis using R v2.9 in order to visualise the spread and structure of these data. Using this same data set we also performed a genetic cluster analysis using the Bayesian approach implemented in Structure 2.2 (Pritchard et al., 2000) and used the program to estimate the most likely number of clusters within the range of 1–6. The MCMC (Markov Chain Monte Carlo) ran for 10^6 iterations with a burn in of 100,000, repeated for 10 chains under a model of admixture and the optimal number for *K* was confirmed based on variance in the log probability of data between successive *K* values. The proportion of ancestry in each individual at the optimal number of clusters found by Structure was plotted as a pie chart onto the PCA plot (Fig. 3B). Pairwise linkage disequilibrium between loci and AMOVA between the optimum number of groups found by the cluster analyses were performed using Arlequin v3 (Excoffier et al., 2005). A median joining tree of the unique nuclear genotypes and of mtDNA haplotypes including all races was created using the software Network v 4.6 (Bandelt et al., 1999). Spatial autocorrelation of races with geographic data was estimated by using Morans I in the software R using the ape package. A Mantel test between genetic distance of individual genotypes and spatial coordinates in was performed R using the ade4 package (Dray and Dufour, 2007) and also using Roussets distance measure $\hat{a}_r$ (analogous to Fst/1-Fst) (Rousset, 2000) in Genepop v4 (Raymond and Rousset, 1995).

3. Results

3.1. Genetic and genotypic diversity

The 13 nuclear loci had two alleles except Pha79 which had three alleles and Pha7 (the microsatellite locus) had 16 alleles with 3.9% missing data overall (due to poor PCR amplification or chromatogram peaks that were difficult to score). The mean expected level of heterozygosity over all loci was 0.47 and the mean observed value was 0.05. The global values for F_{IS} and F_{ST} were 0.78 and 0.65 respectively. From the total of 147 isolates, there were 98 different multi locus genotypes (MLGs) of which 84 were unique (found only once), and 14 were represented in the data set by two or more samples, (details of clones are given in Table 2). In seven of the 14 cases the same clonal genotype included two different races. This means that multilocus genotypes were not unique to a particular race: for example samples races 100 and 304 shared a single genotype among three samples, samples of race 307 had matching genotypes with samples of races 703, 304 matched 100, and 714 matched 710. Of the 14 repeated multilocus genotypes some differed only because of missing data or microsatellite variation, and so discounting the microsatellite locus the 14 repeated genotypes are reduced to six genotypes. For example without the microsatellite MLG 2, 4, 5 and 7 described in Table 2 becomes a single MLG with 24 matching individual samples.

3.2. Principal component analysis

A single representative of each multilocus genotype was included in the Principal Component Analysis (PCA). Where a single genotype represented more than one race, both (or all) samples were included. This resulted in 106 different samples to be analysed. The PCA revealed strong differentiation in the spread of the data, with 42.7% of the observed variation on the first axis and 18.9% on the second axis (Fig. 3a). When plotted, these data formed three distinct groups. Group 1 consisted of races 100, 300 and 304. Group 2 comprised of samples belonging to races 300, 304, 307, 700, 703, 707 and 730. Group 3 was made up of samples mostly from races 314, 334, 704, 710, 714 and 717. The first axis clearly separated Group 2 from 1 and 3, and the second axis separated Groups 1 and 2 from 3 (Fig. 3a). Using this model free approach we were able to detect three main groups within our data set.

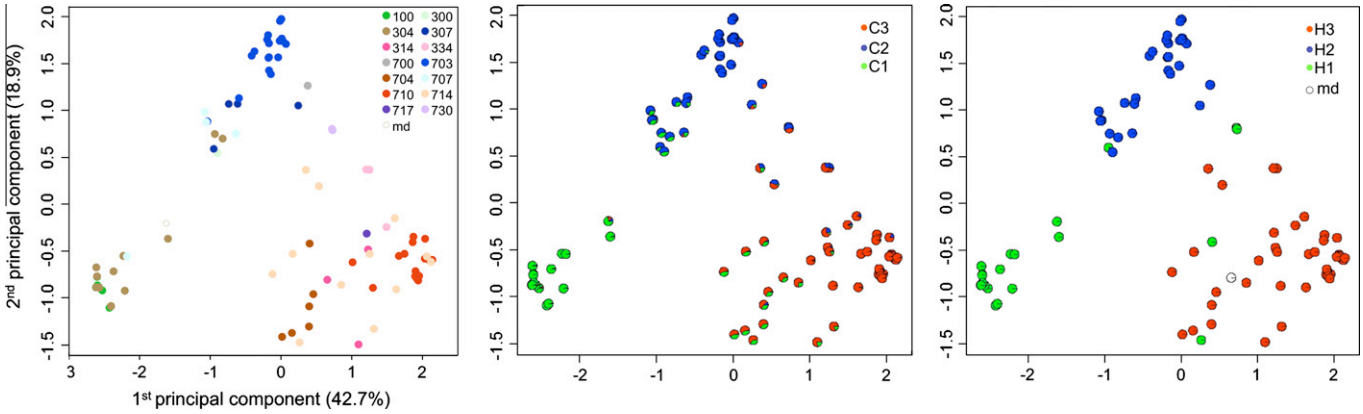

Fig. 3. Plot of the principal component analysis. (A) PCA plot of 106 unique genotypes shown on the first and second axis. (B) PCA plot of 106 samples with the proportion of ancestry from each cluster for each sample indicated by colour. Green shading is Cluster 1, blue shading is Cluster 2, and red shading is Cluster 3. (C) PCA plot for 106 samples with Mitochondrial DNA haplotype for each sample indicated by colour. Green = Haplotype 1, blue = haplotype 2, red = haplotype 3, white = missing data point. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Details of clonal multilocus genotypes (MLG) among the three genetic clusters. The 14 repeated genotypes are listed by nuclear cluster membership, the name of the MLG is denoted by a number, the number of times it was observed in the full dataset, and in which races. Where the same genotype was found in more than one race, the number of observations for each race is given next to the race name in parenthesis.

	MLG name	Number of observations	Race $_{(nb)}$
Cluster 1	83	2	100
	82	3	$300_{(1)}$ $304_{(1)}$ $707_{(1)}$
	83	3	$304_{(2)}$ $100_{(1)}$
Cluster 2	2	16	$700_{(1)}$ 703 $_{(15)}$
	4	2	307, 703
	5	4	703
	7	2	703
	91	4	703
Cluster 3	32	3	710 $_{(3)}$ 714 $_{(1)}$
	35	3	710
	36	8	710
	39	3	$710_{(2)}$ $714_{(1)}$
	42	3	710
	29	6	$334_{(4)}$ 314 $_{(2)}$

3.3. Individual clustering analysis

Cluster analyses of the 106 samples using the program Structure (Pritchard et al., 2000) also suggested an optimum partition of the data into three clusters which (per sample) agreed largely with the PCA. The best estimate for the posterior probability of the data $P(X/K)$ was for $K = 3$ (mean $P = 104.24$) closely followed by $k = 4$ (mean P 96.39). Values of K greater than four led to great fluctuations in the log likelihood values obtained between the 10 chains. Cluster 1 (C1) was made up of mostly 100, 300 and 304, Cluster 2 (C2) mostly consisted of races 300, 304, 307, 700, 703, 707 and 730 and Cluster 3 (C3) was mostly 314, 334, 704, 710, 714 and 717 as for the PCA groupings. A histogram of the proportion of ancestry for each sample in order of race membership is shown with its corresponding mtDNA haplotype above each bar (Fig. 4). The alternative resolutions for the different number of K estimates range 2–5 are shown in Supplemental data (Fig. S1). The histogram shows that when K increases above three that Clusters C1 and C2 remain the same but C3 becomes further fragmented suggesting that there may be some substructure within that group where race 710 becomes separated from the other races in that group.

A posterior probability value of 70% was chosen as an indicator of strong assignment to a particular cluster. If this value was increased to 75% only two samples fell out of the strong assignment category and by increasing it further to 80% just two more samples fell out of the low assignment category, resulting in very few changes to the overall structure. However, if we lowered the posterior probability threshold to 60% a further 17 samples were included in the high assignment category, of which three had incongruent genotype and mtDNA haplotype and the mean proportion of ancestry in the next most likely cluster was 30.8%. We therefore considered the 70% an appropriate cut off point between strong and weak level of assignment.

We found that 75% of the samples had a threshold of 70% or more of their ancestry assigned to one or other cluster. Most of these had assignments of 90% or more to a single cluster (Fig. 4). Individual samples of each race were found to share many alleles, and there was a positive correlation between race and cluster membership. The remaining 25% samples that were assigned to one cluster with less than 70% posterior probability tended to have the main proportion of ancestry in one of the other two clusters (mean value of 0.308) rather than equally between the two. These data were combined with the PCA output to produce a pie plot in order to visualise the proportion of ancestry of each sample and its coordinate in the PCA (Fig. 3b). Summary statistics for the mean value across all loci for allelic richness, gene diversity and F_{IS} where also calculated according to the three clusters assigned by Structure (C1, C2 and C3) (Table 3).

The PCA and Structure analyses were repeated after omission of the highly variable microsatellite locus in order to observe its effect on cluster assignment. However its exclusion did not affect the optimum number of groups detected or the assignment of samples to each group overall but did increase the number of samples with multiple matching genotypes. This was confirmed further by a median joining network of the 106 unique genotypes (Supplementary Appendix B). The races again formed three main clusters where each cluster formed a star like shape around the prominent race member of each cluster (namely 100, 703 and 710 for C1, C2 and C3 respectively as described in Section 3.2). One sample of race 714 appeared to lie between samples of C2 and C3 in the tree, but this sample was narrowly assigned by Structure with 0.45 in C2 and 0.49 in C3. The network revealed many loops especially around the points associated with races 710 and 703 indicating the presence of recombination and/or homoplasy.

The sequence data derived for the fragment of the nad9 – cyt*b* mitochondrial DNA region revealed two synonymous and two non-synonymous changes. Base substitutions were found at positions 324, 455, 1055 and one insertion–deletion at base position

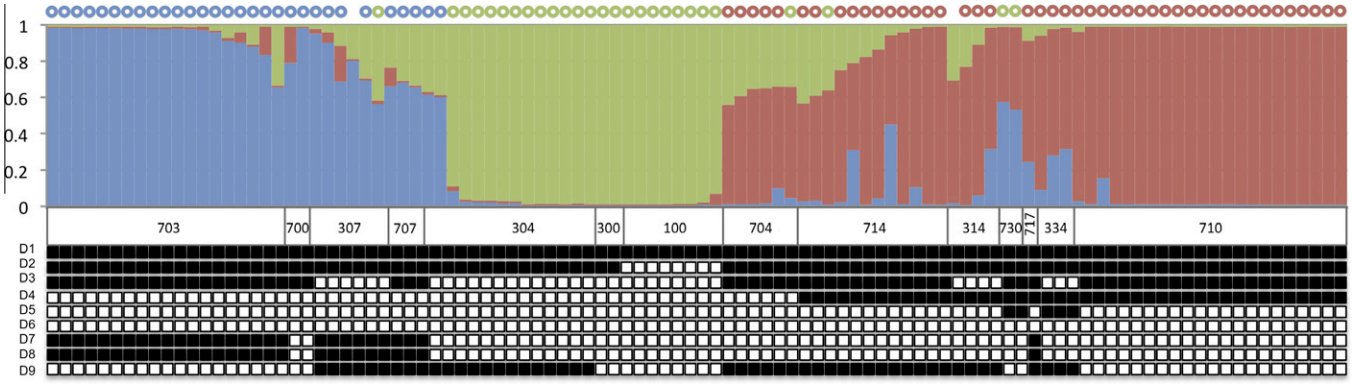

Fig. 4. Histogram showing proportion of membership to each cluster for each unique mutilocus genotype (MLG). The race of each sample is indicated below the corresponding histogram bar. Circles above the bars represent the mtDNA haplotype of each sample. Cluster 1/H1 = green, Cluster 2/H2 = blue, Cluster 3/H3 = red. White squares beneath each histogram bar indicate that host cultivar on the left is resistant to the pathogen race above, black squares indicate susceptibility. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Details and summary statistics for the genetic clusters defined by the program Structure based on 13 nuclear loci. Cluster name, number of samples assigned to each cluster, number of unique genotypes, number of different races found in each cluster, names of races, mean allelic richness, gene diversity, F_{IS} value ($^{*}P < 0.01$). Italicised race names highlight membership in more than one cluster.

Nuclear cluster	Number of samples	Number of unique genotypes	Number of races	Races	Ar	Gd	F_{IS}
Cluster 1	24	22	4	100, *300*, *304*	1.89	0.110	0.656*
Cluster 2	56	35	5	*300*, *304*, 307, 700, 703, 707, 730	2.21	0.198	0.758*
Cluster 3	66	48	7	314, 334, 704, 710, 714, 717	2.45	0.281	0.814*
Global	146	105	14		2.60	0.193	0.780*

339. These mutations defined three different mitochondrial haplotypes as follows (H1 is TACA, H2 is TATT and H3 C-CT) (Giresse et al., 2010) and these haplotypes were associated with races 100, 703 and 710 respectively. The median-joining network tree (shown in Supplementary Appendix C) of the mtDNA haplotypes illustrates that H3 is the most divergent of the three. The haplotypes showed a strong association with the three clusters and PCA groupings (Fig. 3c). Therefore isolates of C1 were almost always associated with H1, C2 samples had H2, and C3 samples had H3. There were five exemptions to this pattern where three samples assigned to C2 had mtDNA H1 (two were members of race 730 and one was a member of race 307) and two samples assigned to C3 had mtDNA H1 (Fig. 4). This is discussed further in Section 3.5.

3.4. Cluster differentiation

The global F_{ST} value for the three groups was high at 0.65 across all loci (F_{IT} 0.94, F_{IS} 0.78) and observed patterns of differentiation were consistent across loci (F_{ST} at individual loci range 0.02–0.88, median 0.65). The genetic diversity within each cluster was measured by the number of alleles per locus, allelic richness and F_{IS} per locus

Table 4
Analysis of Molecular Variance (AMOVA) performed on 106 unique genotypes of *P. halstedii*. Comparisons are by cluster.

Source of variation	Degrees of freedom	Sum of squares	Variance	Percentage of variation
Among groups	2	323.121	2.331 (Va)	56.93
Among samples within groups	103	329.757	1.438 (Vb)	35.12
Within sample	106	34.500	0.325 (Vc)	7.95

(Table 3). C1 had 25 typed alleles in total, C2 had 31 and C3 had 34. C1 was generally less genetically diverse than the other two and eight out of the 13 loci were at fixation. The significance test for the global fixation indices (F_{IS}: 0.81544, F_{ST}: 0.56927, F_{IT}: 0.92051) all had P-value < 0.001 (for random value ⩾ observed value). Values obtained for F-statistics varied slightly to those obtained in Fstat.

Three of the loci (Pha6, Pha74 and Pha79) appeared to have alleles that were associated with a particular cluster. A strong pattern could be seen in Locus Pha79 that had private alleles associated with certain races (allele 3/C1/race 100, allele 2/C2/race 703, allele 1/C3/race 710) and the pairwise F_{ST} values between the three clusters at this locus were between 0.71 and 0.91. This locus, typical of the others, was mostly homozygous.

For the hierarchical analysis of molecular variance (Excoffier et al., 2005), groups were also defined according to the Structure output therefore the comparison provided the variance within and between clusters or groups with the variation being greatest among groups (Va) (Table 4).

3.5. Genetic admixture

Locus Pha79 had seven heterozygotes with genotypes 1/3, and 2/3. Genotype 1/2 was found only once suggesting hybrids between C1 and C2 samples were rare. The presence of heterozygotes carrying alleles 1/3 and 1/2 suggests that there could have been hybridisation events between members of either races 100 and 703 or 710 but that hybrids of 703 and 710 are less likely, probably due to their different spatial distributions. The map (Fig. 1) reveals that these races were rarely found to co-exist.

In the case of matching mtDNA haplotype and nuclear cluster assignment only five samples out of 147 were incongruent. These included two samples that were assigned to C3 and three that were assigned to C2 but all of them had mtDNA H1. For these

mismatches the Structure assignment was weak (range 63–53%) suggesting mixed ancestry/hybridisation for these samples. For example, a race 307 sample that had the maximum proportion of ancestry in C2 had the remaining proportion of ancestry in C1 and mtDNA H1. This pattern was also observed in samples from C3 that were phenotyped as races 704 and 714 but had mtDNA H1. The two remaining incongruent samples that were phenotyped as race 730 were assigned to C2 (with some proportion of ancestry in C3), but had mtDNA H1 (Fig. 4).

The 28 samples that fell below the 70% assignment level (described in Section 3.3) and were considered as admixed were members of races 300 (n = 1/3), 304 (n = 2/14), 307 (n = 3/6), 314 (n = 2/4), 334 (n = 2/3), 703 (n = 1/19), 704 (n = 6/6), 714 (n = 5/12), 707 (n = 3/3), 717 (n = 1/1), 730 (n = 2/2). Races 100, 700, 710 did not have any samples that were assigned at less than 70% of ancestry in a single cluster. Samples of race 703, 304, 307, 707 were found in both C1 and C2 clusters with a bias towards Cluster 2. Samples of race 314, 334, 714 and 730 tended towards shared ancestry between C2 and C3 whereas samples with races 334 and 714 were biased towards C3. Hybrids from C1 and C3 were the most rarely found admixed individuals and this was observed in only three samples (one each from race 314, 704 and 714). The common theme that could be seen throughout the endemic races 304, 307, 314, 334, 704 and 714 was that they had all overcome resistance in differential line D9 (Table 1).

3.6. Spatial genetic structure

The geographic distribution of samples by race and genetic cluster is shown in Fig. 2a and b. Samples of race 710 were typically from the North of France while samples of race 703 were mainly found in the southern part of France. Race 100, although now relatively rare, was ubiquitous across the sunflower growing regions of France. The races that emerged after 1994 were observed in both north-western and south-western France. The central/northern regions comprise mainly of emergent races that were assigned to C3 while south-western France included emergent races assigned to all three genetic clusters.

The test for spatial autocorrelation using Moran's I statistic in R (Paradis et al., 2004) was significant (P = 0.0008) and rejected the null hypothesis of 'no spatial autocorrelation between race and geographic position'. A Mantel test was also performed in R between all individual genetic and geographic distances and was able to reject the null hypothesis of 'no correlation between individual spatial and genetic distance' (observed = 0.2, P = 0.01). A Mantel test based on Roussets $\hat{a}_r$ statistic (Rousset, 2000) performed in Genepop (Raymond and Rousset, 1995) at the individual level including all samples was also significant (fitting $\hat{a}$ statistic to $a + b$ ln(distance), a = 10.549, b = 1.683). However, when this test was performed by genetic cluster the same statistic showed there was no correlation between individual genetic and spatial distance (C1 a = 2.481, b = −0.502, C2 a = 2.840, b = −0.202, C3 a = 5.476, b = −0.753 all with non-significant P values) providing further support for three genetic clusters within our data set.

4. Discussion

We have demonstrated using a data set that combines virulence race profiles with nuclear and mitochondrial haplotype data that samples in our study fell into three distinct groups. This differentiation was focused around the three most important and prevalent races in France namely 100, 703 and 710. This confirms our original hypothesis that multiple introductions of *P. halstedii* have contributed to the genetic diversity and structure currently found in France (Delmotte et al., 2008) and that spatial structure observed across race distributions may reflect the initial areas of introduction. Groups one and two appeared to be more closely associated with one another than the third group in that they shared more alleles and admixture than group three. Two of the races (300 and 304) were present in both C1 and C2, whereas C3 races were never found in other clusters. Population genetic analyses revealed that *P. hasltedii* populations were highly inbred. Although the frequency of identical genotypes in our data set was low, samples of the same race often had multiple matching loci. Therefore the genotypic data suggested the possibility of virulence emergence through clonal reproduction by the existence of identical multilocus genotypes belonging to samples of different race. However, the strongest and most plausible evidence for virulence emergence was via hybridisation as indicated by the Structure output and the pattern of heterozygosity.

4.1. Reproductive system

The global F_{IS} value of 0.78 equated to a selfing rate of approximately 88% (where F_{IS} = S/(2-S), or an outcrossing rate of 12%. This was in line with our findings for the number of possible intermediate samples that may have arisen due to hybridisation between different races (28 samples out of 106 unique genotypes) which makes the coexistence of races easier to envisage. This high selfing rate, reduced genetic variation within cluster and increased linkage disequilibrium found in the three genetic groups with only limited outcrossing, may be a consequence of this species being homothallic with no known self incompatibility mechanism. Heterothallic Oomycetes such as *Phytophthora infestans* (Grünwald and Flier, 2005) and *Plasmopara viticola* (Delmotte et al., 2006) are reported as having lower F_{IS} than observed in this study further suggesting that homothallism may contribute to high inbreeding levels. Sexual reproduction is an obligate part of the life cycle in *P. halstedii*, but when one considers the spatial scale at which this is believed to occur, i.e. within a sunflower crop field, populations often present very low levels of variability, typical of soil-born pathogens.

4.2. Multiple introductions of P. halstedii into France

The results support our hypothesis that the current diversity of races found in France is due to at least three successive introductions of the main races 100, 703 and 710. A single introduction event for this pathogen was not realistic because races 703 and 710 were found in France only 22 years after the first sighting of race 100 in 1966. This would not provide sufficient time for the two more recently found races to have evolved from the first, given how distinct they are from one another in terms of both genotype and virulence profile. Furthermore, races 710 and 703 differ in their spatial distributions which would be difficult to explain had they both evolved from 100.

Eight of the races (100, 703, 710, 300, 700, 707, 717, 730) are also found in other countries, therefore may represent additional introductions above the minimum three that we propose, but the six endemic races (304, 307, 314, 334, 704, 714) provide evidence for evolution *in situ*. However, this is not unexpected since a consequence of the obligate deployment of genetically resistant sunflower varieties across France is strong selection pressure exerted by the host. This is unlike other sunflower producers in Europe where the use of resistant sunflower varieties is not obligatory. Although we have found a clear partition of our data into three groups, we propose that subsequent genetic diversification either by hybridisation or mutation in the pathogen has occurred in response to selection pressure exerted by the host. This finding is in line with studies on others invasive plant-pathogen oomycetes that present evidence for multiple events of introduction, such as in *Phytophthora infestans* (Montarry et al., 2009), *P. ramorum* (Goss

et al., 2009a) or *P. cinnamomi* (Dobrowolski et al., 2003). Population structure as a result of multiple introductions has also been demonstrated in the fungal pathogen *Cryphonectria parasitica* (Dutech et al., 2010).

4.3. How have novel races appeared?

P. halstedii monitoring revealed that between 1987 and 2008 there was a rapid increase in the number of virulent races rising from just 1–14 (Fig. 1a). In some years the number increased by more than one, for example in 2001 and in 2003 three new races were encountered. The high levels of genetic differentiation between the three genetic groups indicated overall limited exchange between them, but our results also suggest that recombination between genotypes of these groups does occur. This is in agreement with other Oomycetes such as *P. ramorum* (Goss et al., 2009b) and *P. infestans* (Miller et al., 1997) where sexual reproduction occurs depending on geographical location and environmental conditions. We also found that members of different genetic clusters do sometimes co-exist, for example the main races of 100, 703 and 710 were all found to be present in a small area of central France. A quarter of our samples had less than the chosen threshold limit of 70% of ancestry assigned to one or other cluster (ranging 48.1–68.9%, mean of 61.3%) and these samples were mainly races 304, 307, 704 and 714. These belong to a group of newly emergent races that have never been recorded outside France and that are able to overcome resistance in the host *Pl*6 locus (found in differential line D9). It can be seen on the map (Fig. 2) that races 307, 704 and 714 are found in central, west and south west France and therefore have greater opportunity for hybridisation with races in different genetic clusters.

It was previously unknown whether field populations of *P. halstedii* hybridise, however there is evidence to suggest this in our data. For example, the admixed samples of race 707 (shown in Fig. 1) first documented in 2004 in France, are probably the product of a hybrid between races 703 from C 2 and 304 from C 1 resulting in a new race able to overcome resistance in sunflower differential lines D7, D8 and D9 as shown in Table 1. Novel pathogen types arising from interspecific hybridisation in natural populations are becoming increasingly documented due to molecular studies (Brasier et al., 1998; Joly et al., 2006; Masneuf et al., 1998). Although we were able to demonstrated how new virulent races may have emerged, we were not able ascertain from these data at what frequency such events occur, or if crosses between certain races are more likely than others.

In converse to admixture, our results suggested the possibility that new endemic races could also arise through clonal evolution, a ubiquitous process in the evolution of many pathogens. Evolution of virulence by accumulation of mutations has been previously described for rusts such *P. striiformis* where 20 different pathotypes were found to have arisen from a single introduction followed by a stepwise mutation process (Steele et al., 2002). Furthermore, recent evidence for race emergence in *P. halstedii* was recently demonstrated by infecting an experimental plot with races 100 and 710. After 5 years six other races were observed (300, 304, 314, 700, 704, 714) that had not been present at the start of the study (Tourvieille de Labrouhe et al., 2010). Although we do not have sufficient data to conclude this mechanism has led to race emergence *in situ*, it appears to be a plausible explanation given that these genotypes have not arisen by recombination.

5. Conclusion

These data provide the first population-wide picture of the genetic structure of *P. halstedii* in France. We maintain the hypothesis that three groups represent at least three separate introductions and propose that increased virulence is a result of admixture between the main introduced races and has led to hybrids with novel virulence. Our findings add to the growing body of work being accumulated on the biology and genetics of this pathogen. A more detailed understanding of the global distribution of this pathogen would be informative since although it is generally accepted that this pathogen is of North American origin, its route of introduction to other countries is not known. Since it is a widespread disease found in many sunflower producing countries reliant on this crop, a more detailed understanding of worldwide pathogen populations may help to control this disease. Furthermore, since it appears to evolve new pathogenicity through both mutation and hybridisation, this species may provide a useful model for further research into the genetics of pathogen evolution. These issues are not only important for informing disease management policy, but also they address key questions in the genetics of adaptation.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fgb.2012.06.012.

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