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# Genetic variation within *Phaeoacremonium aleophilum* and *P. chlamydosporum* in Italy

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**Summary.** Genetic variation among isolates of *Phaeoacremonium chlamydosporum* (*Pch*) and *P. aleophilum* (*Pal*), two hyphomycetous fungi involved in the development of symptoms of esca and in a decline of young grapevines, was studied in eight Italian grapevine growing regions. Genetic variation was estimated by analysis of amplification profiles obtained in RAPD- and RAMS-PCR experiments (RAPD = Random Amplified Polymorphic DNA; RAMS = Random Amplified Micro- or Mini-Satellites). The genetic divergence between the two species suggests their assignment to two different genera. Within each species, a low level of polymorphism was found; however a higher degree of genetic variation was found with RAPD than with RAMS experiments, and more with *Pal* than with *Pch* isolates. No relationship was found for either species between the clustering of isolates in statistically defined groups and the geographic origin of those isolates. A low level of genetic disequilibrium was found in *Pch* and *Pal*, suggesting that sexual reproduction may occur in both fungi.

**Key words:** genetic variation, *Phaeoacremonium*, PCR, RAPD, RAMS.

## Introduction

The mitosporic fungi *Phaeoacremonium chlamydosporum* (*Pch*) W. Gams, Crous, M.J. Wingf. & L. Mugnai (Crous *et al.*, 1996) and *P. aleophilum* (*Pal*) W. Gams, Crous, M.J. Wingf. & L. Mugnai (Crous *et al.*, 1996) have both been found in association with esca-diseased grapevines (Ferreira *et al.*, 1994; Morton, 1995, 1997; Scheck *et al.*, 1998; Graniti *et al.*, 1999; Mugnai *et al.*, 1999). A third fungus that has a primary role in esca is the basidiomycetes *Fomitiporia punctata* (*Fop*) (Fr.) Murrill (Fischer, 1996). This fungus is responsible for white rot of vine wood, while *Pch* and *Pal* cause brown/black wood-streaking and pink to reddish wood discoloration. From the wood so discolored *Pch* and *Pal* are regularly isolated (Mugnai *et al.*, 1996, 1999; Larignon and Dubos, 1997; Larignon, 1999).

*Pch* and *Pal* also cause brown wood streaking in rooted cuttings and very young grapevines (Bertelli *et al.*, 1998; Surico *et al.*, 1998; Larignon, 1999) and are considered the causal agents of a decline of young grapevines previously named "black goo" and now also known as "Petri grapevine decline" (Ferreira *et al.*, 1994; Morton, 1995, 1997; Scheck *et al.*, 1998; Graniti *et al.*, 1999; Mugnai *et al.*, 1999). Another *Phaeoacremonium* species which in California is associated with Petri grapevine decline is *P. inflatipes* (*Pinf)* (Scheck *et*

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*al.*, 1998). All the above mentioned species, and also *P. angustius* (*Pang*), *P. rubrigenum* (*Prub*) and *P. parasiticum* (type species), are members of this new genus which has been proposed by Crous *et al*. (1996) to accomodate a group of fungi intermediate between the genera *Acremonium*, *Cephalosporium* and *Phialophora*. Most of the new *Phaeoacremonium* species, i.e. *Pch*, *Pal*, *Pang* and *Pinf*, include isolates from grapevine plants.

In Italy, from 1992 until now, several studies on esca and related syndromes have been carried out but the only *Phaeoacremonium* species ever isolated from esca-diseased plants have been *Pch* and *Pal*. For each one of these species the isolates, although obtained at different times and from various grapevine plants of different ages and cultivars, did not present morphological differences when grown *in vitro*. Even when some of these isolates from either species were examined for the production of exocellular enzymes (e.g. laccase, peroxidase, phenoloxidase, esterase, endo-glucanase, β-glucosidase, etc.) and other physiological and biochemical characteristics, they were quite uniform (Mugnai *et al.*, 1997). However, apart from this and sporadic observations by other researchers (Yan *et al.*, 1995; Dupont *et al.*, 1998), no exaustive study has ever been done to assess the extent of diversity within *Pch*, *Pal* and/or the other *Phaeoacremonium* species. The present study is a first step towards defining the genetic variation of the populations of *Pch* and those of *Pal* in Italy. The Polymerase Chain Reaction (PCR) technique, and in particular the amplification profiles obtained with RAPD (Random Amplified Polymorphic DNA) and RAMS (Random Amplified Micro- or Mini-Satellites) (Stenlid *et al.*, 1994; Hantula and Müller, 1997; Hantula *et al.*, 1997; Longato and Bonfante, 1997) experiments were used to determine the level of genetic heterogeneity in a representative sample of Italian isolates of *Pch* and *Pal*.

## Materials and methods

### *Phaeoacremonium* isolates and cultural conditions

A total of 44 isolates, 15 of *Pal* and 29 of *Pch*, were analysed. Forty-one came from various Italian regions and were identified in our laboratory; the remaining 3 (1 *Pal* and 2 *Pch*) came from the Centraalbureau voor Schimmelcultures (CBS), Baarn, The Netherlands, and were used for comparison. The Italian isolates were collected from various grapevine cultivars in 1995 (except *Pal* 157, isolated in 1994), in 8 Italian regions. Some isolates came from the same vineyard or even from the same plant. Table 1 shows the geographic origin and the host plant of each isolate.

The isolates were maintained on malt extract agar (MEA) (Difco Laboratories, Detroit, MI, USA) at 4°C, and routinely subcultured on the same medium in Petri dishes at 25°C in the dark. For liquid cultures, Erlenmayer flasks (100 cc) containing 50 ml of Potato Dextrose Broth (PDB, Difco Laboratories) were each sterile inoculated with a mycelial plug (5-mm diameter) each taken from the actively growing margin of MEA fungal cultures. The flasks were then incubated on a rotatory shaker (100 rpm), at 25°C in the dark for about a week, or until the fungal suspension reached a concentration of about $10^9$ cfu/ml.

### *Phaeoacremonium* DNA extraction

Two ml of fungal liquid culture was collected in a sterile Eppendorf tube (1.7 ml), centrifuged at maximum speed for 3 min and the supernatant discarded. DNA extraction was then performed on the fungal pellet, using the "Puragene DNA Isolation Kit" (Gentra System Inc., Minneapolis, MN, USA), according to the manufacturer's instructions. The DNA obtained was further purified by a phenol-chloroform extraction, followed by precipitation in absolute ethanol and 3 M Na-acetate, pH 5.5 (Sambrook *et al.*, 1989). The DNA was resuspended in 10 mM Tris-HCl buffer, pH 8.0, containing 1 mM EDTA (TE buffer) (Sambrook *et al.*, 1989), and stored at 4°C.

### PCR reactions

The PCR reactions were carried out in an automated thermal cycler (Delphy 1000™, Oracle Biosystems™, MJ Research Inc., Watertown, MA, USA), in thin-walled 0.5-ml Eppendorf tubes (Alpha Laboratories Ltd, Eastleigh, Hampshire, UK), with a final reaction volume of 25 μl, and with 10 ng of fungal DNA as template. Sterile distilled water was used instead of DNA as a negative control to test for contamination of reagents. The primers used were listed in Table 2. Each primer was tested indipendently at least three times on each fungal isolate, to verify the reproducibility of the

Table 1. Isolate designation, sampling date, geographic origin, and host plant of *Phaeoacremonium* spp. used in this study.

| Species | Isolate code[a] | Date | Origin[b] | Vine code[c] |
|---|---|---|---|---|
| *P. aleophilum* | CBS 246.91[d] | 1991 | Yugoslavia | –[e] |
| | 999.95 | 1995 | Abruzzi (I) | M2 |
| | 1004.95 | 1995 | Abruzzi (I) | M2 |
| | 1002.95 | 1995 | Abruzzi (I) | M1 |
| | 321.T2.95 | 1995 | Apulia (I) | T |
| | 334.T2.95 | 1995 | Apulia (I) | T |
| | 334.V1.95 | 1995 | Apulia (I) | V |
| | 403.Z.95 | 1995 | Friuli (I) | Z |
| | 98.L.95 | 1995 | Lombardy (I) | L |
| | 93.I.95 | 1995 | Lombardy (I) | I |
| | Natt.3.95 | 1995 | Sicily (I) | Sic |
| | 157 | 1994 | Tuscany (I) | PG |
| | 405.Y.95 | 1995 | Tuscany (I) | Y |
| | 444.J.95 | 1995 | Tuscany (I) | J |
| | 445.J.95 | 1995 | Tuscany (I) | J |
| *P. chlamydosporum* | CBS 161.90 | 1990 | Cape Province (RSA) | – |
| | CBS 239.74 | 1974 | California (USA) | – |
| | 989.95 | 1995 | Abruzzi (I) | S2 |
| | 991.95 | 1995 | Abruzzi (I) | M1 |
| | 1000.95 | 1995 | Abruzzi (I) | M2 |
| | 324.R5a.95 | 1995 | Apulia (I) | R |
| | 325.R7b.95 | 1995 | Apulia (I) | R |
| | 283.U4.95 | 1995 | Apulia (I) | U |
| | 330.U2.95 | 1995 | Apulia (I) | U |
| | 113.I.95 | 1995 | Lombardy (I) | I |
| | 229.I.95 | 1995 | Lombardy (I) | I |
| | 1121.95 | 1995 | Sicily (I) | Sic |
| | 1122.95 | 1995 | Sicily (I) | Sic |
| | 56 (CBS 229.95)[d] | 1995 | Tuscany (I) | PA |
| | LT3 | 1995 | Tuscany (I) | Lat |
| | RM1 | 1995 | Tuscany (I) | Rom |
| | Bb13 | 1995 | Tuscany (I) | P13 |
| | Bb16 | 1995 | Tuscany (I) | P16 |
| | Bb27 | 1995 | Tuscany (I) | P27 |
| | Bb32 | 1995 | Tuscany (I) | P32 |
| | Bb36 | 1995 | Tuscany (I) | P36 |
| | 191.N7.95 | 1995 | Umbria (I) | N |
| | 217.N3.95 | 1995 | Umbria (I) | N |
| | 191.P1.95 | 1995 | Umbria (I) | P |
| | 209.P1.95 | 1995 | Umbria (I) | P |
| | 1091.95 | 1995 | Veneto (I) | Ven2 |
| | 1096.95 | 1995 | Veneto (I) | Ven2 |
| | 1095.95 | 1995 | Veneto (I) | Ven1 |
| | 1101.95 | 1995 | Veneto (I) | Ven1 |

[a] CBS: Centraal Bureau voor Schimmelcultures (Baarn, The Netherlands); Italian isolates are from the culture collection of Dipartimento di Biotecnologie Agrarie - Patologia vegetale, University of Florence, Italy.
[b] I: Italy; RSA: Republic of South Africa; USA: United States of America.
[c] Same code = same plant.
[d] Holotype.
[e] –, unknown.

Table 2. Details of primers used in this study.

| Primer | Sequence[a] |
|---|---|
| CGA | 5'-DHB$(CGA)_5$-3' |
| GT | 5'-YHY$(GT)_5$G-3' |
| M13 | 5'-GAGGGTGGCGGTTCT-3' |
| OP-B12 | 5'-CCTTGACGCA-3' |
| OP-B14 | 5'-TCCGCTCTGG-3' |
| OP-B18 | 5'-CCACAGCAGT-3' |
| OP-B19 | 5'-ACCCCCGAAG-3' |

[a] (B= G,T or C; D= G, A or T; H= A, T or C; Y= A, C or G)

amplification patterns obtained in the reaction conditions chosen. Aliquots of amplification products (2.5 µl) were analysed by electrophoresis in 1.4% (w:v) agarose gels, run in 1x TBE buffer (Sambrook *et al.*, 1989), stained with ethidium bromide (0.5 µg/ml) and photographed under ultraviolet light. The length of the DNA fragments was estimated by comparison with "1 kb Plus DNA Ladder" (GIBCO-BRL, Life Technologies, Gaithersburg, MD, USA).

*RAPD-PCR*. Four primers (OP-B12, OP-B14, OP-B18 and OP-B19) were chosen from the 20 ten-based primers of arbitrary sequence of the Operon Kit B (Operon Technologies Inc., Alameda, CA, USA) to amplify *Phaeoacremonium* DNA according to manufacturer's recommendations, with slight modifications. The reaction components were: 20 mM Tris-HCl (pH 8.0), 50 µM KCl, 2 µM $MgCl_2$, 200 µM of each deoxynucleotide triphosphate (dNTP), 0.8 µM of primer and 0.65 U Taq DNA Polymerase (Polymed s.r.l., Florence, Italy). The samples were denatured at 94°C for 3 min, after which 45 cycles of amplification were performed as follows: 1 min denaturation at 95°C, 1 min annealing at 36°C, 2 min primer extension at 72°C.

*RAMS-PCR*. Amplifications of fungal DNA using the micro- and mini-satellite based primers CGA, GT and M13 were carried out in reaction mixtures containing 20 mM Tris-HCl (pH 8.0), 50 mM KCl, 2.5 mM $MgCl_2$, 200 µM of each dNTP, 2 µM for primers CGA and GT or 0.5 µM for primer M13, and 1 U Taq polymerase. The cycling parameters for CGA and GT were: 3 min denaturation at 95°C, 35 cycles with 30 sec denaturation at 95°C, 45 sec annealing at 61°C for CGA or at 58°C for GT, 2 min extension at 72°C. The amplification parameters for primer M13 were: 3 min at 93°C for denaturation, 45 cycles of amplification with 1 min each for denaturation (93°C), annealing (55°C) and extension (72°C).

#### RAPD and RAMS data analysis

RAPD and RAMS amplification profiles were analysed from photographic prints of the electrophoretic gels. Only reproducible, clear and distinct bands were included. Comparison of each profile for each primer tested was done on the basis of the presence or absence (1/0) of amplification products having the same length. Bands of the same size were scored as identical and the presence or absence of a fragment was considered to indicate one or another of the two alleles of the same locus. The data for the RAPD and RAMS primers with all the isolates were combined in two binary matrices. Data were analysed using TREECON software (Van de Peer and De Wachter, 1994) to obtain a genetic similarity matrix using the Nei's genetic distance coefficient (Nei and Li, 1979), and a dendrogram using the neighbor-joining method. For each species the RAPD and RAMS data were computed with Arlequin software (vers.1.1, Schneider *et al.*, University of Geneva, Switzerland), and subjected to AMOVA (Excoffier *et al.*, 1992) to evaluate the genetic structure among populations with different geographic origin.

## Results

#### RAPD-PCR

The analysis of *Pal* and *Pch* isolates with RAPD primers revealed no banding patterns specific for either species, with the exception of the amplification profile obtained with OP-B 14 on *Pch*, which was characteristic and common to all *Pch* but one isolate and just for one polymorphic band. When data from the RAPD experiments were combined, a total of 122 markers were scored, of which only 3 were common to both *Pal* and *Pch* (data not shown). The number of fragments amplified was always greater in *Pal* (78) than in *Pch* isolates (47), and this occurred with all the RAPD primers tested (Table 3). No substantial variation was found in the number of markers produced by each primer from individual isolates belonging to the same species. As far as *Pal* is concerned, 20 markers

Table 3. Number of haplotypes, and of total and monomorphic fragments obtained with each RAPD primer from *Phaeoacremonium aleophilum* and *P. chlamydosporum* isolates.

| Primer | *P. aleophilum* (15 isolates) | | | *P. chlamydosporum* (29 isolates) | | |
|---|---|---|---|---|---|---|
| | No. of haplotypes | No. of total fragments | No. of monomorphic fragments | No. of haplotypes | No. of total fragments | No. of monomorphic fragments |
| OP-B 12 | 14 | 20 | 5 | 17 | 18 | 11 |
| OP-B 14 | 11 | 21 | 8 | 2 | 5 | 4 |
| OP-B 18 | 11 | 18 | 6 | 3 | 12 | 11 |
| OP-B 19 | 14 | 19 | 8 | 14 | 12 | 3 |
| Total | 15 | 78 | 27 | 24 | 47 | 29 |

were revealed by OP-B 12, 21 by OP-B 14, 18 by OP-B 18, and 19 by OP-B 19, among which the number of monomorphic fragments was 5 for OP-B 12, 8 for OP-B 14, 6 for OP-B 18, and 8 for OP-B 19 (Table 3). *Pch* isolates gave rise to the amplification of 18 fragments with OP-B 12, 5 with OP-B 14, 12 with OP-B 18, and 12 with OP-B 19, and the monomorphic markers were 11 for OP-B 12, 4 for OP-B 14, 11 for OP-B 18, and 3 for OP-B 19 (Table 3). When data from RAPD primers were combined, a total of 27 and 29 monomorphic bands were found in *Pal* and *Pch* isolates respectively. The evolutionary distance among *Pal* isolates ranged from 0.03 to 0.28%, that among *Pch* isolates from 0 to 0.17%. Variation in the RAPD markers revealed 14, 11, 11 and 14 different haplotypes in *Pal* produced by the four primers OP-B 12, OP-B 14, OP-B 18 and OP-B 19 respectively, and 17, 2, 3, and 14 different haplotypes in *Pch* by the same primers respectively (Table 3). When combined, these markers identified 15 different haplotypes on 15 *Pal* isolates, and 24 haplotypes on 29 *Pch* isolates, as shown in Fig. 1. Four statistically different groups were found in *Pal*, but no significant relationship was found between these groups and the geographic origin by AMOVA ($P \leq 0.02$). A similar situation was found with *Pch*, where five statistically different groups were identified. With both species, even when isolates from the same Italian region were clustered together, they always formed a group also including isolates with a different geographic origin. But, *Pal* isolates 321.T.95, 334.T2.95 and 334.V1.95 from Apulia, and *Pch* isolates 1091.95, 1095.95, 1096.95 and 1101.95 from Veneto formed groups by themselves. With *Pch*, isolates from the same group sometimes had the same haplotype but came from a different area. This never happened with *Pal* isolates, each of which corresponded to a different haplotype. *Pal* isolates from the same grapevine plants always clustered in the same groups (444.J.95 and 445.J.95 from Tuscany, 999.95 and 1004.95 from Abruzzi, and 321.T.95 and 334.T2.95 from Apulia). However, they never had the same haplotype and the evolutionary distance between isolates from the same plant could be greater than that between isolates from different plants, as was the case for isolates 999.95, 1004.95 and 1002.95. *Pch* isolates from the same plant always belonged to the same group, and their haplotype was sometimes the same (1091.95 and 1096.95 from Veneto, 113.I.95 and 229.I.95 from Lombardy, and 991.95 and 1000.95 from Abruzzi) and sometimes different (324.R5a.95 and 325.R7b.95, 283.U4.95 and 330.U2.95, all the four from Apulia, 1101.95 and 1095.95 from Veneto, 191.N7.95 and 217.N3.95, 191.P1.95 and 209.P1.95, all the four from Umbria). On the other hand the same haplotype was also identified in isolates from the same region but from different plants (191.N7.95 and 209.P1.95 from Umbria) and in isolates from different regions (991.95 and 1000.95 from Abruzzi, and 113.I.95 and 229.I.95 from Lombardy).

**RAMS-PCR**

The analysis of *Pal* and *Pch* isolates with RAMS primers CGA, GT and M13 revealed banding patterns with which the species could be distinguished. Moreover, primer M13 produced a species-specific banding pattern with *Pch* isolates. When data from

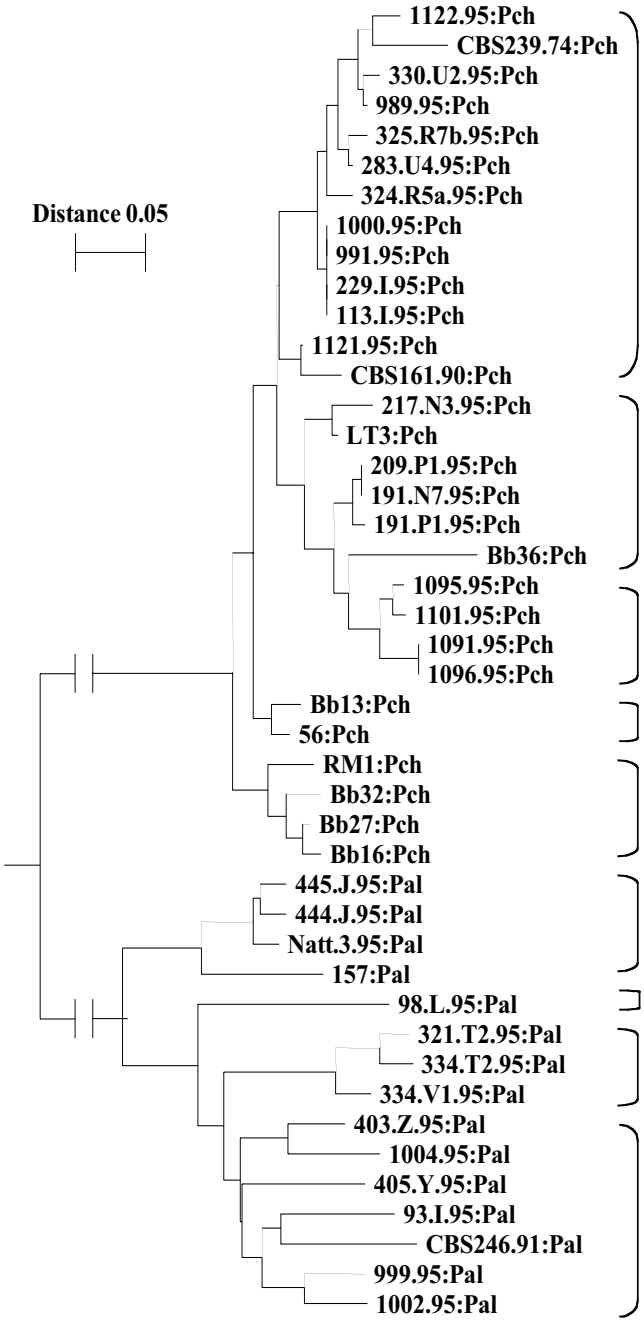


Fig. 1. Dendrogram based on RAPD data showing the relationships among *Phaeoacremonium aleophilum* and *P. chlamydosporum* isolates studied. The dendrogram was constructed by TREECON software using the neighbor-joining method based on Nei's genetic distance values.

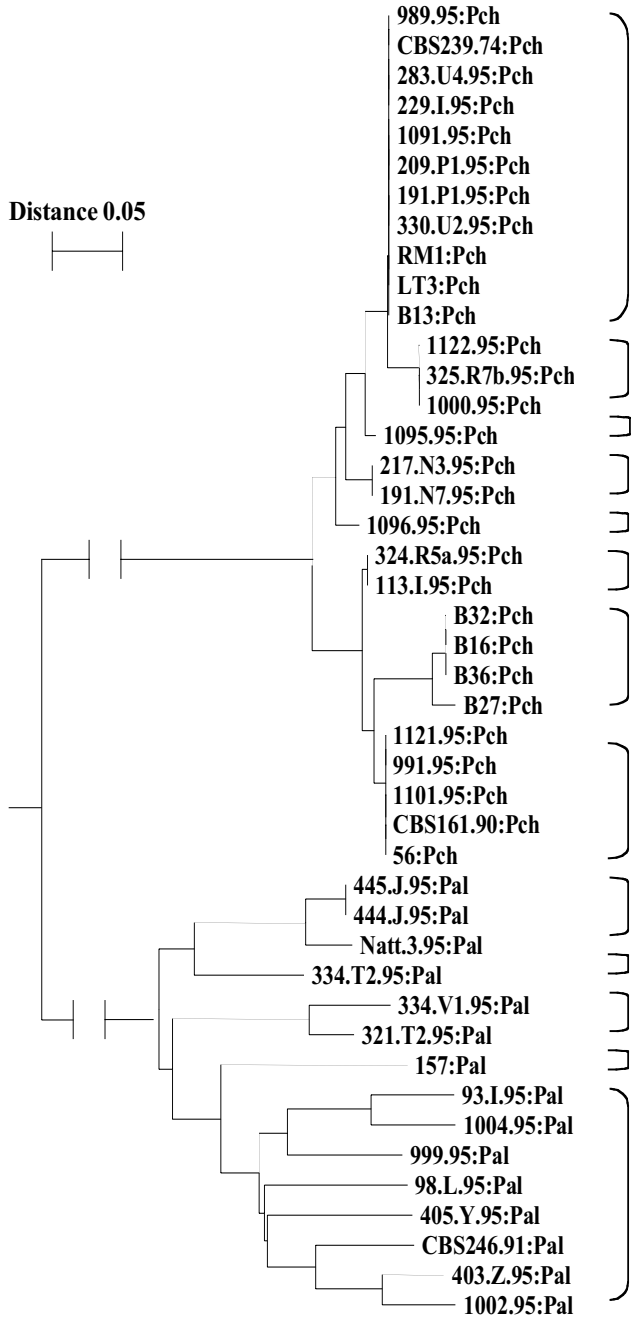


Fig. 2. Dendrogram based on RAMS data showing the relationships among *Phaeoacremonium aleophilum* and *P. chlamydosporum* isolates studied. The dendrogram was constructed by TREECON software using the neighbor-joining method based on Nei's genetic distance values.

Table 4. Number of haplotypes, and of total and monomorphic fragments obtained with each RAMS primer from *Phaeoacremonium aleophilum* and *P. chlamydosporum* isolates.

| Primer | *P. aleophilum* (15 isolates) | | | *P. chlamydosporum* (29 isolates) | | |
|---|---|---|---|---|---|---|
| | No. of haplotypes | No. of total fragments | No. of monomorphic fragments | No. of haplotypes | No. of total fragments | No. of monomorphic fragments |
| CGA | 13 | 14 | 4 | 7 | 8 | 3 |
| GT | 13 | 16 | 1 | 2 | 6 | 4 |
| M13 | 8 | 14 | 7 | 1 | 12 | 12 |
| Total | 14 | 44 | 12 | 9 | 26 | 19 |

the RAMS experiments were combined, a total of 67 markers were scored, only 3 of which were in common to both species (data not shown). The number of fragments amplified was always higher in *Pal* (44) than in *Pch* isolates (26), and this occurred with all the RAMS primers used (Table 4). No substantial variation was found in the number of markers produced by each primer from single isolates belonging to the same species. For *Pal*, 14 markers were revealed by CGA, 16 by GT and 14 by M13, among which the number of monomorphic fragments was 4 for CGA, 1 for GT and 7 for M13 (Table 4). *Pch* isolates gave rise to the amplification of 8 fragments with CGA, 6 with GT and 12 with M13, and the number of monomorphic markers was 3 for CGA, 4 for GT and 12 for M13 (Table 4). When RAMS data were combined, 12 and 19 monomorphic fragments were found from *Pal* and *Pch* isolates respectively. The evolutionary distance among *Pal* isolates ranged from 0.05% to 0.34%, and that among *Pch* isolates from 0 to 0.15%. Variation observed in the RAMS markers by CGA, GT and M13 revealed 13, 13 and 8 different haplotypes in *Pal*, and 7, 2 and 1 haplotypes in *Pch* respectively (Table 4). When combined, these markers identified 14 different haplotypes in 15 *Pal* isolates, and 9 haplotypes in 29 *Pch* isolates (Fig. 2). According to AMOVA, *Pal* and *Pch* isolates were clustered in five and eight statistically different groups, respectively. No statistically significant clustering was found for isolates belonging to the same species and having the same geographic origin ($P \leq 0.02$). Isolates 321.T2.95 and 334.V1.95 formed the only significant group consisting exclusively of *Pal* isolates that came from the same region, Apulia. Isolates from the same grapevine plants could belong to different groups (321.T2.95 and 334.T2.95), or to the same group, as with isolates 999.95 and 1004.95 from Abruzzi, and isolates 444.J.95 and 445.J.95 from Tuscany. These last two isolates also showed the same haplotype; the remaining 13 *Pal* isolates always exhibited different haplotypes.

As regards *Pch* isolates, the only group of isolates from the same region comprised isolates Bb16, Bb27, Bb32 and Bb36 from Tuscany, and of these three (Bb16, Bb32 and Bb36) also exhibited the same haplotype. In the other groups isolates with a different geographic origin or from different plants were found, and nevertheless here too the same haplotype was shared by the isolates belonging to the same group.

*Pch* isolates from the same grapevine plants could belong to different groups (113.I.95 and 229.I.95 from Lombardy, 1101.95 and 1095.95 from Veneto, 1091.95 and 1096.95 from Veneto, and 324.R5a.95 and 325.R7b.95 from Apulia), to the same group but with a different haplotype (283.U4.95 and 330.U2.95 from Apulia) or to the same group and with the same haplotype (191.N7.95 and 217.N3.95, and 191.P1.95 and 209.P1.95, all the four from Umbria).

## Discussion

Genetic variation between and within Italian isolates of *Pal* and *Pch* with a different geographic origin was assessed by analysis of amplification profiles obtained in RAPD- and RAMS-PCR experiments.

The experimental criteria used to test genetic variation between *Pal* and *Pch* were found to be correct. The statistical analysis of data from RAPD and RAMS experiments confirmed the absolute

divergence between the two species. Moreover the evolutionary distance between them suggested that they should be classified into two different genera, as already proposed by Dupont *et al*. (1998).

In Italy the grapevine has been cultivated for thousands of years, in all parts of the country. At present the total area under vineyard cultivation is about 922,000 ha. Esca has been known since antiquity, and the fungi involved, *Pal* and *Pch*, were identified about a century ago although with different names (Petri, 1912). Therefore greater genetic variation might have been expected among isolates from these species, despite their assumed lack of sexual reproduction. Instead, according to the data on the number of monomorphic and polymorphic fragments amplified in RAPD and RAMS experiments in each species, on the genetic distances, and on the number of haplotypes for each species, the level of genetic variation for these fungi was found to be low, although it was higher in *Pal* than in *Pch*.

Analysis of nucleic acid variation in the last half-decade has shown itself to be essential in plant pathology to identify not only fungal species but also specific populations and their distribution during the different steps of an epidemic event or in an endemic situation. Moreover such analytical studies have elucidated the reproductive modes really present in nature for some fungi, making fundamental contribution to the knowledge of the epidemiology of these fungi and of the diseases they cause (Milgroom, 1996; Taylor *et al.*, 1999). What is suggested by the data on the *Pal* and *Pch* populations so far examined is that several sources of inoculum for both fungi may be available in the same vineyard, and that sexual reproduction, as stated by the low level of gametic disequilibrium (in *Pal* isolates, 16.5% and 15.1% of loci combinations were significantly associated with the 15 and 14 different RAPD and RAMS haplotypes, while in *Pch*, 28.1% and 23.8% of loci combinations were significantly associated with the 24 and 9 different RAPD and RAMS haplotypes respectively), may occur in both fungi.

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