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Population genetics of fungi associated with esca disease in French vineyards

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Summary. Among the fungi isolated from vines showing symptoms of esca disease, the ascomycetes *Eutypa lata* (*Ela*), and the hyphomycetes *Phaeoacremonium chlamydosporum* (*Pch*) and *P. aleophilum* (*Pal*) appear to precede the activity of the decay fungus, *Fomitiporia punctata* (*Fop*) which seems to come at the end of the wood degradation process. We carried out studies on the population genetics of these fungi in order to gain more insight into their mode of propagation and life history as well as on their ability to evolve in response to control practices. Random Amplification Polymorphic DNA (RAPD) markers were used to study the genetic relationships among isolates at different geographic levels. Particular attention was given to the diversity existing at vineyard level. Diversity was also studied at regional level and supraregionally among populations from different regions. *Ela* isolates from single vineyards were all of different haplotypes and there was no gametic disequilibrium between RAPD markers. These findings strongly suggested that the population structure of the heterothallic *Ela* is shaped by random mating, being spread only by its ascospores. This population structure was also found at the regional scale with no genetic differentiation among *Ela* populations from different French regions. In *Fop* populations, an exclusive spread by basidiospores seemed to occur and genetic differentiation was very weak. RAPD markers were more difficult to detect for *Phaeoacremonium* species, indicating they had less polymorphism than *Ela* and *Fop*. Several different haplotypes of *Pch* and *Pal* were found at vineyard level, suggesting several outside sources for the primary inoculum. Populations of *Pch* and *Pal* from different regions appeared weakly differentiated. These results indicate that French viticultural regions have to be considered as unique epidemiological unit when considering control measures.

Key words: grapevine, esca, population genetics, *Eutypa lata, Fomitiporia punctata, Phaeoacremonium* spp.

Introduction

Esca of grapevine is a complex disease whose causes are not completely elucidated (Mugnai *et al.*, 1999). Despite very characteristic external symptoms, the analysis of cross sections in the branches and trunks of diseased vines reveals a wide variation in lesion patterns. Several fungi and bacteria are associated with these lesions (Larignon, 1991; Mugnai *et al.,* 1996). Surveys performed in Languedoc-Roussillon, France, revealed that 43% of vines with esca symptoms showed brown lesions caused by *Eutypa lata* (Pers. : Fr.) Tul. & Tul (Jamaux-Despréaux *et al.*, 1997), whereas 33% of vines with eutypa dieback had white rot (Péros *et al.*, 1999) attributed to the basidiomycete *Fomitiporia punctata* (Fr.) Murrill (Mugnai *et al.*, 1999). In addition, the hyphomycetes *Phaeoacremonium chlamydosporum* W. Gams, Crous, M.J. Wingfield & L. Mugnai and *P. aleophilum* W. Gams, Crous, M.J. Wingfield & L. Mugnai have also been frequently isolated from vines showing dieback

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(Mugnai *et al*. 1996; Larignon and Dubos, 1997). *E. lata* (*Ela*), *P. chlamydosporum* (*Pch*) and *P. aleophilum* (*Pal*) were supposed by Larignon (1991) to be the first wood colonizers, while *F. punctata* (*Fop*) come at the end of the degradation process. In this study, isolates of the four fungi most commonly found in vines with dieback (*Ela*, *Fop*, *Pch*, *Pal*) were collected in French vineyards at different geographic levels. The genetic structure of the fungal populations was then analyzed using RAPD (Random Amplified Polymorphic DNA) markers. Such analysis was expected to provide more information on the propagative mode of these fungi and on the genetic differentiation among their populations, and appeared necessary to develop new and durable methods of control.

Materials and methods

Sampling

Isolates of *Ela*, *Fop*, *Pch* and *Pal* were collected in the French regions with different climates: Languedoc-Roussillon (LR, Mediterranean climate); Alsace (AL, temperate); Bordelais (BX, oceanic) and Charentes (CH, oceanic). Populations were sampled either at regional level (Table 1) or at vineyard level (Table 2). Infected vines were cut transversely to look for lesion types A, B, C, E or P as differentiated by Larignon and Dubos (1997). Isolation was performed according to either Larignon and Dubos (1997) or Péros and Berger (1994): small pieces of wood were taken from the margin of the lesion, disinfected in calcium hypochlorite, rinsed and then placed on malt agar or potato-dextrose agar (Difco Laboratories, Detroit,

Table 1. Characteristics of populations of *Eutypa lata* (*Ela*), *Fomitiporia punctata* (*Fop*), *Phaeoacremonium chlamydosporum* (*Pch*) and *P. aleophilum* (*Pal*) collected at regional level.

Region (Code)	No. isolates of				Date isolation[a]	Grapevine cultivar[b]
	Ela	*Fop*	*Pch*	*Pal*		
Region						
Languedoc-Roussillon (LR)	54	40	72	34	1996	Cinsault, Grenache, Carignan
Alsace (AL)	53	8	9	-	1997	Pinot, Riesling, Gewürztraminer
Bordelais (BX)	16	28	18	15	1996	Cabernet-Sauvignon
Charentes (CH)	36	23	18	16	1996	Ugni-blanc
South-West (SW)	-	-	9	-	1996	Baco, Cabernet franc

[a] Populations were obtained by the authors with P. Larignon supplying BX and CH and infected vines from Alsace provided by G. Blaszczyk and G. Cloquemin.
[b] Only the main cultivars are indicated when populations from several cultivars were collected.

Table 2. Characteristics of populations of *Eutypa lata* (*Ela*), *Fomitiporia punctata* (*Fop*), *Phaeoacremonium chlamydosporum* (*Pch*) and *P. aleophilum* (*Pal*) collected at vineyard level.

Region (Code)	Fungus	No. isolates	Date isolation[a]	Grapevine cultivar[b]
Languedoc-Roussillon (LR)	*Ela*	55	1994	Gramon
Charentes (CH)	*Ela*	47	1996	Ugni blanc
Languedoc-Roussillon (LR)	*Fop*	126[c]	1996	Cinsault, Grenache, Carignan
Charentes (CH)	*Phc*	47	1998-99	Ugni-blanc
Charentes (CH)	*Pha*	42	1998-99	Ugni-blanc

[a] See Table 1.
[b] See Table 1.
[c] Corresponding to 40 vineyards with 1-5 isolates per vineyard.

Michigan, USA) plates. Some *Ela* isolates from BX were obtained from a single ascospore, according to Péros and Berger (1994). All isolates were stored as culture disks (5 mm in diameter) in distilled water at 4°C.

RAPD analysis

From *Ela*, *Pch* and *Pal*, DNA was extracted using the CTAB method described by Péros *et al.* (1996). The method was slightly modified for *Fop*, for which fresh mycelium was ground in liquid nitrogen in a chilled mortar followed by incubation in extraction buffer for 1-2 hours. The amplification reactions were carried out according to Péros *et al.* (1996, 1997). One hundred and twenty primers (Bioprobe, Montreuil, France) were screened for reproducible and polymorphic fragments. Amplifications were carried out in a Trio-Thermobloc thermocycler (Biometra, Göttingen, Germany). The analysis was repeated twice from extraction to the electrophoresis of amplification products. Polymorphic and reproducible bands (RAPD markers) were scored as separate putative loci with two alleles, designated 1 for presence and 0 for absence. Jaccard's distance (JD) between isolates was calculated from the binary matrix as $JD = 1 - S_J$, where S_J is the coefficient of similarity defined by Jaccard (1908). In our case, $S_J = [a / (a + b + c)]$, where a is the number of RAPD markers shared by the two isolates, and b and c are the RAPD markers observed in only one of each isolate. The genetic diversity (Nei, 1973) was calculated as the average over all RAPD loci of the quantity $h = 1 - (p^2 + q^2)$, where p and q are the frequencies of the positive allele and the null allele respectively. To test for random mating, the values of gametic disequilibrium among pairs of RAPD loci and their significance were determined using the GENEPOP software (Raymond & Rousset, 1995). Genetic differentiation among populations was studied using Fst, which is the standardized variance in allele frequencies (Wright, 1951), and G^2 which is the likehood ratio chi square. Fst was estimated using GENEPOP, and G^2 using ProcFreq of the SAS package (SAS Institute Inc., Cary, USA).

Results

Diversity in *Eutypa lata*

Seventeen to 32 polymorphic bands (RAPD markers) were used to analyze the genetic structure of *Ela* populations. Nearly all isolates within each local or regional population correspond to different haplotypes, indicating maximum genotypic diversity at that sampling level (Table 3). Moreover there was no gametic disequilibrium between pairs of RAPD markers, suggesting that random mating shaped the genetic structure of *Ela* populations. In the population from Alsace, the average JD between isolates and gene diversity was lower than in other populations (Table 3). Sampling of several populations per region would be necessary to evaluate the significance

Table 3. Genetic structure of six populations of *Eutypa lata* sampled in Languedoc-Roussillon (LR), Charentes (CH), Bordelais (BX and Alsace (AL) respectively.

Population	DG%[a]	No. isolates	No. haplotypes	Mean JD[b]	Hs[c]
LR	3.7	54	53	0.62	0.353
CH	0.7	36	35	0.58	0.345
BX	0.0	16	15	0.54	0.321
AL	2.2	53	51	0.49	0.278
LR vineyard[d]	2.6	55	55	0.64	0.264
CH vineyard[e]	3.9	45	44	0.60	0.340

[a] Percentage of gametic disequilibrium values between RAPD loci significant at $P<0.05$.
[b] Means for Jaccard's distance (JD) was based on (n)(n-1)/2 comparisons, where n was the number of isolates.
[c] Gene diversity corrected for small sample size (Nei and Chesser, 1983).
[d] From Péros *et al.* (1997).
[e] From Péros and Larignon (1998).

of this difference. On the basis of data obtained with the 17 RAPD markers used to analyze the regional populations, the differentiation between populations was very weak. However, the frequency of 4 markers differed between the LR and AL populations, giving a significant G^2 value (Table 4). This corresponded to a very low level of differentiation since the Fst between the two populations was only 0.02. At vineyard level, the genetic differentiation was also not significant (Fst=0.001) between the LR and CH population from single vineyards (Péros and Larignon, 1998).

Diversity in *Fomitiporia lata*

We selected 9 primers to amplify 34 RAPDs markers. All isolates corresponded to different haplotypes, indicating maximum genotypic diversity. In particular, *Fop* isolates from the same vineyard in the LR region were distinct haplotypes. Moreover, the number of significant gametic disequilibrium values was very low, suggesting random mating. Differentiation among populations was also very low. Although 7 markers showed significant differences between LR and the combined BX-CH populations (Table 5), this corresponded to a Fst value of only 0.009 over all the 34 RAPD markers.

Diversity in *Phaeoacremonium chlamydosporum*

RAPDs were more difficult to find in *Pch* and only 7 markers obtained with 6 primers could be used to compare isolates of regional populations. Among the 126 isolates, these markers identified 48 haplotypes. Isolates with identical RAPD patterns were found in different regions. The percentage of significant disequilibrium values calculated for the 48 haplotypes was about 10% indicating that sexual recombination occurred in this fungus. A higher percentage would be expected in case of asexual reproduction alone. Data did not show any differentiation among populations (Table 6). Six RAPD markers identified 20 haplotypes among 47 isolates that were collected in a single vineyard, indicating several inoculum sources at this low spatial scale. This population was not differentiated from the regional populations.

Diversity in *Phaeoacremonium aleophilum*

RAPDs were also difficult to find in *Pal*. Eight markers, detected with 5 primers, were used to compare the regional samples. Among the 65 isolates, the markers distinguished 23 haplotypes. Isolates with identical RAPD patterns were found in different regions. Population LR was differentiated from populations BX and CH (Table 7). Some of the markers identified using a subset of isolates from the LR region showed no polymorphism in the BX and CH regions. As a consequence, the genetic diversity was smaller in the BX and CH regions (Hs=0.19 and 0.23 respectively) than in the LR region (Hs=0.37). Eight RAPD markers (six the same as those used in the regional population analysis) identified 12 haplotypes among 42 isolates collected in a single vineyard, indicating several

Table 4. Genetic differentiation among regional populations of *Eutypa lata* collected in Languedoc-Roussillon (LR), Alsace (AL), Bordelais (BX) and Charentes (CH). The global likelihood ratio chi-square (G^2)[a] is given above the diagonal and the number of RAPDs for which a significant difference in allele frequency exists below the diagonal.

Population	LR	AL	BX	CH
LR	-	39.59**	22.03	20.46
AL	4	-	17.82	25.65
BX	1	0	-	12.38
CH	1	1	0	-

[a] Significant at $^{**}P<0.01$

Table 5. Frequency of RAPD markers showing differences (out of a total of 34 markers) and differentiation between *Fomitiporia punctata* populations from Languedoc-Roussillon (LR) and South-West (CH and BX populations were combined).

RAPD	Population (No. isolates)		G^{2a}
	LR (40)	SW (51)	
A08-1400	0.80	0.61	4.00*
A08-950	0.13	0.31	4.72*
A11-1100	0.33	0.10	7.35**
B18-470	0.65	0.94	13.03**
E16-1500	0.75	0.55	4.00*
E18-420	0.73	0.98	14.07**
P15-290	0.40	0.18	5.63*

[a] Likelihood ratio chi^2, significant at $^{*}P<0.05$, $^{**}P<0.01$. Only RAPDs showing differentiation are shown.

Table 6. Frequency of seven RAPD markers and differentiation in *Phaeoacremonium chlamydosporum* populations from Languedoc-Roussillon (LR), Bordelais (BX), Charentes (CH), South-West (SW), and Alsace (AL).

RAPD	Population (No. isolates)					G^{2a}
	LR (72)	BX (18)	CH (18)	SW (9)	AL (9)	
A8-2070	0.64	0.67	0.56	0.22	0.56	6.35ns
A11-270	0.35	0.33	0.50	0.67	0.33	4.63ns
C8-1100	0.69	0.78	0.56	0.67	0.78	2.49ns
E20-1510	0.86	0.94	0.94	0.89	0.89	1.88ns
E20-850	0.64	0.61	0.50	0.67	0.78	2.27ns
O15-1700	0.53	0.50	0.33	0.44	0.56	2.45ns
O16-1600	0.54	0.44	0.56	0.22	0.33	4.88ns

[a] Likelihood ratio chi^2, non significant at $P>0.05$.

inoculum sources for this vineyard. The population from a single vineyard situated the CH region appeared differentiated from the LR population but not from the combined BX-CH population.

Discussion and conclusions

Using RAPD markers, the analysis of *Ela* and *Fop* population structures in France revealed similar features in the two fungi. Their genetic diversity appeared very large whatever the sampling level, and no gametic disequilibrium was evidenced between RAPD putative loci, indicating that sexual recombination shaped the population structure. Ascospores and basidiospores issued from random mating thus appear to propagate *Ela* and *Fop* respectively. Heterothallism in *Ela* was recently demonstrated in French and Australian vineyards by an analysis of natural progenies with RAPD markers (Péros and Berger, 1999). Cortesi *et al.* (2000) also found very wide diversity and outcrossing in *Ela* populations from single vineyards in Italy and Germany based on vegetative-compatibility grouping. In Italy, the basidiospores of *Fop* also appeared to propagate fungus, as indicated by vegetative incompatibility types and spatial distribution of diseased vines (Cortesi *et al.*, 2000). Thus all populations analyzed for these two fungi currently present the same characteristics. RAPDs were more difficult to find within *Pch* and with *Pal,* for which the intraspecific diversity appeared less pronounced than with *Ela* and *Fop*. The lack of sizeable gametic disequilibrium indicated that sexual reproduction probably occurred in *Pch*, although the sexual stage is unknown. Moreover, analysis at the vineyard level indicated several sources of inoculum since different haplotypes were distinguished at this small spatial scale. The diversity of *Pal* in the western populations appeared lower than in Languedoc-Roussillon; however, this may be due to the differentiation among regions existing for this fungus and to the fact that RAPD markers were selected using a subset of isolates from the LR region. More markers appear necessary to confirm these preliminary observations on *Pch* and *Pal*.

Table 7. Frequency of eight RAPD markers and differentiation in *Phaeoacremonium aleophilum* populations from Languedoc-Roussillon (LR), Bordelais (BX) and Charentes (CH)

RAPD	Population (No. isolates)			G^{2a}
	LR (34)	BX (15)	CH (16)	
A8-2780	0.56	0.67	0.88	5.39ns
D20-940	0.18	0.13	0.13	0.29ns
O15-850	0.21	0.07	0.00	6.57*
O15-540	0.24	0.07	0.06	3.88ns
P1-1380	0.82	1.00	1.00	8.33*
P1-890	0.71	0.73	0.63	0.48ns
P15-1640	0.21	0.00	0.13	5.65ns
P15-810	0.38	0.33	0.31	0.27ns

[a] Likelihood ratio chi^2, non significant at $P>0.05$, significant at $*P<0.05$, $**P<0.01$.

With the four fungi analyzed, there is evidence for low-level genetic differentiation among populations sampled in different French regions, which thus seem to constitute one epidemiological unit. As stated by Milgroom and Lipari (1995), interpreting data on genetic population structure in fungal pathogens is difficult because current levels of gene flow cannot be distinguished from historical effects. The lack of differentiation revealed in this study may be due either to a current gene flow with sufficient unrestricted transport of spores, or to the fact that the populations have not had sufficient time to diverge since their geographic separation.

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