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A hypothesis about the reproductive modes of *Phaeoacremonium aleophilum* and *Phaeomoniella chlamydospora*

Stefania Tegli

Dipartimento di Biotecnologie Agrarie – Patologia Vegetale
Università di Firenze, Piazzale delle Cascine 28, 50144 Firenze, Italy

Summary. The mode of reproduction of *Phaeomoniella chlamydospora* (syn.: *Phaeoacremonium chlamydosporum*) (*Pch*) and *Phaeoacremonium aleophilum* (*Pal*), two mitosporic fungi involved in producing symptoms of esca and a decline of young grapevines, was studied by analysing the amplification profiles obtained in RAPD- and RAMS-PCR experiments (RAPD= Random Amplified Polymorphic DNA; RAMS= Random Amplified Micro- or Mini-Satellites). A low level of polymorphism was found in both species, but no relationship was detected between the genetic variation of isolates and their geographic origin. However, greater variation was found in *Pal* than in *Pch* isolates, and more with the RAPD than with the RAMS experiments, as shown by the percentage of polymorphic fragments and multilocus genotypes obtained. The clonal fraction of the *Pal* and *Pch* populations, as well as the gametic disequilibrium tests suggested the existence of sexual reproduction in both these species; nevertheless the contribution of asexual reproduction to the genetic structure of the natural populations of *Pch* seemed to be greater than that of sexual reproduction. With *Pal* the opposite is thought to be the case.

Key words: *Phaeoacremonium aleophilum*, *Phaeomoniella chlamydospora*, gametic disequilibrium, RAPD, RAMS.

Introduction

In a previous work on genetic variation in *Phaeomoniella chlamydospora* (*Pch*) Crous & W. Gams (syn.: *Phaeoacremonium chlamydosporum*) W. Gams, Crous, M.J. Wingf. & L. Mugnai, and in *Phaeoacremonium aleophilum* (*Pal*) W. Gams, Crous, M.J. Wingf. & L. Mugnai (Crous *et al.*, 1996), two mitosporic fungi associated with some of the most important grapevine diseases, esca and young grapevine decline (Ferreira *et al.*, 1994; Morton, 1995, 1997; Scheck *et al.*, 1998; Graniti *et al.*, 1999; Mugnai *et al.*, 1999), a fairly high degree of homogeneity was found among the genotypes of these fungi, especially *Pch*, as would be expected if these fungi reproduced asexually (Tegli *et al.*, 2000). By contrast, no relationship was ever found between genotypic variation and the geographic origin of the isolates examined, even though a strictly asexual fungal species generally differentiates into clones by means of mutations and according to a particular geographic distribution (Koenig *et al.*, 1997; Zhan *et al.*, 1998). The absence of spatially aggregated clones of *Pch* and *Pal* makes probable two hypotheses, which are not mutally exclusive, and which may hold true for both fungi: i) these fungi spread over long distances with grapevine propagation material, so that there can be multiple sources of infections even in the same vineyard; ii) despite

To correspond with the author:
Fax: +39 055 354786 / 4209208
E-mail: tegli@ipaf.fi.cnr.it

the vascular habit of these fungi, their reproduction is at least in part sexual, recombining the genes of the founder population(s) into new combinations. Unfortunately, up to now no sexual fruiting bodies of these fungi have ever been found and the suggestion that their inoculum has an air-borne dispersal (Larignon, 1999) has been advanced only as a hypothesis.

Knowledge of the genetic variability and mode of reproduction of a phytopathogenic fungus is important to understand the mechanisms that regulate the spread of the disease it causes (Wang, 1997; Zhan *et al.*, 1998; DeScenzo *et al.*, 1999; Et-touil *et al.*, 1999; Toda *et al.*, 1999). Molecular markers as given by Random Amplified Polymorphic DNA (RAPD) (Péros *et al.*, 1996; Delye *et al.*, 1997; Péros *et al.*, 1997; Sicard *et al.*, 1997; Gonzales *et al.*, 1998) and Random Amplified Micro- and Mini-Satellites (RAMS) (Stenlid *et al.*, 1994; Zietkiewicz *et al.*, 1994; Bridge *et al.*, 1997; Hantula and Müller, 1997; Hantula *et al.*, 1997; Longato and Bonfante, 1997; Czembor and Arseniuk, 1999) have been used for the direct assessment of genetic variation (population structure, genetic and genotypic composition) of haploid organisms because they are easy to handle and allow many polymorphic loci to be identified in a single experiment. With a large number of polymorphic markers it is possible to identify the presence of clones in a population and correlate genetic variations with the mode of reproduction (asexual, sexual, or varying proportions of both), by an analysis of gametic disequilibrium. Gametic disequilibrium is the measure of the association among pairs of alleles at different loci, supposed to be physically unlinked on the chromosome, and ranges from 0 to 100%. In organisms that reproduce only asexually all the loci are completely associated in the progeny, in a state called "gametic disequilibrium" (100% association), whereas in organisms that reproduce sexually the unlinked loci are expected to be randomly associated (0% or a low level of gametic disequilibrium).

The aim of this work was to elucidate the source of genetic variation in *Pal* and *Pch*, and thus to understand their mode of reproduction, independently of the fact that no sexual fruiting bodies for these fungi have ever been found.

Materials and methods

Fungal strains, cultural conditions and DNA extraction

The fungal strains used in this study, the growth conditions and the protocol for the extraction of fungal DNA were as described in Tegli *et al.* (2000). As in that study, to ensure a random distribution the strains tested, were isolated from the wood of esca-diseased grapevine plants in different vineyards or from different plants in the same vineyard, located in 8 Italian Regions.

PCR reactions and data analysis

RAPD- and RAMS-PCR reactions were carried out according to Tegli *et al.* (2000). Amplification profiles were analysed from photographic prints of the electrophoretic gels. Only clear, distinct and reproducible bands were included in the analysis. Bands of the same size were scored as identical. Each distinct and reproducible fragment was deemed to be a putative locus, and two alleles were scored for each locus, 1 for the presence and 0 for the absence of the fragment. A locus was considered polymorphic when the frequency of the more common allele was 0.99 or less. Only polymorphic loci were considered in the analysis. They were combined to form a "multilocus genotype", that is the combination for each isolate of all the possible alleles present, one for each polymorpic locus. Isolates having the same multilocus genotype were assumed to belong to the same clone. The clonal fraction of a species was taken to be the number of isolates that were additional members of the same genotype(s) as a percentage of the total number of isolates considered.

Genotypic diversity was calculated according to Sheldon (Sheldon, 1969) with subsequent modifications (Goodwin *et al.*, 1992), and indicated with *Hs*:

$Hs = -\Sigma P_i \ln P_i / \ln N$

where P_i is the frequency of the ith multilocus genotype in a sample size N. Values for *Hs* range from 0, when all the isolates examined belong to the same genotype, to 1, when each isolate has a different genotype.

Differences among RAPD- or RAMS-multilocus genotypes were determined by genetic distance, using AMOVA (Analysis of MOlecular VAriance) (Excoffier *et al.*, 1992). A distance matrix was generated for all possible pairwise comparisons of dif-

ferent RAPD or RAMS genotypes for each species.

Gametic disequilibrium was calculated according to Weir (1990), on all the isolates for each species, and on a single isolate for each of the multilocus genotypes of a species. Contingency tables were calculated for all pairs of loci, and the probability test for each table (Fisher's exact test) was performed using the Markov chain method (Raymond and Rousset, 1995). The association between loci was considered significantly different if Fisher's test gave a probability less than 0.05.

Computations were performed using Arlequin software (vers.1.1, Schneider *et al.*, University of Geneva, Switzerland).

Results

RAPD analysis

When the data from the four RAPD primers were combined, a total of 122 loci were scored, 78 for *Pal* and 47 for *Pch*, with 3 loci being the same between the two species (Table 1). The percentage of polymorphic loci out of the total number of fragments amplified was higher in *Pal* (65.4%, 51 polymorphic loci out of 78), than in *Pch* (38.3%, 18 polymorphic loci out of 47). The number of polymorphic loci obtained with each RAPD primer varied from 11 to 15 for *Pal*, and from 1 to 9 for *Pch* (Table 1). Examples of the RAPD profiles generated by primer OP-B12 with *Pal* and *Pch* isolates are presented in Fig.1. When genotypes for each species and at each RAPD locus were combined, a total of 15 multilocus genotypes were identified among the 15 *Pal* isolates, and 24 among the 29 *Pch* isolates (Table 2). For *Pal*, each genotype was obviously detected only once (*Hs* = 1), but among the 24 *Pch* multilocus genotypes 21 (87.5%) were detected once and the remaining three more than once, but nevertheless with low frequencies (*Hs* = 0.91), 2 multilocus genotypes being detected twice and 1 genotype four times. In total these 3 more frequently occurring multilocus genotypes comprised 8 isolates, of which 5 (= 8-3) were additional members of the 3 genotypes. Assuming that isolates with an identical multilocus genotype were clones, the clonal fraction of the *Pch* isolates examined in this study was 17.2% (5 out of 29 isolates). No clonal fraction was identified in the *Pal* sample studied.

The Euclidean distance between pairs of genotypes was calculated for all the pairwise combinations among the 15 multilocus genotypes of *Pal* and the 24 of *Pch*. The pairwise genotype comparisons amounted to 105 for *Pal* and 276 for *Pch*. A graphic depiction of the distance matrix obtained for each species is shown in Fig. 2. As regards *Pal* combinations, no pair of multilocus genotypes differed by 1 or 2 fragments. Only 1.9% of the combinations differed by 3 fragments, (*i.e.* 5.9% of 51 loci). Most genotypes were very different from each other, and for example 54.3% differed by 20-30 fragments (39.2-58.8% of 51 loci). With *Pch*, 2.2% of the pairs of genotype combinations differed by 1 fragment (5.5% of 18 loci). About 63.3% of combinations differed by 5 to 9 fragments (27-50% of 18 loci), while the maximum observed difference was 13 fragments (72.2% of 18 loci), which occurred in about 1.8% of possible combinations.

Table 1. Total number of fragments, number and percentage of polymorphic fragments, as amplified by four RAPD primers from isolates of *Phaeoacremonium aleophilum* and *Phaeomoniella chlamydospora*.

Primer	*Phaeoacremonium aleophilum* (15 isolates)			*Phaeomoniella chlamydospora* (29 isolates)		
	Total no. of fragments	No. of polymorphic fragments	% of polymorphic fragments	Total no. of fragments	No. of polymorphic fragments	% of polymorphic fragments
OP-B 12	20	15	75.0	18	7	38.9
OP-B 14	21	13	61.9	5	1	20.0
OP-B 18	18	12	66.7	12	1	8.3
OP-B 19	19	11	57.9	12	9	75.0
Total	78	51	65.4	47	18	38.3

Table 2. Number (n) and frequency (P) of multilocus genotypes occurring (x) times and detected in *Phaeoacremonium aleophilum* and *Phaeomoniella chlamydospora* with RAPD and RAMS experiments. For each species and primers, the genotypic diversity (Hs) is shown. N = sample size.

Fungal species	Molecular marker	Genotypes occurred x times x_i	No. of genotypes occurred x times n_i	Frequency of genotypes occurred x times P_i
Phaeoacremonium aleophilum	RAPD			
		1	15	1/15
		$N = \mathrm{S}\, x_i\, n_i = 15$	Total no. of genotypes = 15	$Hs = 1$
Phaeomoniella chlamydospora	RAPD			
		1	21	1/29
		2	2	2/29
		4	1	4/29
		$N = \mathrm{S}\, x_i\, n_i = 29$	Total no. of genotypes = 24	$Hs = 0.91$
Phaeoacremonium aleophilum	RAMS			
		1	13	1/15
		2	1	2/15
		$N = \mathrm{S}\, x_i\, n_i = 15$	Total no. of genotypes = 14	$Hs = 0.97$
Phaeomoniella chlamydospora	RAMS			
		1	3	1/29
		2	2	2/29
		3	2	3/29
		5	1	5/29
		11	1	11/29
		$N = \mathrm{S}\, x_i\, n_i = 29$	Total no. of genotypes = 9	$Hs = 0.55$

RAMS analysis

When data from the RAMS primers were combined, 67 loci were identified, 44 for *Pal,* 26 for *Pch,* and three being the same between the two species, at least in terms of size (Table 3). In *Pal* isolates, 32 polymorphic loci were identified (32/44=72.7% of amplified fragments), but only 7 polymorphic loci were found in *Pch* isolates (7/26=26.9% of amplified fragments). The number of polymorphic loci obtained with each RAMS primer varied from 7 to 15 for *Pal*, and from 2 to 5 for *Pch*. No polymorphic fragments were obtained using the M13 primer with *Pch* isolates. Examples of the RAMS profiles generated by primer M13 with *Pal* and *Pch* isolates are presented in Fig. 3.

When genotypes for each species and at each RAMS locus were combined, a total of 14 multilocus genotypes were identified among the 15 *Pal* isolates, and 9 among the 29 *Pch* isolates (Table 2). With *Pal*, one genotype was detected twice, the remaining 13 genotypes (13/14= 92.8%) only once. Therefore the clonal fraction of the *Pal* isolates examined in this study was 6.6%, and it was represented by the isolate that was the additional member of the multilocus genotypes detected two times (1/15=6.6%). The genotypic diversity was calculated to be Hs=0.97. Among the 9 *Pch* multilocus genotypes, 3 (33.3%) were detected once. Of the remaining 6, 1 genotype (11.1%) was represented by 11 isolates, and 5 genotypes were detected

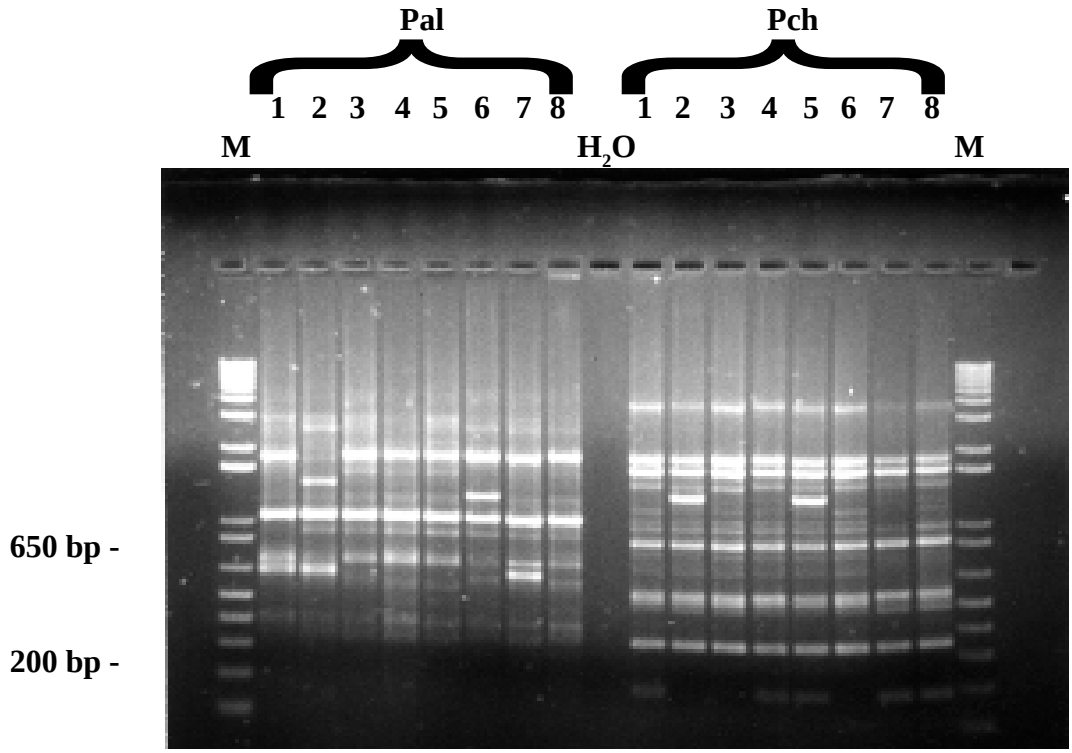

Fig. 1. Gel electrophoresis of the PCR products obtained with primer OP-B12 with *Pal* and *Pch* isolates. Lanes M, 1Kb Plus DNA Ladder (Gibco-BRL, Life Technologies); lane H_2O, negative control with sterile distilled water.

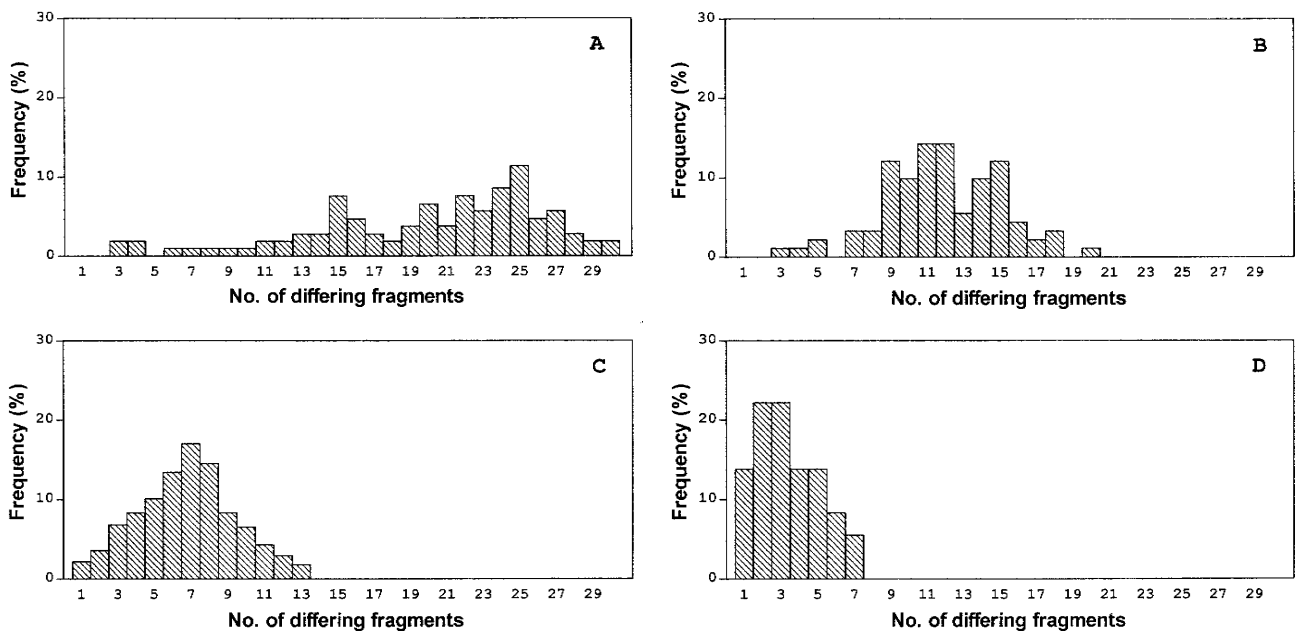

Fig. 2. Frequency of pairwise combinations of multilocus genotypes differing for the number of polymorphic fragments. A and B, *Phaeoacremonium aleophilum* samples, tested with RAPD and RAMS primers respectively; C and D, *Phaeomoniella chlamydospora* samples, tested with RAPD and RAMS primers respectively.

Table 3. Total number of fragments, and number and percentage of polymorphic fragments, as amplified by three RAMS primers from isolates of *Phaeoacremonium aleophilum* and *Phaeomoniella chlamydosporum*.

Primer	*Phaeoacremonium aleophilum* (15 isolates)			*Phaeomoniella chlamydospora* (29 isolates)		
	Total no. of fragments	No. of polymorphic fragments	% of polymorphic fragments	Total no. of fragments	No. of polymorphic fragments	% of polymorphic fragments
CGA	14	10	71.4	8	5	62.5
GT	16	15	93.7	6	2	33.3
M13	14	7	50.0	12	0	0.0
Total	44	32	72.7	26	7	26.9

from 2 to 5 times in the sample, for a total of 15 isolates. In all, these 6 multilocus genotypes comprised 26 isolates, of which 20 isolates (=26-6) were additional members of these genotypes, and so the clonal fraction of the *Pch* isolates was about 68.9% (20 out of 29 isolates). The total genotypic diversity of *Pch,* as revealed by RAMS data, was *Hs* = 0.55.

The Euclidean distance between pairs of genotypes was calculated for all the pairwise combinations among the 14 multilocus genotypes for *Pal* and 9 for *Pch*. The pairwise genotype comparisons amounted to 91 for *Pal* and 36 for *Pch*. A graphic depiction of the distance matrix obtained for each species is shown in Fig. 2. As regards *Pal* combi-

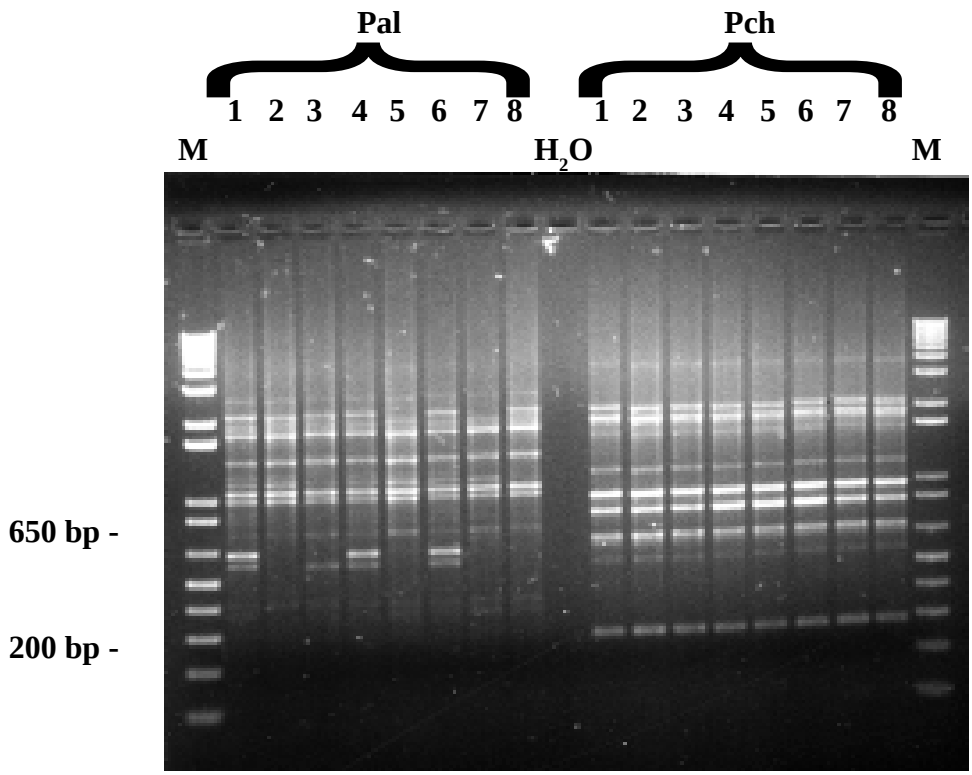

Fig. 3. Gel electrophoresis of the PCR products obtained with primer M13 with *Pal* and *Pch* isolates. Lanes M, 1kb Plus DNA Ladder (Gibco-BRL, Life Technologies); lane H_2O, negative control with sterile distilled water.

nations, no pair of RAMS multilocus genotypes differed by 1 or 2 fragments. Only 1 (i.e. 1/91=1.1% of combinations) differed for 3 fragments (9.4% of 32 loci). About 28.6% of genotypes differed by 11 or 12 fragments (34.5-37.5% of 32 loci), and these combinations were most frequent. The maximum observed difference was 20 fragments (62.5% of 32 loci) and it occurred with about 1.1% of possible pairwise genotype comparisons. For *Pch*, five multilocus genotypes (13.8% of pairwise comparisons) differed by 1 fragment (14.3% of 7 loci). About 44.4% of genotypes differed by 2 or 3 fragments (28.5-42.8% of 7 loci), and these combinations were most frequent. The maximum observed difference was 7 fragments (100% of 7 loci) and it occurred with about 5.5% of possible combinations.

Gametic disequilibrium

Gametic disequilibrium was tested for both species and for all pairwise RAPD and RAMS loci combinations. With *Pal*, among the 51 RAPD polymorphic loci 43 were significantly associated (84.3%) (Table 4). The number of loci combinations was 1275 [51x(51-1)/2] and the pairs of loci significantly associated (Fisher's exact test, $P<0.05$) was 211 out of 1275 (16.5%). The same results were obtained when only a single isolate of each genotype was used in the analysis. Among the 32 RAMS polymorphic loci detected with *Pal* isolates, the number of those significantly associated was 30 (93.8%) when all *Pal* isolates were included, or 32 (100%) when a single representative for each genotype was considered. The 32 polymorphic loci gave rise to 496 possible loci combinations: the pairs of loci having a significant non-random association were 53 (53/496=10.7%) if all *Pal* isolates were included in the analysis, or 75 (75/496=15.1%) if only a single isolate per genotype was included. *Pch* isolates generated 18 polymorphic loci with RAPD and 7 polymorphic loci with RAMS primers, and gametic disequilibrium tests demonstrated that all these loci were significantly associated (Table 4). The loci combinations with RAPD analysis were 153, and among these the pairs of loci with significant association were 55 (55/152=35.9%) when all *Pch* isolates were considered, but only 43 (43/153=28.1%) when the analysis was restricted to a single isolate for each of the 24 multilocus genotypes. The same phenomenon occurred with the 21 RAMS loci combinations: the pairs of loci non-randomly associated were 16 (16/21=76.1%) if the clonal fraction of *Pch* was included in the test, 5 (5/21=23.8%) if it was not.

Discussion

The levels of genotypic and genetic variation of *Pal* and *Pch*, measured as the number of multilocus genotypes and as the genetic distance among multilocus genotypes, respectively, appeared quite different, and the results obtained with RAPD and RAMS primers were perfectly consistent. *Pal* exhibited a greater level of variation than *Pch*, as measured by the higher percentage of polymorphic

Table 4. Total number of polymorphic loci and of all pairwise loci combinations, and number of polymorphic loci and of pairwise loci combinations with significant non-random association, from RAPD and RAMS data on *Phaeoacremonium aleophilum* and *Phaeomoniella chlamydospora*. For each species the parameters evaluated included all the individuals of the sample or one isolate for each multilocus genotype (between parentheses).

Molecular marker	*Phaeoacremonium aleophilum*				*Phaeomoniella chlamydospora*			
	15 isolates (1 isolate/multilocus genotype)				29 isolates (1 isolate/multilocus genotype)			
	Polymorphic loci	Pairwise loci combinations	Polymorphic loci associated[a]	Pairwise loci combinations associated[a]	Polymorphic loci	Pairwise loci combinations	Polymorphic loci associated[a]	Pairwise loci combinations associated[a]
RAPD	51	1275	43 (43)	211 (211)	18	153	18 (18)	55 (43)
RAMS	32	496	30 (32)	53 (75)	7	21	7 (7)	16 (5)

[a] Values shown are statistically significant ($P \leq 0.05$, according to Fisher's exact test).

fragments, and by the number of multilocus genotypes on the total number of isolates examined. Moreover, when the clonal fraction was calculated, values obtained in RAPD and RAMS experiments, respectively, ranged from 0 to 6.6% for *Pal* and from 17.2 to 68.9% for *Pch*. When the genetic distance among the multilocus genotypes of each species was calculated, as the number of polymorphic fragments not shared between the compared genotypes, the analysis confirmed previous observations (Tegli *et al.*, 2000) that the genetic distance was always higher with RAPD than with RAMS data, and with *Pal* than with *Pch* genotypes. When these data were graphically represented it became more clear that in both species, though to different degrees in each, most genotypes were not closely related: for example, about 54.3% of *Pal* multilocus genotypes differed by 20-30 fragments, representing 39.2-58.8% of the total number of polymorphic fragments. The high percentage of unrelated multilocus genotypes in both species, but particularly in *Pal*, and the fact that most such genotypes comprised just a few members was consistent with the low values of the clonal fraction in the samples examined, and suggested that most individuals of *Pal* and *Pch* resulted from sexual reproduction.

These conclusions were confirmed by the gametic disequilibrium tests. The statistical analysis of the loci associations revealed a significant disequilibrium in 16.5% of the pairwise loci combinations of the 51 RAPD loci, that showed 15 different multilocus genotypes among the 15 *Pal* isolates. The disequilibrium was also statistically significant when the RAMS data for *Pal* were considered: 10.7% of the loci combinations showed association with all the 15 isolates of *Pal,* and 15.1% did so with 1 isolate for each multilocus genotype. With RAPD and RAMS primers there was significant disequilibrium for 35.9% and 76.1% respectively of the pairwise combinations of the 29 *Pch* isolates. When the clonal fractions were removed from both sets of data, these disequilibrium values strongly decreased to 28.1% for RAPD and 23.8% for RAMS, suggesting that in *Pch* many of the significant associations among loci in the sample examined were strictly dependent on the presence of clones. In conclusion, the low levels of gametic disequilibrium found in the non-clonal part of both *Pal* and *Pch* samples were consistent with the existence of sexual reproduction in these species. On the other hand both direct multilocus genotype analysis and the indirect gametic disequilibrium tests suggested different degrees of sexual *vs* asexual reproduction in *Pal* and in *Pch*. With *Pch*, the low level of genotypic variation and the small genetic distances among the genotypes seemed consistent with the hypothesis that the contribution of asexual reproduction to the structure of the natural populations in this species is greater than that of sexual reproduction. With *Pal* the opposite is thought to be the case. Moreover, two other factors of fundamental importance in the genetic structure of a population or a species should not be forgotten: the mode of dispersal of the inoculum and the source of infection. About these aspects of the epidemiology and biology of *Pal* and *Pch* almost nothing is yet known, although such information would be very useful to help control the disease these fungi are thought to cause. Since these species, particularly *Pch,* have been shown to be present in grapevine propagation material (Bertelli *et al.*, 1998; Surico *et al.*, 1998), but the existence and importance of sexual reproduction in *Pal* and *Pch* were not known until that study, further research is needed on the genetic structure of populations of these fungi in propagating material, and on how this structure changes in the years after this material is planted. A similar study would give information on the source and the spread of the inoculum both in the vineyard and on a larger scale. Our results would be consistent with the hypothesis that *Pal* and *Pch* are dispersed over long distances by vegetative propagating material. This would explain the absence of any geographic pattern in the genetic and genotypic diversity (Tegli *et al.*, 2000). In the vineyard both sexual and asexual cycles would occur, and would give rise to an air-dispersed inoculum, as suggested by Larignon (1999) and by our data on the presence of widely distributed clones (Tegli *et al.*, 2000).

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