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The genus *Phaeoacremonium* from a molecular point of view

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Summary. A molecular approach combined with phenotypic and morphological observations was used to delimit *Phaeoacremonium* species involved in esca disease and grapevine declines. *P. aleophilum* and *P. angustius* were synonymised, a new species *P. viticola* was reported and preliminary results concerning the delimitation of *P. inflatipes* and *P. parasiticum* were presented. The incubation of isolates at 35°C appeared to be a discriminant character to identify these two species, *P. inflatipes* having very low growth in comparison with *P. parasiticum*. Specific restriction profiles were determined using the type strain of each species, and the method was applied to screen unidentified isolates.

Key words: taxonomy, esca disease, *Phaeoacremonium*, phenotypic characters, molecular approach.

Introduction

The genus *Phaeoacremonium* described by Crous *et al.* (1996) comprises six species□*P. parasiticum, P. aleophilum, P. angustius, P. chlamydosporum, P. inflatipes* and *P. rubrigenum*. The type species of the genus *P. parasiticum,* and also *P. inflatipes* and *P. rubrigenum* have been isolated from humans as well as from diseased plants, the other three species were first described from isolates coming from esca-diseased grapevines. To date, five species have been reported from grapevines in decline and with esca symptoms: *P. chlamydosporum, P. aleophilum* and *P. angustius* seem to have a worldwide distribution (Crous *et al.*, 1996; Larignon and Dubos, 1997; Morton, 1997; Mugnai *et al.*, 1999; Pascoe, 1998); *P. inflatipes*, frequently found in Californian vineyards (Morton and Larignon, personal communication) could also be present in South America (Chile, personal observation), and *P. parasiticum* occurs in Argentina and Turkey (personal observation).

In 1998, using partial rDNA sequencing, we established the heterogeneity of the genus, showing that *P. chlamydosporum* was related to the Chaetothyriales while the other five species were members of the Magnaporthaceae (Diaporthales) (Dupont *et al.*, 1998). Among these, two lineages were observed, one consisting of the closely related *P. parasiticum* and *P. rubrigenum* with *P. inflatipes* in a basal position, and the other grouping *P. aleophilum* and *P. angustius* with a very high nucleotidic similarity, suggesting their possible conspecificity since they are morphologically very much alike also in other respects. In this paper we retain the name *P. chlamydosporum* as it has not yet been formally removed from the genus *Phaeoacremonium*.

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Building on these informative results we used DNA sequencing and restriction analysis (PCR-RFLP) to screen a large number of *Phaeoacremonium* isolates from a worldwide range of sources to evaluate their genomic polymorphism and to define the limits of each existing species.

Materials and methods

Fungal strains and cultural methods

Type strains of each *Phaeoacremonium* species were used as reference material for the morphological and molecular identification of isolates collected from esca-diseased grapevines in Europe and America (Tables 1, 3). The isolates were grown on 2% malt agar (MA) at 25°C under near-ultraviolet light to promote sporulation for microscopic examination. Radial growth was determined after 15 days on three replicate MA plates incubated in the dark at 25, 30 and 35°C.

DNA extraction, amplification and sequencing methods

Genomic DNA was extracted from fresh mycelium grown on MA for 5 days using a CTAB micropreparation method (Rogers and Blendich, 1995). Dilutions of 100X stock DNA were used as template for the polymerase chain reaction (PCR). PCR was performed in 50 µl reactions, using 25 µl of DNA template, 1.25 units of AmpliTaq DNA Polymerase (Roche Molecular Systems, Inc., Branchburg, NJ, USA), 5 µl of 10X Taq DNA Polymerase buffer, 5 µl of 50% glycerol, 2 µl of 5 mM dNTPs (Eurogentec, Seraing, Belgium) and 2 µl of each 10 µM primer. The oligonucleotide primer set ITS4 and ITS5 (White *et al.*, 1990) was used to amplify the ITS region of the rDNA, and the primer set Bt2a and Bt2b (Glass and Donaldson, 1995) was used to amplify a part of the 5' end of the ß-tubulin gene. Amplifications were performed on a Perkin Elmer Cetus thermal cycler model 2400 using the following parameters: an 8 min step at 94°C, followed by 30 cycles of 20 s at 94°C, 20 s at 58°C and 25 s at 72°C, and then a final 8 min extension step at 72°C. DNA sequencing was performed on both strands using the ABI PRISM TM Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems Foster City, CA, USA) with

Table 1. Isolates of *Phaeoacremonium* used in this study.

Species	Isolate number	Origin	Host	GenBank number (ITS, ß-tubulin)
P. aleophilum	CBS 246.91 [a]	Yugoslavia	*Vitis vinifera*	AF017651, AF192390
P. aleophilum	CBS 631.94	Italy	*Vitis vinifera*	
P. angustius	CBS 249.95 [a]	USA, California	*Vitis vinifera*	AF118138
P. angustius	LCP 97 4088	France	*Vitis vinifera*	
P. inflatipes	CBS 391.71 [a]	USA, California	*Quercus virginiana*	AF118140, AF192393
P. inflatipes	LCP 96 3899	Chile	*Vitis vinifera*	
P. parasiticum	IMI 181 115 [a]	USA, California	Human	U31841, AF192392
P. parasiticum	IMI 192 879	Tunisia	*Prunus armeniaca*	
P. rubrigenum	CBS 498.94[a]	USA	Human	AF118139, AF192389
P. rubrigenum	LCP 97 4002	France	*Vitis vinifera*	
P. viticola	LCP 93 3886[a]	France	*Vitis vinifera*	AF118137, AF192391
P. viticola	LCP 93 3551	USA, California	*Vitis vinifera*	
P. viticola	LCP 96 3897	USA, California	*Vitis vinifera*	
P. viticola	LCP 97 4003	France	*Vitis vinifera*	
P. viticola	LCP 97 4004	France	*Vitis vinifera*	
P. viticola	LCP 97 4009	France	*Vitis vinifera*	
P. viticola	LCP 97 4014	France	*Vitis vinifera*	
P. viticola	LCP 97 4016	France	*Vitis vinifera*	
P. viticola	LCP 97 4017	France	*Vitis vinifera*	
P. viticola	LCP 97 4018	France	*Vitis vinifera*	

[a] Type strain of the species

Table 2. Nucleotide differences between *Phaeoacremonium viticola* and other species of the genus within ITS and partial ß-tubulin sequences. Type strain of each species was used (see Table 1).

Species	ITS1 (180 bp)	ITS2 (170 bp)	ß-tubulin (440 bp)
P. viticola / *P. aleophilum*	15	3	60
P. viticola / *P. inflatipes*	20	9	105
P. viticola / *P. parasiticum*	22	12	113
P. viticola / *P. rubrigenum*	24	10	107

amplification primer sets and internal primers ITS2 and ITS3 for the ITS region (White *et al.*, 1990). Sequencing assays were analysed on an automated DNA Sequencer ABI PRISM 377 (Applied Biosystems). The nucleotide sequences were aligned manually and edited using the MUST computer package (Philippe, 1993).

Restriction enzyme digestions

Aliquots of 10 µl of the amplified DNA were digested with 2 units of *Dde*I, *Hha*I or *Taq*I (Appligène, Strasbourg, France), *Hae*III, *Msp*I or *Rsa*I (Eurogentec). All the enzymes gave complete digestion of the DNA in the PCR buffer, making it unnecessary to purify the DNA prior to digestion. The restriction fragments were separated on 3% "small fragments" agarose gels (Eurogentec), stained with ethidium bromide (10µg/µl) and photographed under UV light. The molecular size marker was the "Superladder-low 20 bp Ladder" (Eurogentec).

Results and Discussion

Conspecificity of *P. aleophilum* and *P. angustius*

The sequencing of the type strains of *P. aleophilum* (CBS 246.91) and *P. angustius* (CBS 249.95) was extended to ITS1 and to the 5' end of

Table 3. Comparison of colony diameters and restriction patterns of 5 unidentified "grey isolates" from grapevines with reference to *Phaeoacremonium* species.

Isolate number	Morphological identification	Origin	Host	Colony diameter (mm)		Restriction pattern type
				30°C	35°C	
CBS 391.71[a]	*P. inflatipes*	USA, Texas	*Quercus virginiana*	56	5	*P. inflatipes*
LCP 96.3899	?[b]	Chile	*Vitis vinifera*	55	5	*P. inflatipes*
CBS 246.91[a]	*P. aleophilum*	Yugoslavia	*Vitis vinifera*	35	10	*P. aleophilum*
CBS 249.95[a]	*P. angustius*	California	*Vitis vinifera*	38	10	*P. aleophilum*
IMI 181 115[a]	*P. parasiticum*	California	Human	55	42	*P. parasiticum*
CBS 736.94	*P. inflatipes*	Finland	Human	52	42	*P. parasiticum*
CBS 139.69	*P. inflatipes*	Tahiti	Soil	53	34	*P. parasiticum*
CBS 651.85	*P. inflatipes*	Venezuela	Human	57	42	*P. parasiticum*
CBS 694.88	*P. inflatipes*	Hawaii	Human	58	18	Unique
CBS 428.95	*P. inflatipes*	Germany	*Sorbus intermedia*	12	0	Unique
CBS 408.78	*P. inflatipes*	California	Human	58	30	Unique
Arg. 0	?[b]	Argentina	*Vitis vinifera*	50	44	Unique
Arg. 2	?[b]	Argentina	*Vitis vinifera*	-	-	Unique
Arg. 3	?[b]	Argentina	*Vitis vinifera*	-	-	Unique
Arg. 5	?[b]	Argentina	*Vitis vinifera*	47	14	Unique
T16	?[b]	Turkey	*Vitis vinifera*	60	5	Unique

[a] Type strain of the species.
[b] Unidentified strain.

the ß-tubulin gene. Thirty-four additional isolates collected in Argentina, California, France, Italy and Turkey, were observed, checked for their optimal growth temperature, and compared using restriction analysis of their whole ITS region with *Dde*I, *Hae*III, *Msp*I, *Rsa*I and *Taq*I.

Within the ITS rDNA region (500 bp), only two nucleotidic differences (0.3%) were observed in the ITS1 between the *P. aleophilum* (GenBank No.: AF 017651) and the *P. angustius* (AF 118138) type strain sequences, one of them generating a supplementary and easily visualized *Rsa*I site in *P. angustius* (Dupont *et al.*, in press). The 34 additional isolates digested with *Rsa*I were all of the *P. angustius* pattern type. No polymorphism was observed among the 36 isolates with the other enzymes.

The sequences of the 5' end of the ß-tubulin gene (440 bp) of the two type strains were strictly identical (GenBank No.: AF192390 for *P. aleophilum* CBS 246.91) even within the introns which are often variable at the interspecific level (Geiser *et al.*, 1998; Aoki and O'Donnell, 1999) and were also observed in the other species of the genus *Phaeoacremonium* (Dupont *et al.*, in press).

Morphologically, we agree with Crous *et al.* (1996) that the type strains of the two species are distinguished by their conidial size and shape and we found both conidial types among the other 34 isolates. However, we observed uniform optimal growth for all the isolates at 30°C. This finding was not consistent with the original diagnosis, where optimal growth was described as occurring at 35°C for *P. aleophilum* and 25°C for *P. angustius*. We noted a high sensibility of some isolates, including the *P. aleophilum* type strain, to daylight.

Considering the totality of morphological, cultural and genomic characters, we concluded that the two species were conspecific and retained the designation *P. aleophilum* (Dupont *et al.*, in press).

P. viticola, a new species

Among the numerous isolates collected from diseased grapevines that we received for identification, 2 from California and 8 from France were microscopically similar to existing *Phaeoacremonium* species but differed by the light colour of their mycelium and the bluish-red colony reverse. Significant physiological differences we found between these isolates and the other species of the genus□: their optimal growth was at 25°C instead of at 30°C, and they exibited a characteristic inability to grow at 35°C. Their colony diameters at 25°C (28-34 mm) after 15 days incubation were comparable to those of *P. aleophilum* (24-32 mm) and consistently smaller than those of *P. parasiticum* (38-42 mm), *P. rubrigenum* (36-40 mm) and *P. inflatipes* (44-45 mm). Like *P. aleophilum*, they diffused a yellow pigment in the medium and had cylindrical conidia, while conidia from the other species were ellipsoidal.

From the molecular point of view, the phylogenetic analysis placed *P. viticola* within the Magnaporthaceae lineage, closely related to *P. aleophilum*, from which it differed by 15 nucleotides within ITS1, 3 within ITS2 and 60 within the ß-tubulin region sequences. The differences observed with the other species are detailed in Table 2. The 10 isolates we used to describe this species (Table 1) showed a perfect genomic homogeneity in the restriction patterns of the two DNA regions, using *Dde*I, *Hha*I, *Hae*III, *Msp*I, *Rsa*I and *Taq*I.

We designated this species as *Phaeoacremonium viticola* J. Dupont (Dupont *et al.*, in press).

Molecular analysis of "grey *Phaeoacremonium* isolates"

To identify the grey isolates that we received from Argentina (Arg. 0, Arg. 2, Arg. 3 and Arg. 5), Chile (LCP 96.3899) and Turkey (T16), but never found in French vineyards, we compared the morphology, growth at different temperatures and ITS restriction profiles of these isolates with isolates of *P. parasiticum* and *P. inflatipes* to which they appeared phenotypically similar (*P. aleophilum* was also included to have an overview of the *Phaeoacremonium* species involved in esca, though it was clearly morphologically distinct by its brownish colonies diffusing a yellow pigment on MA medium). The reference strains used for comparison are listed in Table 3 with the results.

As a first molecular approach, restriction analysis of ITS using *Dde*I, *Hha*I and *Taq*I distinguished *P. inflatipes* from *P. parasiticum* isolates (and from *P. aleophilum*, see *Hha*I patterns in Fig. 1). The restriction patterns obtained were consistent with the theoretical profiles deduced from the sequences using the DNA Strider computer program. *Bgl*I, *Msp*I and *Rsa*I were tested but were not discriminant. The patterns which did not cor-

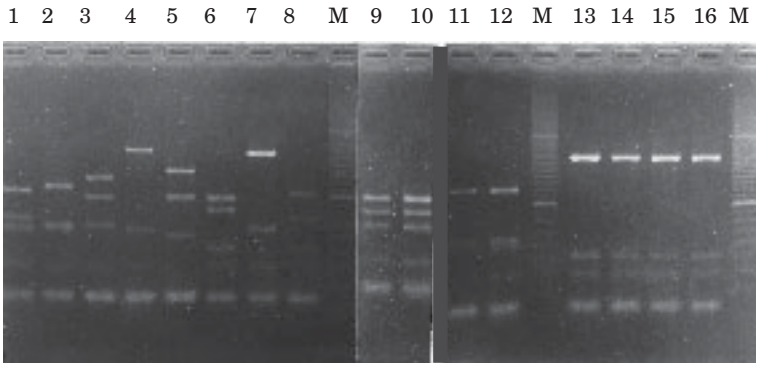

Fig. 1. ITS-RFLP patterns of *Phaeoacremonium aleophilum*, *P. inflatipes*, *P. parasiticum* and unidentified isolates using restriction endonuclease *Hha*I. Lanes 1-8: unique patterns (CBS 694.88, T16, Arg.2, Arg.0, Arg.3, CBS 408.78, CBS 428.95, Arg.5). Lane 9: *P.aleophilum* (Type strain CBS 246.91). Lane 10: *P. angustius* (Type strain CBS 249.95). Lanes 11 and 12: *P. inflatipes* (Type strain CBS 391.71, LCP 96 3899). Lanes 13-16: *P. parasiticum* (Type strain IMI 181 115, CBS 736.94, CBS 139.69, CBS 651.85). M, size marker (high-intensity bands: 200 and 500 bp).

respond to those of *P. parasiticum* and *P. inflatipes* were recorded as unique and ITS sequencing will be necessary to determine the exact nucleotidic divergence of these isolates. However, isolate Arg. 0, differing from *P. parasiticum* only by the *Hha*I pattern, was classified as unique in Table 2, although it could be closely related to this species, as was suggested by its morphology. The Turkish isolate T16 was also morphologically similar to *P. parasiticum,* although its restriction patterns were different. Arg. 2 was characterized by short cylindrical phialides, atypical of *Phaeoacremonium* but related to those of *Phialophora*. Arg. 5 was morphologically similar to CBS 408.78 but different in its ITS profiles and is still unidentified. The culture of Arg. 3 on MA plate revealed a contamination by an *Acremonium* species.

Among the *P. inflatipes* CBS strains (Crous *et al.*, 1996), 3 were re-interpreted as *P. parasiticum* by the molecular approach. This result was corroborated by the microscopic examination and by the growth behaviour of the strains at different temperatures. *P. inflatipes* was characterized by low growth at 35°C (5 mm after 15 days) compared with *P. parasiticum*, which achieved growth of 42 mm at that temperature. More analysis is in progress to confirm this identification.

Moreover it seems there is often misidentification between *P. inflatipes* and *P. aleophilum* concerning Californian isolates (all isolates we received from the USA were confirmed as *P. aleophilum* by restriction analysis). The results suggest that *P. inflatipes* has a minor incidence in esca.

Conclusion

The delimitation of species is a dynamic process in taxonomy, in which the boundaries must be continually adjusted as more isolates are analysed and more methods devised to search for phylogenetically informative characters (molecular, physiological, morphological, ecological, …). Some species were restricted, like *P. aleophilum* and *P. angustius,* for which similar genotypes were observed. *P. inflatipes* appeared to be rare and more isolates are needed to investigate this species. Conversely, genomic variability was observed within *P. parasiticum*, as already noticed by Yan *et al.* (1995), and more sequencing work is in progress to better delimit this species. Among fungal species previously defined on the basis of their morphological characters, the intraspecific nucleotidic variability observed within the ITS rDNA was large, ranging from almost nil in *Fusarium oxysporum* (Avelanche, 1994), to 16% in *Puccinia recondita* (Zambino and Szabo, 1993) and 11% in *Beauveria brongniartii* (Neuvéglise *et al.*, 1994) (for a review see Biju-Duval, 1994). The differences observed were largely dependent on the number of morphological characters available to describe the species and on their phylogenetic value. It is important to remember

that the number of isolates studied needs to be large enough to permit a good statistical analysis.

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