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# AFLP markers reveal two genetic groups in the French population of the grapevine fungal pathogen *Phaeomoniella chlamydospora*

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**Abstract** *Phaeomoniella chlamydospora,* (Chaetothyriales, Herpotrichiellaceae) is one of the main causal agents of Petri disease and esca on grapevines. We have used AFLP markers to study the population genetic structure of 74 isolates collected at different spatial scales: 56 isolates originated from vines with esca disease sampled from four French vineyards (Poitou-Charentes, Aquitaine, Languedoc-Roussillon, Alsace); 18 isolates were collected from a single plot (Aquitaine vineyard). Significant linkage disequilibrium indicated that *P. chlamydospora* populations are not panmictic, whereas the level of haplotypic diversity observed, 72 single multilocus haplotypes identified in total among the 74 isolates analysed, suggest that reproduction in this species may not be strictly clonal. Clustering analyses suggests the presence of two genetically differentiated but sympatric clusters of isolates. The level of differentiation between the two clusters is high ($F_{ST}$=0.23) and significant at 13 out of the 21 loci analyzed. The most plausible explanation for this pattern of admixture is the coexistence in *P. chlamydospora* French populations of two predominant clonal lineages. Finally, the low level of spatial genetic differentiation in this study is consistent with the spread of this fungus through the transport of infected plant material by human activities.

**Keywords** AFLP · Esca disease · Fungal plant disease · Population genetic structure

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## Introduction

*Phaeomoniella chlamydospora* (Chaetothyriales, Herpotrichiellaceae) is associated with esca and Petri disease, two of the most important trunk diseases of grapevine, responsible for significant losses, premature decline and dieback in vineyards worldwide (Larignon and Dubos 1997; Borie et al. 2002). Despite the clear implication of *P. chlamydospora* in early stages of grapevine wood disease, the biology and epidemiology of this fungus remain poorly understood. An understanding of its mode of reproduction and the source of inoculum responsible for *P. chlamydospora* dispersal is, however, essential for the efficient management of these diseases in vineyards.

To date, no sexual fruiting bodies of *P. chlamydospora* have ever been found in vineyards. However, sexual reproduction may be transient in vineyards or may occur on currently unidentified alternative host plants. Similarly, the source of *P. chlamydospora* inocula remains unknown. It has been suggested that asexual sporulation occurs on dead wood in the vineyard. Spores of this fungus have also been obtained from the air in vineyards in California and France, indicating that fungal conidia may be dispersed through the air and penetrate the host through pruning wounds (Eskalen et al. 2007). Another potential source of inoculum is the infection of grapevines in nurseries, as *P. chlamydospora* has been reported to infect grapevine rootstocks (Fourie and Halleen 2002). *P. chlamydospora* has been detected in both symptomatic and asymptomatic cuttings (Bertelli et al. 1998) and might therefore disseminate into rootstock canes in the absence of external symptoms. Infection may also take place during the propagation and storage of young plants in nurseries (Bertelli et al. 1998). Consistent with these hypotheses, several recent studies have confirmed the presence of *P. chlamydospora* in Australian and Spanish grapevine nurseries (Edwards et al. 2007).

Population genetics approaches provide useful tools for evaluating the level of recombination in populations of *P. chlamydospora* and gene flow between vineyards. As a first approach, the detection of clonal or partially clonal reproduction within fungal populations can be assessed by the repeated sampling of multilocus haplotypes. Other indicators such as heterozygote deficiency might also help in the detection of shifts in the genetic population structure caused by clonal reproduction (Halkett et al. 2005). However, heterozygote deficiency is not an applicable concept in populations of haploid fungal species such as *P. chlamydospora*. Because clonal dominance may leave a signature of selection with respect to linkage of loci, linkage disequilibrium (LD), i.e. nonrandom associations of alleles at two or more gene loci, can then be used to detect clonal or partially clonal reproduction modes (Halkett et al. 2005). Therefore, population genetic studies not only allow the identification of clonal lineages and the extent of LD, but also allow one to infer population genetic processes such as the admixture of genetically differentiated subpopulations by using individual-based approaches, for example Bayesian clustering methods or multivatiate analyses of genetic data.

Molecular tools have been little used for the analysis of *P. chlamydospora*, probably because it is very difficult to obtain the number of isolates required for population genetic studies. Sampling for this fungus requires the destruction of the plant, because it grows inside the trunk or in the branches of the grapevine. Studies of the genetic variability of *P. chlamydospora* populations have reported a low level of genetic and genotypic diversity, as demonstrated by random amplified polymorphic DNA (RAPD), random amplified micro-and minisatellite (RAMs) and amplified fragment length polymorphism (AFLP) techniques (Borie et al. 2002; Mostert et al. 2006; Pottinger et al. 2002; Tegli et al. 2000). The lack of polymorphism of molecular markers may have reduced the power of the linkage disequilibrium tests used to detect recombination and limited the chances of detecting spatial genetic structure within *P. chlamydospora*. Therefore, this makes it impossible for the authors of these studies to draw firm conclusions concerning the mode of reproduction, spatial genetic structure and relatedness of isolates within this species (Borie et al. 2002). Nevertheless, a comparison of the results obtained for *P. chlamydospora* with different molecular techniques showed that AFLPs revealed the highest level of genetic variability (Pottinger et al. 2002).

In this study, we used AFLP markers and hierarchical sampling to describe the population genetic structure of French populations of *P. chlamydospora*. We addressed three main questions: (1) How genetically diverse is the French *P. chlamydospora* population when assessed with AFLP markers? (2) Does *P. chlamydospora* reproduce strictly asexually? (3) How structured is the population at both the local (plot) and regional (vineyard) scales?

## Materials and methods

### Fungal collection

Isolates of *Phaeomoniella chlamydospora* from esca-diseased vines were sampled from four French regions: Alsace (5), Poitou-Charentes (17), Aquitaine (18) and Languedoc-Roussillon (16) in 1997. Isolates were coded by two letters corresponding to the region name (AL for Alsace, PC for Poitou-Charentes, AQ for Aquitaine, LR for Languedoc-Roussillon) and a

number corresponding to the single-spore isolation (Table S1). Isolates from the Languedoc-Roussillon were sampled by J. P. Péros, isolates from Alsace, Poitou-Charentes and Aquitaine were sampled by P. Larignon. In addition, 18 isolates of *P. chlamydospora* were obtained from a single vineyard in Aquitaine (Medoc localities) in 2005. These isolates were sampled from 18 vines displaying symptoms of esca in a 0.4 ha plot.

Vines with external symptoms of esca were transported to the laboratory for the isolation of *P. chlamydospora*. Vines were cut transversally and small pieces of tissue were cut from the darkened vascular tissues. Diseased pieces of tissue (5 mm) were surface-sterilized by incubation for 1 min in calcium hypochlorite solution (3%, *w*:*v*), then placed on malt agar (3:5, *w*:*w*) and incubated at room temperature. The *P. chlamydospora* isolates were identified on the basis of their morphology, as described by Larignon and Dubos (1997). A single spore was generated from each *P. chlamydospora* isolate to ensure that the individuals obtained on subculture on malt agar were genetically uniform. Disks (5 mm) of single-spore isolates were stored in 1 ml distilled water at 4°C.

## DNA extraction

Mycelia were cultured for two weeks at 22°C on malt-agar medium covered with a porous film (Hutchinson, Chalette/Loing, France) and were then collected by scraping. Mycelia were freeze-dried and DNA was extracted in 400 µl of CTAB and one volume of chloroform/isoamyl alcohol (24:1, *v*:*v*, Sigma, Germany). The suspension was mixed and centrifuged for 10 min at 4°C. The aqueous phase was extracted with 300 µl CTAB and one volume of chloroform/isoamyl alcohol (24:1, *v*:*v*). DNA was precipitated overnight in 75% cold isopropanol at −20°C. The pellet was washed with 70% ethanol and dissolved in 50 µl of ultra-pure water. DNA concentration was estimated by spectrophotometry (UV-1605, Shimadzu, Germany).

## Amplification and sequencing of ITS

The identification of *P. chlamydospora* was confirmed by nucleotide sequencing of the ITS region in a subset of 37 isolates reflecting the different geographic origins of the samples studied. The ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) primers were used to amplify the internal transcribed spacers (ITS) rDNA region, which includes the 3′ end of the small subunit rRNA gene, the first internal transcribed spacer (ITS1), the complete 5.8 rRNA gene, the second ITS (ITS2) and the 5′ end of the large subunit. Polymerase chain reaction was carried out in a reaction volume of 25 µl containing 200 µM of each deoxynucleotide triphosphate, 150 nM of each primer, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl and 1U of *Taq* polymerase. The PCR parameters were set as follows: initial denaturation at 94°C for 4 min, followed by 30 cycles 1 min at 94°C, 1 min at 56°C and 90 s at 72°C, with a final extension period for 10 min at 72°C. PCR products were sequenced, on one strand, by GATC services (Germany). Sequences were checked manually and aligned using the ClustalX 1.83 package. The sequences obtained were compared with the *P. chlamydospora* ITS1 and 2 sequences available from the EMBL international nucleotide database.

## AFLP analysis

For the AFLP analysis, the primary template for preamplification reactions consisted of 250 ng of DNA digested with *Eco*RI and *Tru*I (5 units, Fermentas) for 2 h at 37°C and then ligated for 3 h (0.5 units of T4 DNA ligase, 0.1 pmol EcoRI-adapter, 1 pmol *Tru*I-adapter in a final volume of 20 µl) at room temperature. The ligation products were diluted 1:10 in ultrapure water and 5 µl of the resulting dilution was used for preselective amplification, with *Eco*RI-A and *Tru*I-0 primers (30 ng), 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 1 unit of Taq DNA polymerase (SilverStar™, Eurogentec) and buffer (50 mM Tris-HCl, pH 7.5, 50 mM magnesium acetate, 250 mM potassium acetate) in a total volume of 20 µl. Amplification was performed in an Eppendorf Mastercycler Gradient thermocycler (Eppendorf) with the following cycle profile: 20 cycles of 30 s at 94°C, 60 s at 56°C and 60 s at 72°C. The products of this preamplification step were diluted 1:500 with water and subjected to selective amplification with 30 ng of *Eco*RI-ANN (two additional bases) and *Tru*I-NNN (three additional bases). We added 5 µl of diluted preamplification products to amplification primer

mix, which included 0.2 nM dNTPs, 1.5 mM $MgCl_2$, 0.02 units of *Taq* polymerase and buffer (Eurogentec). Amplification was carried out in a thermocycler, over 36 cycles. The first thirteen cycles consisted of DNA denaturation at 94°C for 30 s, annealing for 60 s at various temperatures and extension at 72°C for 60 s. The annealing temperature for the first cycle was 65°C. After the first cycle, and each of the following 12 cycles, the annealing temperature was decreased by 0.7°C. The remaining 23 cycles, for the completion of amplification, consisted of 30 s at 94°C, 60 s at 56°C and 60 s at 72°C. Following amplification, 10 µl of formamide dye (98% formamide, 10 mM EDTA pH 8.0, and bromophenol blue and xylene cyanol, 10 mM NaOH) was added and the reaction mixture was heated at 95°C for 5 min and cooled on ice. An aliquot of the amplification products was loaded subjected to electrophoresis in a denaturing 6% polyacrylamide gel containing 7 M urea (Euromedex), in TBE buffer (100 mM Tris base, 100 mM boric acid, and 2 mM EDTA, pH 8.0). Electrophoresis was carried out for 2 h at a constant power of 80 W. The gels were then silver-stained as follows. DNA was fixed by incubation with 10% ethanol for 5 min, oxidized by incubation with 1% nitric acid for 3 min, rinsed for 30 s in water and stained by incubation with silver nitrate (1 g/l) for 20 min. The gel was then rinsed twice, each time for 10 min, in sodium carbonate solution (30 g/l).

A total of 66 AFLP primer pairs on a random subset of 10 isolates was tested, with the aim of selecting the most polymorphic combination of primers. Only bands ranging in size from 150 to 600 base pairs (pb) were scored. We retained ten primer combinations (Table 1) with the greatest number of polymorphic bands. The ten primers pairs yielded a total of 268 scorable bands and these were retained for the final genotyping of all *P. chlamydospora* isolates. Of the 268 loci, 216 (81%) were monomorphic and 52 (19%) were polymorphic when tested in a subset of ten isolates. In order to reduce the possibility of scoring errors, we excluded all polymorphic bands that were faint and may therefore have been present but remained undetected. The final set of loci selected had 22 bands which produced the most intense signal after polyacrylamide gel electrophoresis and subsequent staining. The reproducibility of the 22 polymorphic marker bands obtained with the 10 AFLP primer pairs was assessed on subset of 20 samples consisting of two different DNA extracts from each of the same 10 isolates of *P. chlamydospora*. No difference was found for the 22 markers retained between the results obtained in the two independent analyses, demonstrating the reproducibility for the 22 banding patterns that had been selected to analyze the *P. chlamydospora* French isolates.

**Table 1** Primer combinations and sequences of the two or three bases additional to the *Eco*RI (5′GACTGCGTACCAATTC3′) or *Tru*I (5′GATGAGTCCTGAGTAA3′) adaptor used for AFLP analysis

| Primers | *Eco*RI | *Tru*I |
|---|---|---|
| 1 | AA | CGT |
| 2 | AC | CAT |
| 3 | AC | CTA |
| 4 | AC | CGA |
| 5 | AG | CTC |
| 6 | AG | TTA |
| 7 | AG | CAT |
| 8 | AG | CAG |
| 9 | AG | CTA |
| 10 | AT | TTA |

## Genetic data analysis

*AFLP markers* Fragments were scored manually and it was assumed that bands with the same molecular size in different individuals corresponded to homologous loci. AFLP fragments giving a strong signal were scored as binary characters for each isolate: "1" for the presence of the band and "0" for its absence. Polymorphic and monomorphic bands were determined for each AFLP primer pair, but only polymorphic bands were included in the analysis. The resolving power of AFLP markers was evaluated with Multilocus version 1.3 by calculating the percentage of discriminated multilocus haplotypes commensurate to the number of combined loci after 1,000 resamplings.

*Multilocus genotype analyses* We calculated the ratio of distinct haplotypes (G/N) by dividing the number of distinct haplotypes by the total number of isolates. We assessed the likelihood that repeated multilocus haplotypes resulted from asexual reproduction, using GenClone (Arnaud-Haond and Belkhir 2007) to calculate *P*(sex)—the probability of obtaining the observed (or a larger) number of isolates with identical multilocus haplotypes in a panmictic population with the frequency of alleles estimated for the

sample. This index provides some indication of the confidence with which we can assume that isolates with the same multilocus haplotypes result from sexual reproduction.

To compare the ratio of distinct haplotypes obtained in different genetic studies on *P. chlamydospora* that present different sample sizes, we used a rarefaction method following Grunwald et al. (2003). Rarefaction was performed using the software Analytic Rarefaction 1.3 available at http://www.uga.edu/strata/software/. Using this software we calculated the number of expected haplotypes in a random sample corresponding to the smallest sample size of all populations being compared using a rarefaction ($N$=29, Tegli et al. 2000). This provides a corrected ratio of distinct haplotypes (G/N) according to sample size by dividing G obtained with the rarefaction method by the sample size of the smallest population compared ($N$=29).

*Clustering analyses* Isolates were clustered on the basis of their genetic relatedness (rather than their geographic origin). This was achieved by investigating the genetic structure of *P. chlamydospora* using two different individual-based clustering methods, a Bayesian approach and multivariate analyses.

The Bayesian approach to genetic mixture analysis was performed using the software Structure v2.2 (Falush et al. 2003). This method can be used to estimate parameters independently of the posterior probability distribution of allele frequencies. Parameter estimation under the null model of panmixia, where each locus is at Hardy–Weinberg equilibrium and independent of the others is presumed. Nonetheless, this Bayesian approach is robust to some deviations from these assumptions (Falush et al. 2003; Halkett et al. 2005) and only physical linkage of loci can lead to spurious results (Kaeuffer et al. 2007). Therefore, this approach to genetic admixture has been successfully used in partially asexual organisms such as bacteria (Falush et al. 2003), aphids (Halkett et al. 2005), fungal and oomycete plant-pathogens (Delmotte et al. 2008; Barhi et al. 2009; Dutech et al. 2009). Using the admixture model, we estimated the number of genetic clusters, K, between 1 and 5 with ten repeats to which the isolates should be assigned. We performed a burn-in of 100,000 iterations and a run length of $10^6$ iterations following the burn-in. For each run, the natural logarithm (ln) likelihood of each model was calculated.

A principal component analysis (PCA) was performed using the procedure available in the package "adegenet" for the R software (Jombart 2008). PCA has an important advantage over other methods such as the Bayesian clustering approach implemented in Structure v2.2 (Falush et al. 2003), in 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. 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.

*Population genetics summary statistics* Population divergence was estimated using Wrights $F_{ST}$ implemented in Genepop v3.3 (Raymond and Rousset 1995) and by analysis of molecular variance (AMOVA) in Arlequin 2.001 (Schneider et al. 2000). This method was used to partition genetic variance between clusters (as defined by Structure and PCA), between geographic regions within clusters, and within geographic regions. Levels of significance were determined through 1,000 random permutation replicates. The Arlequin software was also used to estimate average gene diversity ($H$) over loci. For haploid species, gene diversity is the probability that two randomly chosen homologous alleles are different in a given population.

We evaluated the evidence for recombination by performing linkage disequilibrium tests. The modified association index $r_d$ (Agapow and Burt 2001) and its distribution in a randomized data set were calculated with Multilocus software. This modified index provides an estimate independent of the number of loci analyzed, making it possible to compare our results with those of other studies (0=random association of alleles; 1=absence of recombination between loci). We assessed the genetic relatedness of multi-locus haplotypes, using the shared allele distance (DAS) to calculate the genetic distance between isolates belonging to the same cluster.

*Maximum parsimony analysis* Maximum parsimony analysis was performed, using the AFLP markers considered to be phylogenetically informative, based on their presence or absence in at least two phenotypes. The number of steps required to resolve the phylogeny of the most parsimonious tree was calcu-

lated with Mix and Consense in Phylip (Phylogeny Inference Package version 3.5c, Felsenstein 1989). The number of "homoplasies" (convergence due to double mutation events) was calculated as the difference between the number of polymorphic loci and the number of steps in the tree generated. These calculations were carried out for the total phylogeny and for the phylogeny inferred for each of the clusters defined by Structure.

## Results

### Haplotypic diversity

Use of the selected combination of 22 genomic markers distinguished 72 different multilocus haplotypes among the 74 isolates analyzed (two repeated multi-locus haplotypes occurred twice). Mean overall haplotypic diversity was high (G/*N*=0.97). Using 15 loci (randomly chosen among the 22 loci) was sufficient to discriminate 90% of the multilocus haplotypes in the dataset, demonstrating the strong resolving power of the AFLP markers used in this study (Fig. 1).

The two repeated multilocus haplotypes corresponded to isolates belonging to different regions: AL217 (Alsace) and PC187 (Poitou-Charentes) and isolates PC31 (Poitou-Charentes) and AQ37 (Aquitaine). No repeated haplotypes were found at local scale in the Médoc vineyard (Aquitaine region). The probability *P* (sex) of the two pairs of isolates sharing identical haplotypes being derived from a sexual reproduction event (as opposed to clonal reproduction) was determined. For the two repeated haplotypes (AL127/PC187 and PC31/AQ37), *P*(sex) was very low, at $1.7\times10^{-4}$ and $4.9\times10^{-5}$, respectively, indicating that these repeated haplotypes are unlikely to have resulted from sexual reproduction events.

We compared AFLP and RAPD resolving power by genotyping 32 isolates previously analyzed with RAPD techniques by Borie et al. (2002). The findings of Borie et al. (2002) reported the presence of 20 haplotypes for this dataset, while we found that each of these 32 isolates had a different haplotype when genotyped with AFLP markers. Moreover, the repeated haplotype that originated from a remote geographic location detected by Borie et al. (2002) was differentiated into different haplotypes with AFLP markers.

Finally, we have compared our results for genotypic diversity with those obtained in previous populations genetic studies performed on *P. chlamydospora* (Table 2). The mean genotypic diversity estimated was 0.54 (ranging from 0.18 to 0.83) using RAPD, and 0.70 with AFLP markers (ranging from 0.51 to 0.99—this study). Table 2 confirmed that the genotypic diversity estimated in this study with the 10 AFLP primers was the highest found until now for *P. chlamydospora*.

Loci 20 and 21 presented a perfect statistical association among the 74 isolates genotyped and we therefore decided to remove one of these loci (locus 20) from the dataset for subsequent analyses.

### Clustering analyses

The Structure output revealed a single splitting solution for *K*=2 when including the entire dataset, using the method described by Evanno et al. (2005).

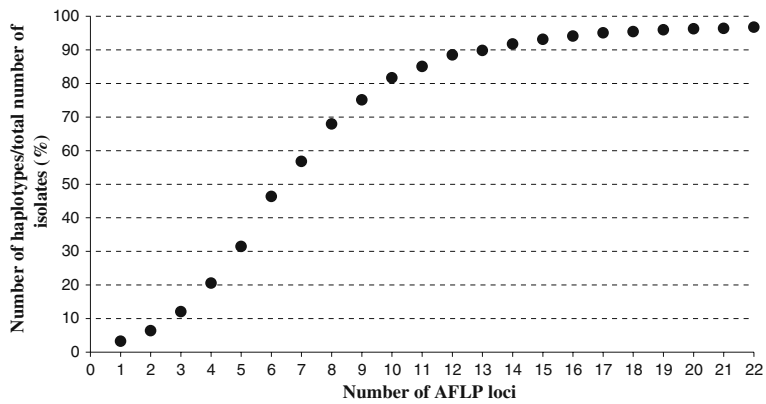


**Fig. 1** Relationship between the percentage of distinct multilocus haplotypes of *P. chlamydospora* found in this study (*N*=74) and the number of AFLP loci randomly assayed

**Table 2** Genotypic diversity obtained in five studies of *Phaeomoniella chlamydospora* genetic structure

| Markers | Geographic origin of isolates | Nb of primers[a] | N[b] | G[c] | G/N[d] | G/N corrected[e] | References |
|---|---|---|---|---|---|---|---|
| RAMS | Italy (27), USA (1), South Africa (1) | 3 | 29 | 9 | 0.31 | 0.31 | Tegli et al. 2000 |
| RAPD | Italy (27), USA (1), South Africa (1) | 4 | 29 | 24 | 0.83 | 0.83 | Tegli et al. 2000 |
| RAPD | France | 6 | 117 | 44 | 0.38 | 0.59 | Borie et al. 2002 |
| RAPD | Intra-vineyard sampling, Charentes, France | 6 | 47 | 20 | 0.42 | 0.54 | Borie et al. 2002 |
| RAPD | New Zeland (39), Italy (6) | 4 | 45 | 6 | 0.13 | 0.18 | Pottinger et al. 2002 |
| AFLP | New Zeland (39), Italy (6) | 2 | 45 | 21 | 0.54 | 0.51 | Pottinger et al. 2002 |
| AFLP | South Africa (63), Italy (9), Australia (5), New Zeland (5), France (3), Iran (1), Slovenia (1), USA (1) | 2 | 88 | 40 | 0.45 | 0.61 | Mostert et al. 2006 |
| AFLP | France | 10 | 74 | 72 | 0.97 | 0.99 | This study |

[a] Number of primers used in the study

[b] Number of isolates analysed

[c] Number of haplotypes

[d] G/N: Number of different haplotypes/N

[e] G/N corrected by rarefaction methods (Grunwald et al. 2003)

Cluster 1 comprised 48 isolates, whereas cluster 2 comprised 26 isolates. The posterior distribution of $q$ for the whole dataset was mostly bimodal, indicating a high level of divergence between the two clusters. Assuming an arbitrary threshold of $q=0.8$ for assignment to clusters, 55 of the 74 isolates (74.3%) were assigned to one of the clusters.

Principal component analysis (PCA) followed by clustering based on Ward's method also revealed two clusters of isolates. Axis 1 and 2 of the PCA accounted respectively for 17.3% and 11.3% of total genetic variability (Fig. 2). Except for four isolates (AQ96, AQ58, AL12 and PC40), the clusters discriminated using the multivariate analysis were in agreement with the clusters inferred using the Bayesian approach (Fig. 2). It is worth noting that these four isolates are typical 'intermediate genotypes" that cannot confidently be assigned to one group or another. Since the Bayesian 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 the Bayesian method.

## Population differentiation

An analysis of the partitioning of molecular variability based on AMOVA revealed that 73.2% of the genetic variability was observed within regions, 23% between clusters (obtained with Structure) and only 3.82% between regions within the same cluster (Table 3). AMOVA confirmed the high level of genetic and genotypic variability of *P. chlamydospora* isolates. It also confirmed the high level of genetic differentiation between clusters: $F_{ST}=0.23$ ($P<0.001$). While no private alleles within clusters were found, a significant differentiation was observed at 13 AFLP loci (over 21), with $F_{ST}$ values of 0.08 to 0.67 (Table 4). By comparison, $F_{ST}$ across geographic regions (Aquitaine, Alsace, Poitou-Charentes, Languedoc-Roussillon), estimated without taking into account assignment to clusters, was an order of magnitude lower ($F_{ST}=0.029$, $P<0.001$). Moreover, $F_{ST}$ within clusters was low: cluster 1 $F_{ST}=0.04$ ($P<0.001$); cluster 2 $F_{ST}=0.05$ ($P<0.001$), excluding populations with fewer than five isolates (i.e. Alsace) and including isolates from the Medoc vineyard in the Aquitaine population (Table 5).

The spatial distribution of clusters was very similar in the four regions: cluster 1 included 61% of samples originating from Aquitaine, 60% of those originating from Alsace, 59% of those originating from Poitou-Charentes and 62% of those originating from Languedoc-Roussillon (Fig. 3). The genetic clusters were also found at metres scale within a single plot. Due to the small number of isolates sampled ($N=18$), we did not carry out a statistical analysis of the spatial

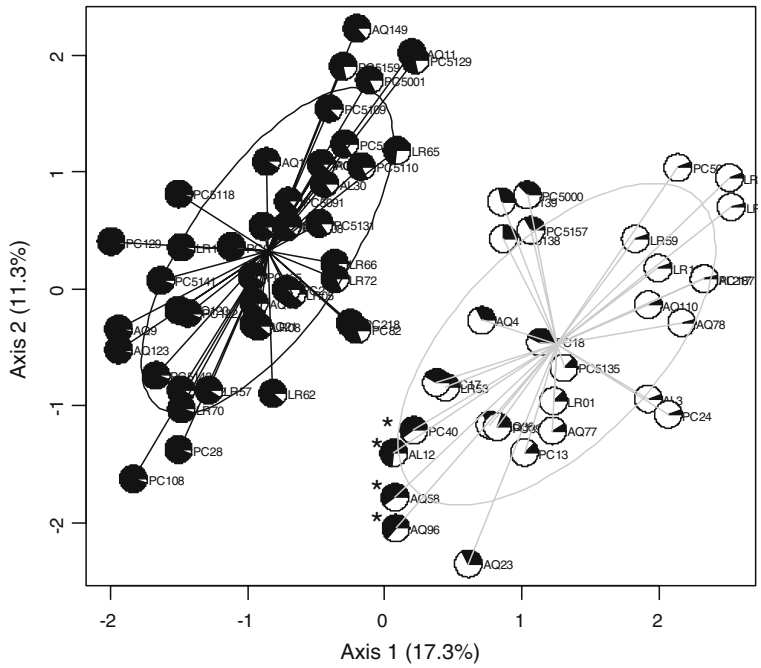


**Fig. 2** Principal component analysis (PCA) performed on the 74 multilocus haplotypes of *P. chlamydospora* based on 21 AFLP markers followed by a non-hierarchical classification with clustering based on Ward method. The percentage of variance explained is indicated under bracket for each axis. The two groups obtained with clustering based on Ward method are represented by black and gray lines on the figure. Ellipses summarizing the scatterplots are centered on the gravity center of the two groups of isolates. In addition, for each *P. chlamydospora* isolate the probability of assignment to each of the two clusters obtained with the Bayesian approach (Structure v2) is reported on the PCA analysis using pie-chart legend: cluster 1=black; cluster 2=white. The four intermediate isolates (AQ96, AQ58, AL12 and PC40) are indicated by an *asterisk*

distribution of genetic clusters in this case, but visual inspection of the results within this plot suggest a lack of spatial aggregation of genetic clusters. Finally, we found no significant relationship between genetic cluster and the cultivar from which the isolates were collected (data not shown).

## Genetic diversity and linkage disequilibrium within clusters

As we identified two genetically differentiated clusters of isolates, we performed intrapopulation genetic analysis for each of these clusters rather than for groups based on the geographic origin of isolates. Within-cluster haplotypic diversity (as measured by G/N) was high, with only one multilocus haplotype repeated once in each cluster (Table 5). The mean genetic distance (DAS) between isolates of the same cluster was 0.206 for cluster 1 and 0.156 for cluster 2, indicating that haplotypes differed by a mean of 4.32 (cluster 1) and 3.28 (cluster 2) alleles, respectively, over the 21 AFLP loci. Within-cluster gene diversity averaged across loci was higher in cluster 1 than in cluster 2 (0.41±0.21 and 0.33±0.17, respectively). It

**Table 3** AMOVA of *P. chlamydospora* haplotypes with genetic clusters consisting of three regions: Aquitaine, Languedoc-Roussillon, Poitou-Charentes. The Alsace region has been removed from this analysis because it included only five isolates

| Source of variation | d.f. | Sum of squares | Variance components | Percentage of variation |
|---|---|---|---|---|
| Among clusters | 1 | 32.95 | 1.186 | 23.0 |
| Among regions within clusters | 4 | 21.82 | 0.197 | 3.82 |
| Within region | 45 | 170.1 | 3.780 | 73.2 |

**Table 4** Frequencies of the positive allele for each locus (L1-L21) in the two clusters of *P. chlamydospora*. $F_{ST}$ between clusters and its associated probability obtained from Genepop, are given for the 21 AFLP loci

| Population | Cluster 1 | Cluster 2 | $F_{ST}$ | $P^a$ |
|---|---|---|---|---|
| L1 | 0.33 | 0.89 | 0.44 | 0 |
| L2 | 0.04 | 0.54 | 0.51 | 0 |
| L3 | 0.44 | 0.58 | 0.01 | 0.122 |
| L4 | 0.06 | 0.04 | −0.02 | 0.713 |
| L5 | 0.48 | 0.15 | 0.18 | 0 |
| L6 | 0.4 | 0.5 | −0.01 | 0.229 |
| L7 | 0.58 | 0.35 | 0.08 | 0.01 |
| L8 | 0.63 | 0.62 | −0.03 | 1 |
| L9 | 0.83 | 0.92 | 0 | 0.207 |
| L10 | 0.5 | 0.15 | 0.2 | 0 |
| L11 | 0.13 | 0.04 | 0.01 | 0.14 |
| L12 | 0.67 | 0.12 | 0.44 | 0 |
| L13 | 0.6 | 0.77 | 0.03 | 0.047 |
| L14 | 0.17 | 0.69 | 0.44 | 0 |
| L15 | 0.35 | 0.31 | −0.03 | 0.591 |
| L16 | 0.69 | 0.92 | 0.12 | 0.001 |
| L17 | 0.5 | 0.92 | 0.3 | 0 |
| L18 | 0.44 | 0.77 | 0.17 | 0 |
| L19 | 0.54 | 0.19 | 0.2 | 0 |
| L20 | 0.77 | 0.04 | 0.67 | 0 |
| L21 | 0.65 | 0.31 | 0.18 | 0 |

[a] *P*: probability for rejecting $H_0$ (the allelic distribution is identical across clusters)

is worth noting that gene diversity is evaluated on the basis of the 21 polymorphic markers selected in the study and that this value would dramatically decrease if we included a random sample of AFLP loci since many of them would be monomorphic. The multilocus index of association ($r_d$) was significantly different from zero in the two clusters, suggesting that there was no random association of alleles in either of these clusters.

All the polymorphic AFLP fragments were phylogenetically informative in the parsimony analysis. We found that 211 steps were required in total for the resolution of any of 100 equally parsimonious trees. Homoplasies (i.e. double mutation events), which were observed for the 21 informative fragments, accounted for 191 of the 211 steps of the consensus tree. The level of homoplasy was 128 steps for cluster 1 and 38 steps for cluster 2.

## ITS sequence variation

Analysis of the internal transcribed spacers (ITS) rDNA region confirmed that the sequences of the 37 isolates belonged to the *P. chlamydospora* species. The sequencing of the 37 isolates revealed the presence of two single nucleotide polymorphisms at positions 385 and 453 over the 590 bp analyzed. This nucleotide polymorphism defined two 'haplotypes', $ITS_A$ and $ITS_B$, which have been deposited in Genbank under accession numbers FJ530942 and FJ530943 (Table 6).

The $ITS_A$ haplotype was identified for 25 of the 37 isolates (isolates AL30, AL217, AQ04, AQ09, AQ21, AQ22, AQ23, AQ36, AQ58, AQ110, LR32, LR59, LR92, PC13, PC18, PC28, PC82, PC187, PC5000, PC5118, PC5129, PC5131, PC5135, PC5138, PC5139). The remaining 12 isolates analysed had $ITS_B$ haplotypes (AL12, AQ10, AQ37, AQ96, AQ120, AQ149, PC97, PC129, PC165, PC218, PC5091, PC5157). The $ITS_A$ sequence was 100% identical to AF197973, and $ITS_B$ was 100% identical to AF197986, both these accessions corresponding to the ITS of *Phaeoacremonium chlamydosporum*, which was recently renamed *Phaeomoniella chlamydospora*.

**Table 5** Number of isolates (N), number of multilocus haplotypes (G), G/N, mean intra-cluster genetic distance, gene diversity across loci and haplotype linkage disequilibrium in the two clusters of *P. chlamydospora*

| Population | *N* | *G* | *G/N* | Intra-cluster mean DAS (*SD*) | *H* (*SD*) | $r_d$ (*P*) | $F_{ST}$ between geographic regions within cluster |
|---|---|---|---|---|---|---|---|
| Cluster 1 | 48 | 47 | 0.98 | 0.206 (*0.056*) | 0.41 (*0.21*) | 0.010 (*0.001*) | 0.04 (*0.001*) |
| Cluster 2 | 26 | 25 | 0.96 | 0.156 (*0.053*) | 0.33 (*0.17*) | 0.016 (*0.012*) | 0.05 (*0.001*) |

*N*: number of isolates; *G*: number of multilocus haplotypes; *DAS*: allele shared distance; *H*±*SD*: average diversity calculated over the 21 polymorphic loci retained in the study; $r_d$: haplotype linkage disequilibrium (LD) with locus 20 removed; *P*: probability for rejecting $H_0$ (absence of LD)

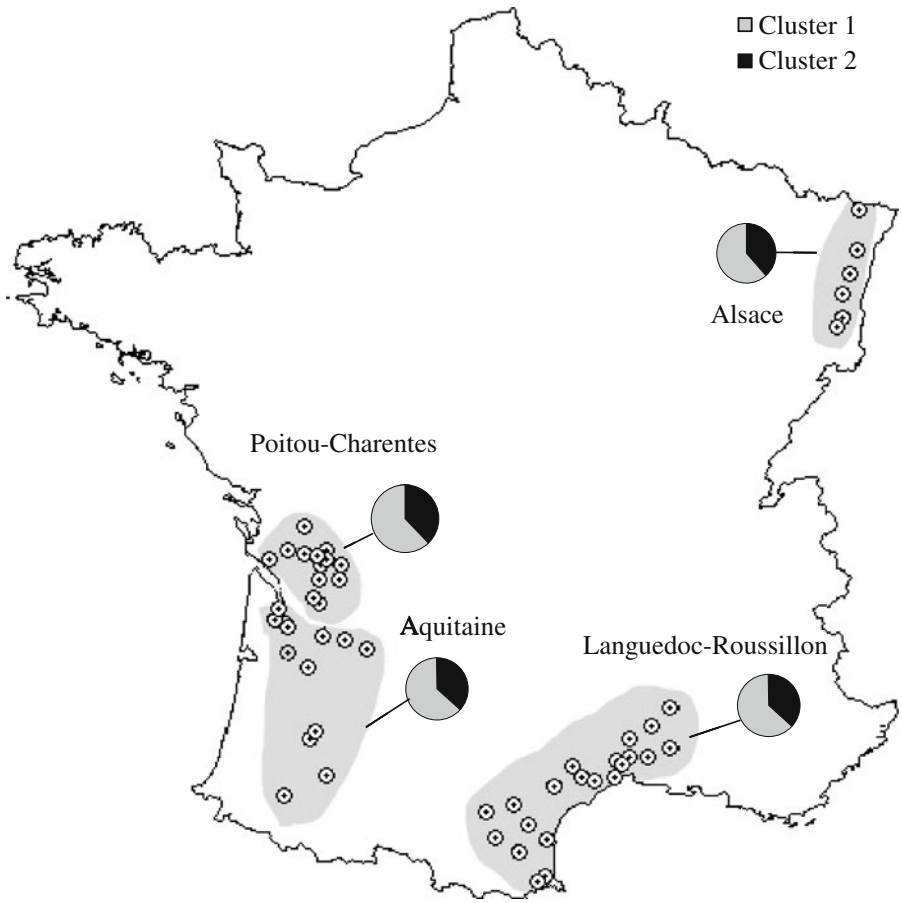


**Fig. 3** Geographic distribution of the French *Phaeomoniella chlamydospora* isolates analyzed with the 21 AFLP markers. The results of the Bayesian analysis are reported on the map: in *gray*, isolates assigned to cluster 1, in *black*, isolates assigned to cluster 2

Twenty of the 37 isolates, belonged to cluster 1 and 17 belonged to cluster 2. The frequencies of $ITS_A$ and $ITS_B$ differed considerably between clusters. The isolates of cluster 1 presented both $ITS_A$ and $ITS_B$ sequences, in similar proportions (45% $ITS_A$, 55% $ITS_B$). By contrast, all but one of the cluster 2 isolates had an $ITS_A$ haplotype (94%). Given the frequencies of $ITS_A$ and $ITS_B$ sequences in each of the two clusters, we estimated the $F_{ST}$ between clusters at 0.42, which was highly significant ($P<0.001$). This result confirms the strong genetic differentiation already observed between clusters with AFLP markers (Table 5).

## Discussion

In the absence of other available species-specific markers, we have used AFLPs to genotype our samples as these are known to generate numerous markers in many species with relative ease. However the final number of polymorphic AFLP bands we found is relatively low and this suggests that the level of polymorphism in *P. chlamydospora* is limited. Our analysis of French populations of *P. chlamydospora* has revealed significant population substructure and significant deviations from expectations in a panmictic population. Yet, the level of diversity observed in haplotype data could indicate rare recombination events within and, to a lesser extent, between genetic clusters. Integrating all our findings, these patterns of genetic variation suggest several lines for future genetic studies of *P. chlamydospora*.

### Evidence for genetic admixture

Clustering analyses (PCA and Bayesian) identified two highly differentiated genetic clusters. The substructuring of the French *P. chlamydospora* population is further illustrated by the high global $F_{ST}$ among clusters ($F_{ST}=0.23$, $P<0.001$) which is underpinned by significant allelic differentiation at 13

**Table 6** Alleles ($IST_A$, $ITS_B$) of the ITS region identified in 37 *P. chlamydospora* isolates collected in France. The number and the frequency of the two ITS alleles are given for each cluster

| | $ITS_A$ | $ITS_B$ |
|---|---|---|
| Accession | FJ530942 | FJ530943 |
| SNP[a] 385 | A | T |
| SNP[a] 453 | C | T |
| N[b] | 25 | 12 |
| Fq[c] in cluster 1 | 0.44 | 0.56 |
| Fq[c] in cluster 2 | 0.94 | 0.06 |

[a] SNP: Single Nucleotide Polymorphism

[b] N: number of isolates sequenced

[c] fq: frequency of isolates

AFLP loci. The isolates assigned to each cluster were distributed evenly between the four geographic regions (Aquitaine, Languedoc-Roussillon, Poitou-Charentes, Alsace) and the two genetic clusters were found to occur in sympatry, within a grapevine field. The presence of significant genetic subdivision within the several geographically isolated vineyards could be explained by admixture of isolates from genetically differentiated clonal lineages (i.e. the asexual progeny derived from a meiotically produced individual). According to this interpretation, the two defined subgroups would correspond to an established asexual lineage intermingled with individuals derived from another asexual lineage that were recently admixed. Providing that sexual reproduction is a rare and episodic event in *P. chlamydospora*, such admixture processes are expected to maintain their signature in populations for several generations.

Our results revealed that recombination events between the divergent clusters were infrequent, since only a few genotypes could be interpreted as intermediate genotypes among the two main clusters. However, intermediate haplotypes may suggest possible recombination events between isolates from the different genetic clusters, and this is discussed below. If sympatric clonal lineages are able to interbreed, then the maintenance of the differentiation between clusters needs to be addressed. The genetic isolation of clusters could then result from prezygotic isolation, e.g. mating preferences for the same cluster, or from a postzygotic isolation, e.g. lower fitness of recombinant isolates. Additional work is thus needed to explore the proximal factors explaining such a low rate of recombination between individuals of the genetic clusters identified in France.

The detection of genetic admixture of clonal lineages in *P. chlamydospora* came from the resolution of the markers used and the power of the clustering analyses carried out. Indeed, for fungal species for which the range of the population is difficult to assess, individual-based methods are powerful tool for the clustering of isolates in the absence of assumptions about the spatial scale at which gene flow occurs. Previous studies on *P. chlamydospora* may have failed to highlight genetic admixture precisely because isolates were grouped as a function of geographic origin rather than genetic relationships (Borie et al. 2002; Mostert et al. 2006; Tegli et al. 2000). Moreover, genetic relationships were analyzed using distance-based clustering that suffer from many disadvantages in that they are heavily dependent on both the distance measure and graphical representation chosen. Additionally, admixed populations that interbreed among differentiated clusters may lead to hybrids that have intermediate positions within a tree. This pattern may sometimes obscure genetic structure and hamper the identification of the source populations, where subtle structure and cryptic gene flow may be important components of fungal plant-pathogen populations.

It is worth noting that this observation of genetically differentiated clusters in *P. chlamydospora* is relevant to an emerging issue in molecular plant pathology, as many fungal diseases of plants have been shown to be caused by a "cloud" of closely related lineages (Crous 2005). There are indeed several other documented examples of asexual plant pathogens consisting of genetically differentiated clusters, such as *Phythophtora ramorum* (Goss et al. 2009), *Puccinia striiformis* Barhi et al. 2009, *Cryphonectria parasitica* (Dutech et al. 2009). Future studies will need to address the genetic or ecological factors responsible for the generation of genetic differentiation within the French population of *P. chlamydospora*. In addition, the discovery of genetically differentiated lineages with high levels of phenotypic similarity raises important questions about their spatial and temporal dynamics. Studies are required to investigate the epidemiological aspects of the diseases caused by each lineage, with the aim of assessing possible differences in virulence.

*How clonal is P. chlamydospora?* This study has revealed a high level of haplotype diversity in the French *P. chlamydospora* population. Despite the different types of markers used (RAMS, RAPD, AFLP), previous studies reported low levels of haplotype diversity in Italian, South African, New Zealand and French populations (0.18 to 0.61, Borie et al. 2002; Mostert et al. 2006; Pottinger et al. 2002), with the notable exception of the study by Tegli et al. (2000), which reported a haplotype diversity of 0.83 using RAPD markers (see Table 2). This highlights the possible underestimation of haplotype diversity in this species in previous studies, likely resulting from the low resolving power of RAPD markers (Borie et al. 2002) or the too few number of primers used in AFLP studies (Pottinger et al. 2002; Mostert et al. 2006). Our results thus provide new insights into the mode of reproduction of *P. chlamydospora*. The significant index of association indicates that the French *P. chlamydospora* population is not panmictic. This finding is reinforced by the difficulty demonstrating the presence of linkage disequilibrium where sample sizes are small and also the presumed predominance of clonal reproduction in this species (Tegli et al. 2000). This result is consistent with the findings of Tegli et al. (2000) and Smetham et al. (2008) who also reported significant linkage disequilibrium in European and Australian *P. chlamydospora* populations. Although we rejected the hypothesis of a panmictic French *P. chlamydospora* population, it should be stressed that caution is required in the interpretation of association index (Maynard Smith 1993). Indeed, linkage disequilibrium may also be influenced by physical linkage between loci, epigenetic effects, and admixture of differentiated genetic isolates, rapid population expansion and bottlenecks. As this fungus is thought to be propagated via rootstocks, we cannot exclude the possibility that demographic fluctuations also contribute to the genetic disequilibrium detected in *P. chlamydospora* populations.

Populations of strictly asexual species are commonly found to have low levels of genotypic diversity (Halkett et al. 2005). By contrast, we observed a high level of haplotype diversity, as only two isolates were found to represent one identical haplotype in this dataset of seventy six samples. Under conditions of strict clonal reproduction, both a large effective population size combined with high levels of gene flow would be required to account for such haplotype diversity. Assuming that new haplotypes arise by successive accumulation of mutation in a diverging clonal lineage (strict clonality of *P. chlamydospora*), our results from the parsimony analyses are not consistent with these expectations. Our detection of sexual recombination must be treated with some caution as we are not able to completely eliminate the possibility of size homoplasy Sexual recombination in the field has not generally been seen as a major reproductive mechanism in *P. chlamydospora,* as the teleomorph of this species has not yet been found in natural conditions. However, *P. chlamydospora* would not be the first fungal species to be presumed to be asexual but subsequently shown to have a cryptic sexual phase. Indeed, population genetic analyses are increasingly providing evidence of sexual reproduction in ascomycete fungi previously thought to be strictly asexual, such as *Coccidioides immitis* (Burt et al. 1996) and *Botrytis cinerea* (Giraud et al. 1997). Moreover, based on a distribution of *MAT* genes consistent with sexual reproduction, recombination has also been documented in clonal species of fungi, such as *Rhynchosporium secalis* (Linde et al. 2003), *Fusarium* spp. (Kerenyi et al. 2004) and *Aspergillus* spp. (Paoletti et al. 2005). Partial recombination due to parasexuality might also allow the exchange of genetic material between isolates without sexual reproduction (see Milgroom et al. 2009, for an evidence of parasexuality in natural population of *C. parasitica*). For *P. chlamydospora*, this hypothesis could be supported by the observation that different haplotypes of *P. chlamydospora* belong to the same mycelial compatibility group (Pottinger et al. 2002). The presence of a single mycelial compatibility group in different isolates of *P. chlamydospora* would favour anastomosis followed by a parasexual cycle. In any case, the role of genetic recombination in the life cycle of *P. chlamydospora* populations requires further investigation to distinguish between cryptic sexual reproduction and somatic recombination.

Evidence for recombination in *P. chlamydospora* as is inferred above must be further elucidated by the use of other markers. Alternate explanations such as the combination of large effective population size with the occurrence of size homoplasy in AFLP markers may indeed be contributing to the main patterns of haplotype diversity observed in our data (Caballero et al. 2008). Validating this hypothesis

would require one to confirm the results of haplotype diversity using species specific markers that are expected to present a low level of homoplasy at the intraspecific evolutionary scale.

## Gene flow within clusters

Our data provide evidence of an absence of significant geographic structuring of the *P. chlamydospora* population, at both the regional (vineyards) and local (plot) spatial scales. It is difficult to distinguish between historic and contemporary gene flow on the basis of fixation indices but the low level of geographic genetic structure indicates a large effective population size and/or high levels of migration. A high migration rate is consistent with the hypothesis of fungal spread through the transport of infected plant material. Bertelli et al. (1998) detected *P. chlamydospora* in rooted cuttings and suggested that young vines may become infected during the propagation process in grapevine nurseries. This finding has recently been confirmed by several studies in which *P. chlamydospora* was isolated from grapevine nurseries in different countries (Edwards et al. 2007; Retief et al. 2006; Ridgway et al. 2002). Given the presence of the fungus in nurseries, these authors suggested that *P. chlamydospora* might be dispersed over long distances through the transport of infected plants from nurseries. Finally, it remains unclear how the fungus is dispersed at the spatial scale of the plot. Our results could suggest that secondary infections may also be due to the dispersion of sexual spores. However, the dispersion by asexual spores of the fungus that can infect pruning wounds after rain cannot be ruled out, as suggested by Eskalen et al. (2007). The microsatellite markers recently isolated from *P. chlamydospora* (Smetham et al. 2008) will be required to distinguish between these hypotheses by carrying out assignment tests on data from a larger number of isolates collected at a fine geographic scale.

In conclusion, this study revealed the existence of genetic admixture, with the presence of two genetically well-differentiated sympatric clonal lineages. Haplotype diversity together with linkage disequilibrium suggests that *P. chlamydospora* may be an asexual species possibly undergoing rare recombination. These data also provide evidence for high levels of gene flow within genetic clusters that are consistent with the spread of this fungus through human activities.

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