

## Emerging races arising from recombination facilitated by multiple introductions of the sunflower downy mildew *Plasmopara halstedii* in France

François Delmotte<sup>1</sup>, Denis Tourvieille de Labrouhe<sup>2</sup>, Sophia Ahmed<sup>1</sup>

<sup>1</sup> INRA, UMR1065 SAVE, ISVV, F-33883 Villenave d'Ornon, France, [delmotte@bordeaux.inra.fr](mailto:delmotte@bordeaux.inra.fr)

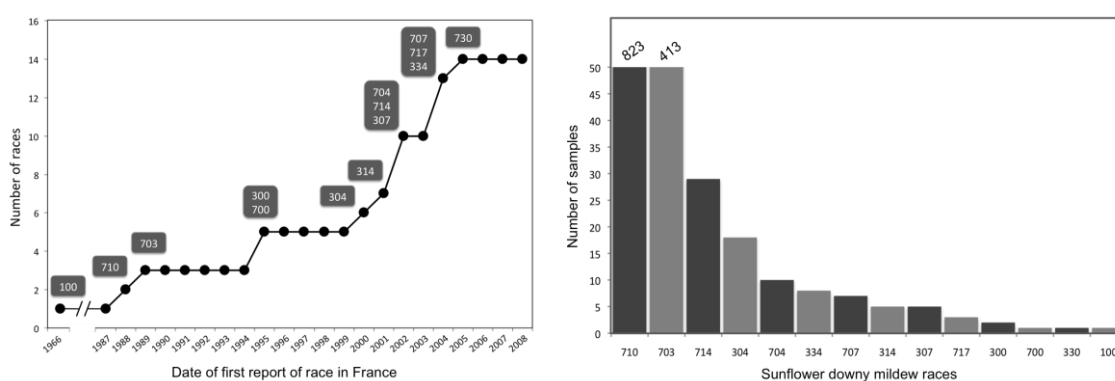
<sup>2</sup> INRA, UMR1095 GDEC, 63100 Clermont-Ferrand, France ; <sup>3</sup>

- **Background to the research and aims.** French pathologists have been intensively monitoring the prevalence of sunflower downy mildew in France over a 20 years period. Despite efforts to produce resistant varieties of sunflower, this monitoring has revealed that there has been a recent and rapid emergence of new and endemic (found only in France) races, that has led to an increase from one original founding race in 1966 to 14 races found today (Moinard et al. 2006).
- We have taken advantage of having access to this unique data set of information and samples in order to identify factors that drive the emergence of new virulence. In a first genetic study, Delmotte *et al.* (2008) used SNP markers to analyse 24 individual isolates that included the 14 races found in France, and observed a strong correlation between genetic and phenotypic structure. We have built on that early work by performing new population genetic analyses using both nuclear and mitochondrial markers.
- **Overview of the methods.** We used one mitochondrial (Giresse et al. 2010) and 13 nuclear DNA markers (Giresse et al. 2006) to genotype a total of 146 samples collected in France. The data were analysed by using classical *F*-statistics and by performing bayesian clustering and discriminant principal component analyses.
- **Key results.** The frequency of different races in France was found to be highly variable across sunflower growing regions with a significant positive correlation between race and geographic location. Samples of the same race were genetically similar and multiple matching genotypes were found among samples of more commonly found races. Cluster analyses using unique genotypes only, revealed that the samples form three distinct groups that are genetically differentiated ( $F_{ST}$  0.65) and characterized by high inbreeding coefficients ( $F_{IS}$  0.78). We believe this infers at least three separate introductions of *P. halstedii* into France. Mixed ancestry was suggested for samples of more recently emergent races of which six of these are endemic, suggesting that they have arisen in situ due to hybridization. Among five samples that had conflicting mtDNA and nuclear DNA profiles, three belonged to these new endemic races, further indicating that admixture events are important for the evolution of new virulence.
- **Main conclusion.** We maintain the hypothesis that three groups represent at least three separate introductions of this pathogen into France and we propose that the increase in virulence observed is a result of admixture between the main races that has led to hybrids with novel virulence.
- **Nature of the contribution to current knowledge.** To our knowledge these data provide the first population-wide picture of the genetic structure of *P. halstedii* in France where the evolution of virulence is of major concern. Further research in order to understand how hybridization of different races of *P. halstedii* occurs would provide valuable information for predicting the rate of future possible race emergence. These issues are not only important for addressing key question in the subject of evolution and adaptation, but also for informing and designing relevant disease management policy.

**Keywords:** Pathotype, race evolution, virulence

## Introduction

The most effective method to control Sunflower downy mildew is introgression in host cultivars of dominant major resistance genes denoted *Pl*. However, the efficacy of sunflower resistant varieties is challenged by the rapid emergence of new virulent races of the pathogen. Race 100 was the only pathotype known in France until race 710 was discovered in central France in 1988, closely followed by race 703 in 1989 in south-western France. A further 11 races able to overcome the *Pl* loci were found over the following years (Figure 1a) in varying numbers (Figure 1b). The different races were found in distinct geographical regions with 710, 704, 714 and 314 predominantly in the central part of France and 703, 307, 300 and 304 found predominantly in south-west France and 100 in both. Among the 14 races found in France, six (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).



**Figure 1.** A. Graph showing number of races found at each point along the time line. The name of each new race found is labelled at the appropriate point. B. Histogram of number of samples collected in each race. The number of samples for each race collected during the 25 years of monitoring of *P. halstedii* in France.

Early studies of *P. halstedii* typically found low levels of genetic diversity using RAPD markers (Komjati et al., 2004), ISSR (Intelmann, 2002) or ITS sequences (Spring & Zipper, 2006). Roekel-Drevet et al. (2003) analysed 77 samples from twelve different countries of six virulence races using 21 RAPD primers and found low levels of differentiation within and between samples grouped by race and country. By contrast, Delmotte et al. (2008) analysed 24 individuals that were reference strains of the 14 races of known in France. Cluster analysis of the genotypes revealed a strong pattern indicating that samples clustered into three groups. However, the limited number of samples in this study provided little information about the relationships among the 14 races, or if genotypes of the same race consistently follow a pattern of strong assignment when assessed in more than one sample. In order to investigate this in more detail we expanded our analyses to include multiple samples (where possible) for each virulence profile and we included mtDNA haplotype data for each sample. Using these and original data obtained by Delmotte et al (2008) on the reference strains, together with the race and spatial information we examined population structure in these samples. We wanted to know if the samples fall into the previously defined clusters demonstrated in reference virulence profiles and if endemic and more recently emerged races follow strong clustering patterns. Do they appear to be newly introduced genotypes from other countries? Or have they arisen from existing races via mutations in standing genetic variation or hybridization?

## Material and methods

**Sampling.** Because *P. halstedii* is a quarantine pathogen in France, ANSES pathologists have monitored the disease in the 21 year period from 1988 to 2008 (Moinard J, 2006) and during that time collected and

identified almost 1200 *P. halstedii* isolates (Figure 1b). All of the samples were collected by harvesting infected sunflower leaves taken from one or two plants in each field. The *P. halstedii* spores from these infected leaves were then propagated onto nine different host lines in order to determine the virulence profile (race) of each sample. 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.

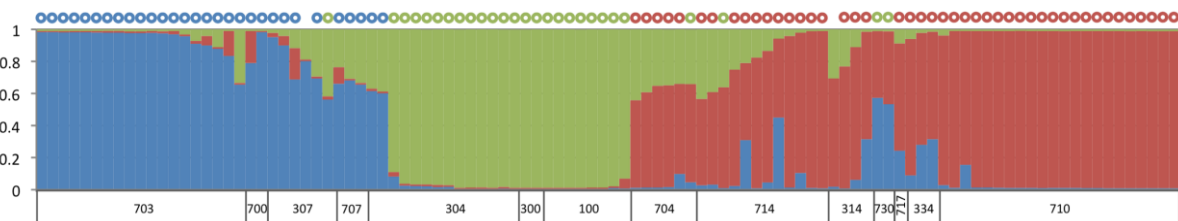
Race phenotyping details. Race phenotyping was determined according to the ability of sporangia to infect differential lines of resistant sunflowers. Resistance tests were performed as described by Cohen and Sackston (1974).

Genotyping. A subset of 147 samples were genotyped at twelve EST derived SNP nuclear markers (Giresse et al., 2007) and one microsatellite marker (Genbank accession number JF914932). 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.

## Results

From the total of 147 isolates, there were 98 different multi locus genotypes (MLG) of which 84 were found only once, and 14 were represented by two or more samples. Multilocus genotypes were not unique to a particular race: for example races 100 and 304 shared a single genotype among three samples, race 307 had matching genotypes with samples of races 703, 304 matched 100, and 714 matched 710.

We used a single representative of each multilocus genotype for clustering analysis. We were able to detect three main groups within our data set (Figure 2). Cluster 1 included races 100, 300 and 304. A second cluster is comprised of samples belonging to races 300, 304, 307, 700, 703, 707 and 730. Cluster 3 included samples mostly from races 314, 334, 704, 710, 714 and 717. The mitochondrial haplotypes showed a strong association with the three clusters. Therefore isolates of cluster 1 were almost always associated with H1, cluster two samples had H2, and cluster three samples had H3. The global  $F_{ST}$  value for the three clusters was high at 0.65 across all loci ( $F_{IT}$  0.94,  $F_{IS}$  0.78). The observed patterns of differentiation were consistent across loci.



**Figure 2.** Histogram of proportion of membership to each cluster for each unique multilocus genotype. Circles above represent mitochondrial haplotypes. Cluster 1/H1 =green, Cluster 2/H2=blue, Cluster 3/H3=red.

## Discussion

We have demonstrated using a combination of virulence profiles together with nuclear and mitochondrial genotype data that the 14 races fall into three distinct groups. Each group included one of races 703, 710 and 100, and 75% samples showed strong membership in one of the three clusters. This supports our hypothesis that the current diversity of races found in France is a result of at least three successive introductions of the main races 100, 710 then 703 into France.

Our data show that groups one and two appear to be more closely associated with another than the third group in that they shared more alleles and more admixed individuals than did group three. Two of the races were also found to have samples in both cluster one and two, whereas group three races were not found in other groups. Their distributions were found to be spatially structured and this may reflect the initial areas of introduction. Although we did not find a high rate of clonality in our data set, samples within race were genetically similar with a number of matching multilocus genotypes among samples of

the same race. Our population genetic analyses also reveal a highly inbred population denoted by the high global  $F_{IS}$  value (0.78). This value equates to a selfing rate of approximately 88% (where  $F_{IS}=S/(2-S)$ ), or an outcrossing rate of 12%. A quarter of our samples had less than our threshold limit of 70% of ancestry assigned to one or other cluster (ranging 48.1% to 68.9%, mean of 61.3%) and these samples were mainly members of races 304, 307, 704 and 714. These races belong to a group of newly emergent races that have never been recorded outside France and that are capable of overcoming resistance to the Pl6 locus. Therefore, our genotype data also suggested that the races that are unique to France could be the result of hybridization between the main races that first introduced, namely races 100, 710 and 703.

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