MOLECULAR AND MORPHOLOGICAL CHARACTERIZATION OF PHAEOMONIELLA ISOLATES ASSOCIATED WITH EUTYPA DIEBACK AND ESCA OF GRAPEVINE IN NORTH ALGERIA

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Résumé

Les maladies du bois, en particulier l'esca et l'eutypiose, sont considérées parmi les plus graves affections de la vigne, car elles affectent la productivité et la longévité des vignobles. Les champignons responsables de ces maladies sont lignivores et se repartissent en deux séquences dans le processus de dégradation du bois. Phaeomoniella chlamydospora est l'une des espèces pionnières qui colonisent le bois sain. C'est aussi, l'agent causal de la «maladie de Petri », un dépérissement touchant les jeunes vignes. Récemment, plusieurs espèces appartenant au genre Phaeomoniella ont été identifiées sur de nombreuses autres cultures ligneuses.

La présente étude a pour but la caractérisation morphologique et moléculaire d'une collection de 84 isolats appartenant au genre Phaeomoniella, isolés à partir de ceps présentant des symptômes typiques d'esca et d'eutypiose. En vue d'une approche globale de caractérisation taxonomique, un génotypage par la technique MSP-PCR (Microsatellite-Primed PCR) a permis d'analyser la diversité génétique de l'ensemble de la collection, en regroupant les isolats en fonction de leurs empreintes génétiques. Les isolats représentatifs de chaque groupe ont ensuite été sélectionnés pour le séquençage et l'analyse phylogénétique basés sur la région ITS. Deux méthodes ont été utilisées pour cet effet, la méthode du Neighbor-Joining (NJ) et celle du Maximum de Parcimonie (MP).
Dans la présente étude, seule l'espèce Phaeomoniella chlamydospora s'est avérée être associée aux symptômes d'esca et d'eutypiose, ce qui confirme son implication et son importance dans les maladies du bois de la vigne, dans le monde et plus particulièrement en Algérie.

Mots clés : *Eutypiose, esca, taxonomie, génotypage, phylogénie*

INTRODUCTION

Eutypa dieback and esca are very destructive grapevine decline diseases that occur in most countries where grapevine is cultivated. They are a major concern because they decrease the lifespan of vineyards. Symptoms of these diseases are well-characterized. Symptoms of Eutypa dieback appear in early spring as stunted shoots with small, chlorotic cup-shaped leaves with a necrotic margin. Cross-sections of arms and trunks of infected vines show wedge-shaped discoloured sectors [1]. Esca can typically be identified by internal wood decay, and by symptoms on leaves, and in some cases on the berries [2]. The disease can appear in a mild form, which is characterized by leaf alterations [3] or a severe form, characterized by sudden wilt of the plant often called “Apoplexy”. The internal symptoms include black spots and dark brown to black streaking of the xylem tissues.

The damage has been reported in grapevines wherever they are grown worldwide with increased severity year by year [3]. Several studies have shown that several different fungi are associated with Eutypa dieback [4-5] and with esca [5-6-7]. The most frequent fungi are *Eutypa lata*, the cause of Eutypa dieback [8], several species of *Phaeoacremonium* [9-10-11], *Phaeomoniella chlamydospora* [12], several species of *Botryosphaeriacea* [13], and the Basidiomycete *Fomitiporia mediterranea* [14]. The survey performed by Berraf and Péros [15] and Berraf-Tebbal [16] revealed that the fungal community in decaying vines in Algeria was similar to the fungal communities observed in other countries. *Phaeomoniella chlamydospora* has been one of the most studied as the causal agent of "Petri disease".

Recent studies have shown that several *Phaeomoniella* species also cause disease on many other woody crops, such as forest trees and woody ornamentals. Two new species, *Phaeomoniella zymoides* H.B. Lee, J.Y. Park, R.C. Summerbell et H.S. Jung, and *Phaeomoniella pinifoliorum* H.B. Lee, J.Y. Park, R.C. Summerbell et H.S. Jung, were isolated from the needle surface of *Pinus densiflora* Sieb. et Zucc. in Korea [11].

The aim of this study was to identify the different *Phaeomoniella*-like isolates associated with eutypa dieback and esca in North Algeria. For that objective, we examined a large sample of decaying vines and strains were identified based on their morphology and a comparison of DNA sequences data for ITS rDNA.

Materials and methods

Analysis of internal symptoms and isolation

Branches and trunks displaying symptoms of dieback were collected from vineyards of Cinsaut cultivar, in the main production areas in the north of Algeria. Cross and longitudinal sections of the trunks and arms of each vine were examined to record the type and localisation of wood necrosis. Isolations were made from each type of necrotic tissues. For each lesion detected, 10 pieces of wood (10 x 5 x 5 mm) were cut from the margin of the soft white rot, the sectorial and the central brown zone and the black spots as described by Larignon and Dubos, [6] and Berraf and Péros [15]. The pieces of wood were surface disinfested with calcium hypochlorite (3% active chlorine) for 10 min, rinsed twice in sterile water and then placed on potato-dextrose agar (PDA, Difco Laboratories, Detroit, Michigan, USA) plates. Plates were incubated at room temperature and observed every 2–3 days for two months. Colonies emerging from wood pieces that were morphologically recognized by their yeast-like growth with dark green mycelium as *Phaeomoniella* species [12] were transferred to fresh PDA plates and incubated at 25°C. Morphological characters included conidiophores morphology, phialide type and shape, size of hyphal warts were noted after 16 days of incubation at 25°C on PDA. Colony colors were defined after 16 d using the color charts of [17].

DNA isolation

Genomic DNA of all isolates identified morphologically as *Phaeomoniella* was extracted from fresh mycelium grown on PDA plates in darkness at 25°C for 2–3 weeks according to Abdollahzadeh [18].

MSP-PCR profiles

The *Phaeomoniella* isolates were initially characterized on the basis of their microsatellite primed-PCR (MSP-PCR) profiles as described by Zhou *et al.* [19], Primers used for the MSP-PCR were the M13 (5'– GAG GGT GGC GGT TCT– 3') [20]. The reaction mix in a final volume of 25 µl contained 1X PCR buffer (20 pmol of primer, 200 µm of each of dNTP's, 1.25U of Taq DNA polymerase (Fermentas Life Science, Ontario, Canada), $MgCl_2$ at 3 mM and 10 ng of template DNA. The cycling conditions were: 2 min at 94°C, followed by 40 cycles of 30 s at 93°C, 3 s at 53°C and 2 min at 72°C, then a final step of 10 min at 72°C. The amplification products were separated by electrophoresis in 1.5% (W/V) agarose gels in 0.5X TBE (Tris Borate EDTA) for 3h 30min at 80V. Gel electrophoresis images were acquired under UV illumination with the Molecular Imager Gel Doc XR System, Bio-Rad, after staining with Gel Red (Biotium, Hayward, USA). DNA banding patterns were analyzed with GELCOMPAR (version 4.1, Applied Maths 1998 Belgium) using Pearson's correlation coefficient and the dendrogram was computed using the UPGMA clustering method.

Sequence analysis

Representative isolates from all separated clusters in the MSP-PCR profiles were selected for sequencing and phylogenetic analyses. The ITS region was amplified and sequenced with primers ITS1 and ITS4 [21], Polymerase chain reaction (PCR) mixtures and amplification conditions were conducted as described by [16]. PCR products were purified according to the manufacturer's instructions using a commercial Kit (Nucleo Spin Extract II, Macherey-Nagel). The ITS region was sequenced by STAB Vida, Lda (Portugal).

Sequences for the DNA region were retrieved in GenBank using the BLAST (Basic Local Alignment Search Tool) [22].

The sequences of *Phaeoacremonium aleophilum* (CBS 100397; GenBank ITS: AF197981 and CBS 246.91; GenBank ITS: AF017651) were used as outgroups. Sequences were edited with Bio Edit Alignment Editor V.7.0.9.0 and aligned with Clustal X version 1.83 [23]. Alignments were checked and manual adjustments were made when necessary. Phylogenetic analyses were carried out using PAUP v4.0b10 [24] for maximum-parsimony (MP) and Neighbour joining (NJ) analyses. The trees were visualized with TreeView [25].

Results

MSP-PCR

A total of 84 isolates of *Phaeomoniella* species were obtained from vines with esca and eutypa dieback symptoms. All isolates were typical *Phaeomoniella* anamorphs with slow-growing colonies that gave visible growth after up to 15 days of incubation and recognized by their yeast-like growth. A variability analysis was done to assess genetic diversity within the *Phaeomoniella* isolates. The bands produced by MSP-PCR profiles divided the strains into 3 clusters and 5 singletons with a reproducibility level of 80% (Fig. 1). Representative isolates from each group and, when possible, isolates from Eutypa dieback and esca symptoms were selected for the phylogenetic analysis.

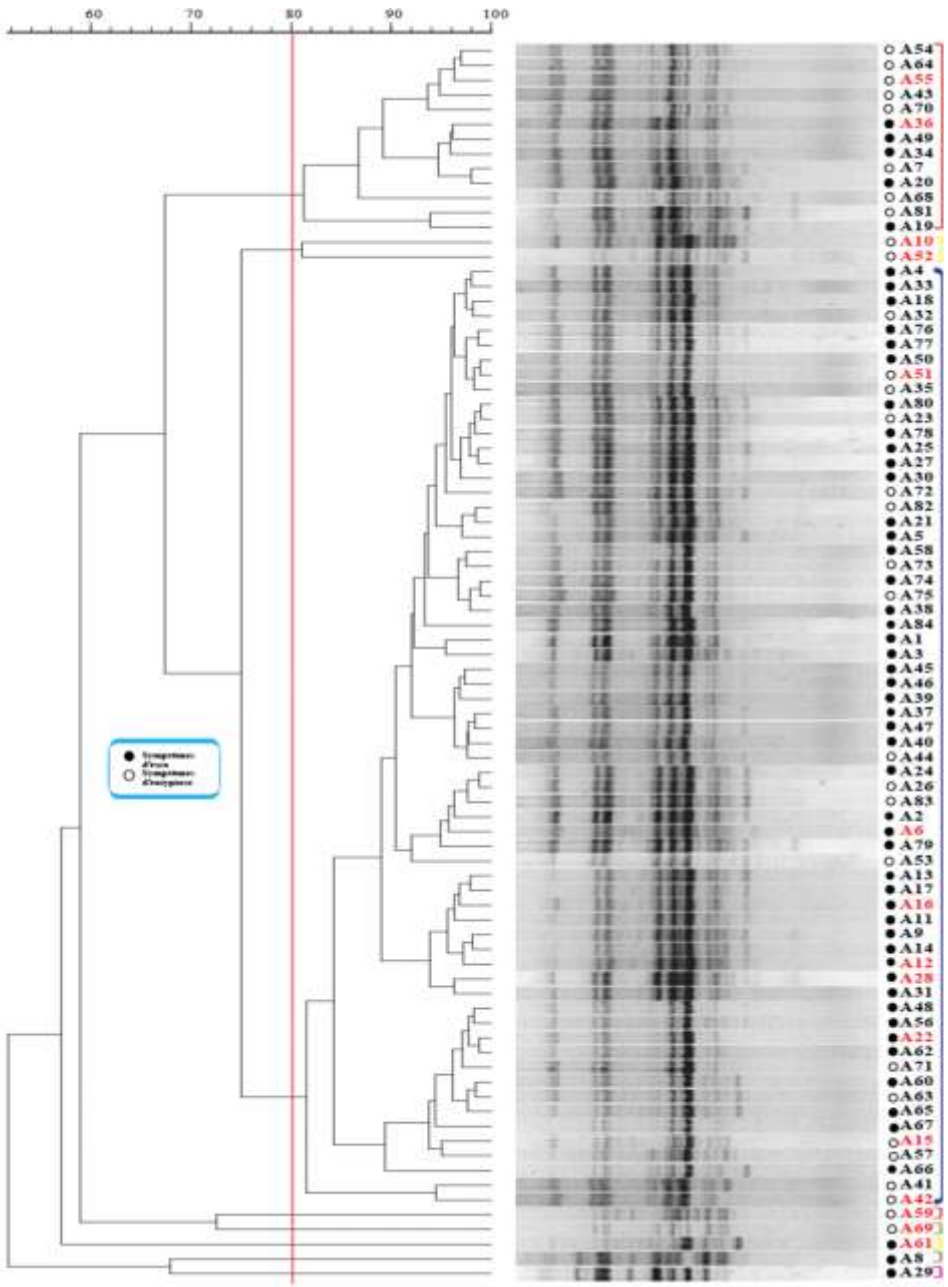

Fig. 1 Consensus dendrogram from MSP-PCR profiles obtained with primer M13. The vertical dashed line corresponds to the reproducibility level (80%) from which nine groups of isolates are inferred (indicated by numbered circles). In each group, isolates highlighted in boldface were selected for phylogenetic analysis. All fingerprints were grouped by similarity using the Pearson correlation coefficient and UPGMA. Isolates obtained in this study from vines with eutypa dieback or esca symptoms are indicated by white and black circles, respectively.

Phylogeny

The ITS sequences for the 16 isolates selected from the MSP-PCR profiles were combined and aligned with sequences of 18 isolates retrieved from GenBank, representing a selection of all known *Phaeomoniella* species. The combined alignment consisted of 577 characters (including alignment gaps). Of these, 279 were parsimony informative, 30 were variable and parsimony uninformative and 268 were constant. After a heuristic search 2 parsimonious trees with the same overall topology were retained (CI = 0.756; RI = 0.858, HI = 0.244). One of the trees is shown in Fig. 2. DNA sequences were compared with those available in GenBank using Neighbor-joining (NJ) and Maximum-parsimony (MP) analyses. The phylogenetic trees of the ITS region revealed that the *Phaeomoniella* isolates clustered with *Phaeomoniella chlamydospora* reference sequences with a bootstrap support of 100 %.

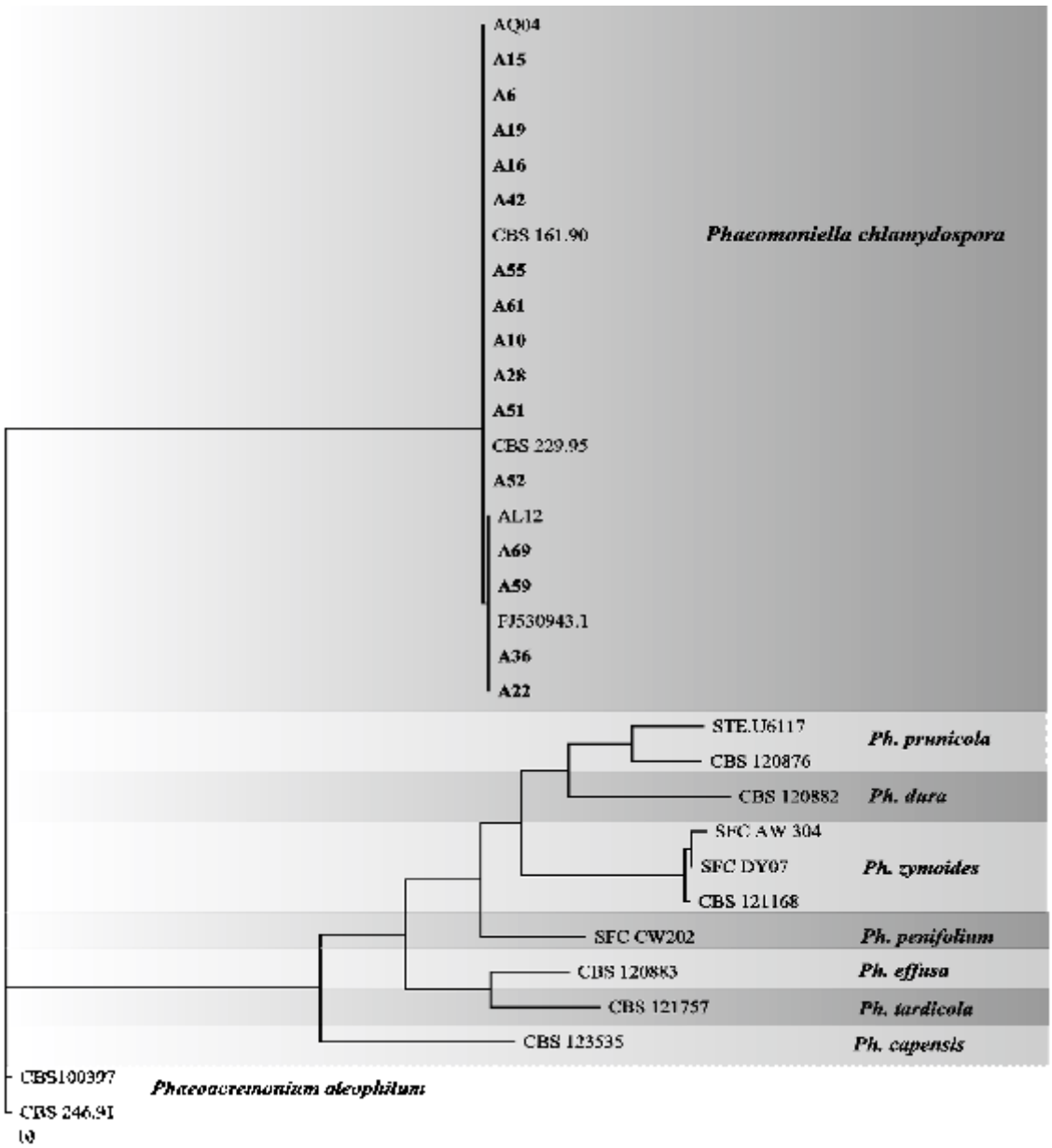

Fig. 2 One of 2 equally parsimonious trees resulting from the alignment of 577 characters of ITS sequences. Consistency index (CI) = 0.756; retention index (RI) = 0.858; homoplasy index (HI) = 0.244. All positions containing gaps and missing data were excluded from the dataset. Newly generated sequences are highlighted in boldface. Bootstrap values from 1000 replications are shown for Maximum Parsimony (MP) and Neighbour-Joining (NJ) at the tree nodes (MP/NJ). Branches marked with a minus (–) are not present in NJ tree. *Phaeoacremonium aleophilum* (CBS 100397; GenBank ITS: AF197981 and CBS 246.91; GenBank ITS: AF017651) were included as outgroups.

Morphology

On PDA, the colonies are first Buff (19" f) and Dark Green (33 m) to become Leaden Black (47"" 'k). They are circular with smooth texture (Fig. 3a). The mycelium is branched, greenish brown (Fig. 3b.c) showing conidiophores simple, short, thick and dark. Phialides are paler (Fig. 3f-l). The conidia are hyaline, ellipsoid to oblong, clustered at the apex of phialide (Fig. 3k.m.n) and measuring (2.23 -) 3.06-3.33 (-4.45) x (1.05) 1.41-1.53 (-2.25) µm; the mean and standard deviation of 50 conidia are 1.47 ± 0.29 x 2.21 ± 0.41.

Chlamydospores appear on old cultures (more than one month); they are abundant, globose to sub-globose, mostly solitary (Fig. 3c.f.) but find also in chains (Fig. 3d). They are characterized by their thick-walled, olive-green color. The colony grows very slowly. It reaches a diameter of 2.5 mm after eight days of incubation at 25 ° C on PDA. An optimum growth is achieved at 25 ° C, a minimum of 15 ° C and maximum 35 ° C.

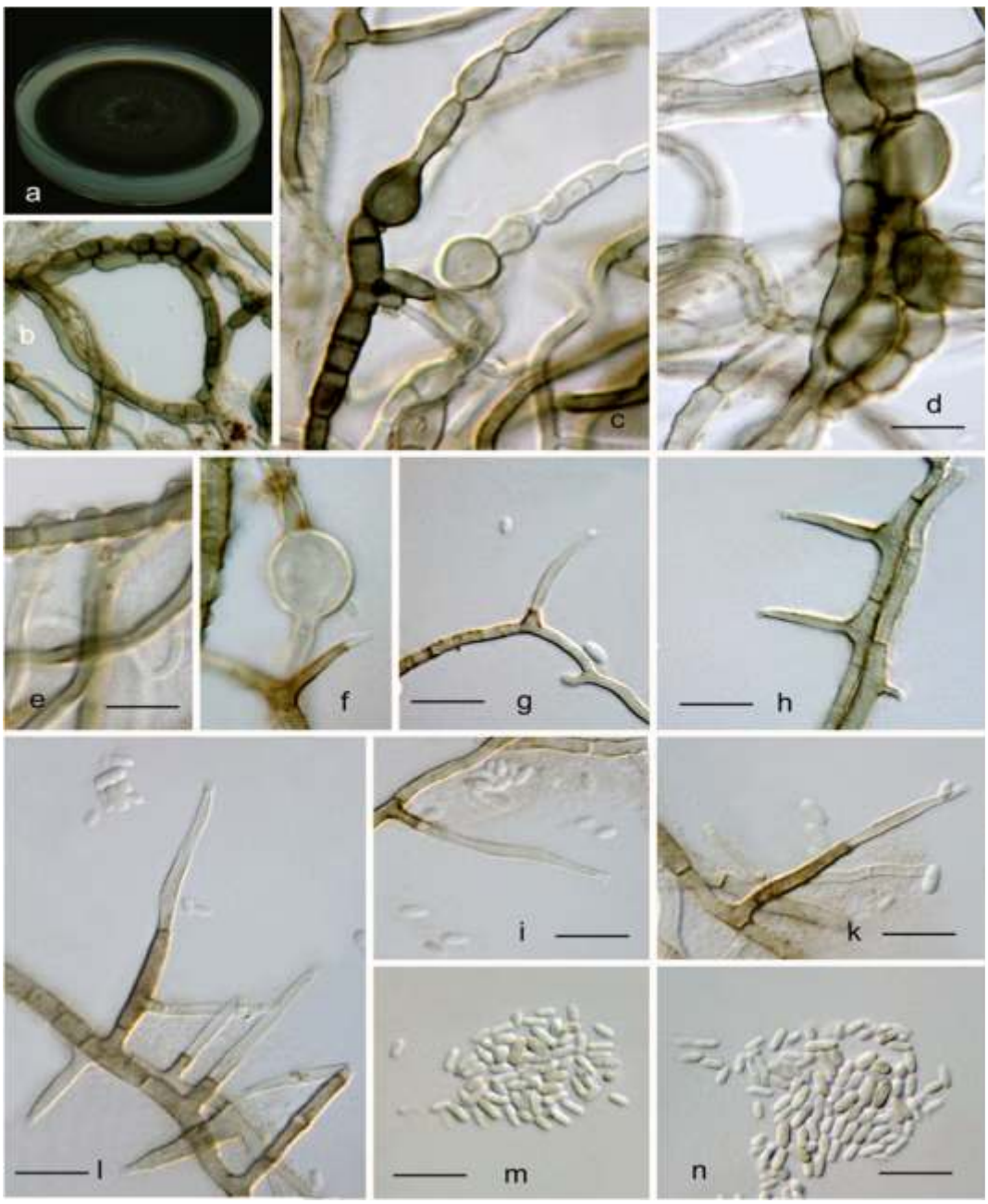

Fig. 3 *Phaeomoniella chlamydospora* a. colony on PDA (one month old). b-d. mycelium with chlamydospores. e. mycelium with exsudats, f. g.i.k. phialides. h. i. branched conidiophores; m.n. conidia. Bars= 10µm.

Discussion

In this study a large collection of *Phaeomoniella* isolates associated with grapevine decline in North Algeria was studied. It constitutes the first attempt to assess the diversity of *Phaeomoniella* species on grapevines showing eutypa dieback and esca symptoms. The identification was based on morphological characters and phylogeny analysis.

The isolates initially were grouped according to their MSP-PCR profiles, and representative isolates of each group were selected for sequencing of the ITS. As a result, only *Phaeomoniella chlamydospora* was identified among species of *Phaeomoniella* genus. This species is involved in esca syndrome and Petri disease, dieback affecting young vines [26]. Péros et al. [27] also indicate its presence in vines showing symptoms of eutypa dieback in France. In Australia, *P. chlamydospora* is found in most grapevines expressing symptoms of esca and Petri diseases [28]. This species is recognized as the most common one on grapevines worldwide. In the case of esca, it would play a pioneering role in promoting the installation of *Fomitiporia mediterranea*, the causal agent of white rot [6]. *Phaeomoniella chlamydospora* may enter in several ways in the trunks, but it could also be a revealing pathogenic endophyte under stress [2]. *P. chlamydospora* is a mitosporic fungus originally described as a species of *Phaeoacremonium* [29] but, upon further examination, it was found to be morphologically and phylogenetically different from other species in the genus, so that a new genus denoted *Phaeomoniella* Crous et W. Gams was established. *P. chlamydospora* is distinct from *Phaeoacremonium* in having yeast-like growth in culture, prominently darkened conidiophores in the basal part, and subhyaline and straight conidia [12]. No teleomorph directly connected to the genus *Phaeomoniella* is known yet, and its disease cycle remains largely unknown. The complexity of the pathosystems vine-eutypa dieback and vine-esca shows clearly the need to identify unambiguously the fungal component in order to allow a better understanding of the etiology of these diseases and justify the establishment of control strategies against these fungal agents.

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