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Molecular and phenotypic characterization of *Phaeoacremonium* and *Phaeomoniella* populations from esca diseased grapevines

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To
my dear father

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Chapter 1

General Introduction and Outline of Thesis

GENERAL INTRODUCTION

In recent years, vineyards have experienced a dramatic increase in grapevine trunk diseases, that cause a drastic decline in the health of grapevines, affect the viability of newly planted vineyards, limit the long-term sustainability of wine grape production and inhibit the productivity of, and in many cases kill, mature vines and consequently lead to important losses in the majority of the grapevine producing countries of the world.

In this thesis, special interest was given to Esca diseases complex, the most destructive trunk diseases of grapevine worldwide, which present a great concern to most producers, viticulturists and researchers.

ESCA DISEASES COMPLEX: STATE OF THE ART

Esca disease, up to the end of the eighties, was known as a wood decay disease. The wood dysfunction was assumed to alter water transport affecting plant growth. The name Esca indirectly refers to the fruiting bodies of certain wood-rotting fungi. For example, *Fomes*, which in Latin means “tinder” is the name of a genus of basidiomycetes once used to make a dry, easily ignited material suitable to start fires when using flints. Wood decayed by these fungi, including rotted grapevine wood, burned slowly and was used to keep fires aglow without a flame. Therefore the term Esca, a Latin word meaning food, aliment, or, figuratively, bait, was used to indicate the disease that changed the wood in a tinder to start fires. The disease has long been known wherever grapes are grown. Studies on the disease started at the end of the nineteenth century in France, however, only in the 1990s, research on esca and its etiology have been intensified. This came after a dramatic increase in the disease, especially in Germany, Italy, and Greece, where the arsenites, traditionally used to keep esca under control, have been banned and were replaced by less effective fungicides. Nevertheless, even in France, Portugal, and Spain, where restricted use of sodium arsenite was still allowed, esca has been widespread in all vine-growing regions (Mugnai *et al*., 1999).

After this upsurge of the disease, research started in France and soon after in Italy (Larignon & Dubos, 1987; Mugnai *et al*., 1996; Larignon & Dubos, 1997; Mugnai *et al*., 1999; Surico *et al*., 2001) and showed that the disease has a much more complex aetiology. According to Surico *et al*., (2008), esca develops as a complex of five syndromes, all of which are related, and which form the esca disease complex. These syndromes are:

1. Brown wood streaking of rooted cuttings;
2. Petri disease;
3. esca (young esca);

4. white rot;

5. esca proper (young esca plus white rot).

Brown wood streaking disease

This disease name is only used to designate a wood discoloration – brown streaking – following a vascular infection, by the vascular pathogens involved in the esca complex that affect only rooting, grafted or not grafted, cuttings ready for planting. There is no foliar sign of the infection while the plantlet is in the nursery, but the latent wood infection can lead to further diseases when the plant is in the field, in its following life stages (Petri disease and/or esca). The cuttings show in longitudinal section, mainly in the rootstock, brown to black streaking, vessels full of tyllosis and black gum exudates (Bertelli *et al*., 1998; Surico *et al*., 2006; Mostert *et al*., 2006) (Fig. 1).

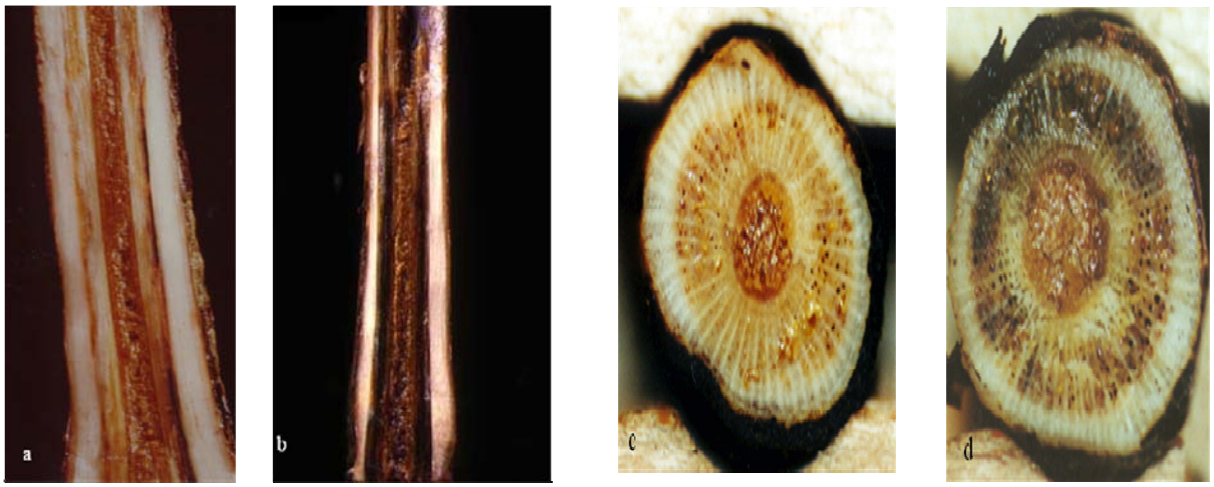

Fig. 1. Symptoms associated with Brown wood streaking disease; a & b. Longitudinal section made in the rootstock showing brown and black streaking. c & d. Cross section showing vessels full of tylosis, gums and phenolic compounds secreted by the host as a reaction to the presence of the fungus in the xylem tissue.

Petri disease of grapevine

Petri disease was first noticed on diseased Italian vines in 1912 (Petri 1912). It is a vascular disease associated with decline and dieback of young grapevines, which was, formerly also known as black goo, young vine decline, slow dieback, *Phaeoacremonium* grapevine decline, and affecting young vines (2-7 yr old), in many newly planted vineyards (Ferreira *et al.,* 1999, Morton, 2000; Mugnai *et al.,* 1999; Pascoe & Cottral, 2000; Scheck *et al.,* 1998).

Field symptoms associated with Petri disease include stunted growth, vine decline, shoot dieback and gradual death of young grapevines. Other field symptoms include a high failure rate in the first year of planting; apparent normal growth the first and second year and then failure to reach normal size; weak plants at the row ends; abnormally low resistance to water

stress, low soil fertility or low crop loads and inconsistent disease expression of the same plant from year to year (Morton, 2000).

Internal symptoms can normally be seen in the trunk and shoots. These include black spots when vines are cut transversely, and dark brown to black streaking when trunks or shoots are cut longitudinally. The damaged xylem vessels often ooze black sap and therefore, the popular name "black goo" was commonly used to describe this disease (Fig. 2).

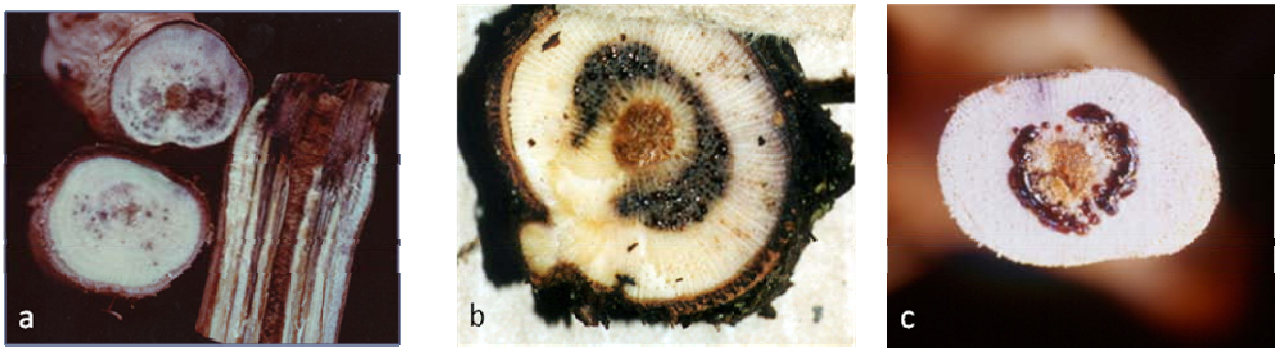

Fig. 2. Typical internal symptoms of Petri disease; a. Longitudinal and transversal cuts showing black spots and streakings. b & c. Cross section showing black gummy masses in the xylem.

The black discolouration of the xylem tissue is caused by the formation of tylosis, gums and phenolic compounds by the host as a reaction to the presence of the fungus in the xylem tissue (Mugnai *et al.*, 1999). The blocking of the xylem tissue prohibits the normal uptake of water. During times of high water demand the host is predisposed by water stress (Ferreira *et al.*, 1999) leading to an increase in Petri disease symptoms. According to Petri, brown wood streaking predisposed the vine plant to wood decay or even represented the first signs of esca.

Petri disease has been recorded in grape-growing regions all over the world, for example in South Africa (Ferreira *et al*., 1994), France (Larignon & Dubos, 1997), the USA (Scheck *et al*., 1998), Australia (Pascoe, 1999), Italy (Mugnai *et al*., 1999), Argentina (Gatica *et al*., 2000), Austria (Reisenzein *et al*., 2000), Portugal (Chicau *et al*., 2000; Rego *et al*., 2000) and Turkey (Erkan Ari, 2000). It is caused by *Phaeomoniella* (*Pa.*) *chlamydospora* (W. Gams, Crous & M.J. Wingf. & L. Mugnai) Crous & W. Gams and several species of *Phaeoacremonium* (*Pm.*) W. Gams, Crous & M.J. Wingf. (Groenewald *et al.*, 2001; Mugnai *et al.*, 1999; Scheck *et al.*, 1998). *Pa. chlamydospora* has been more often associated with typical Petri disease symptoms than species of *Phaeoacremonium* (Mugnai *et al*., 1999; Chicau *et al*., 2000; Edwards & Pascoe, 2004). A major means of spread of the causal organisms, *Pa. chlamydospora* and *Phaeoacremonium* spp., is via infected propagation material. Since no curative control measures are known, proactive measures must be taken in grapevine nurseries to manage this disease.

Esca (young esca)

This disease affects 2-3 yr. and older vines, which exhibit the typical leaf symptoms of esca, and internally the dark streaks, colonized by *Pa. chlamydospora* and sometimes also by *Phaeoacremonium aleophilum*.

Symptoms inside trunk and main branches

Various other types of wood deterioration are visible :

- Small, dark brown or black spots in cross section appear as deep brown or black streaks or columns in longitudinal section. In cross section, the spots are sparsely distributed or arranged in groups around an annual growth ring or in the woody tissues close to the pith.
- Pink-brown or dark red-brown areas often develop from black spots, mainly in the core of the trunk or on the margin of discolored or necrotic tissues, separating such tissues from apparently healthy tissue.
- Brown areas of varying shade and texture are intermingled with the above types of discoloration, often in sectors connected with large wounds.

The various types of wood discoloration are the result of a number of structural and physiological changes:

- Physical and chemical changes caused by the introduction of air and water into wounds and the host reactions to such wounding (e.g., degradation, oxidation, and darkening of tissue components)
- Changes caused by cellulolytic and ligninolytic enzymes produced by the fungi associated with esca
- Tylosis induced by growth-regulating substances, and vascular occlusion due to gels and gums secreted by the diseased xylem parenchyma cells, or even to high-molecular weight compounds produced by the pathogen(s)
- Necrosis of xylem parenchyma cells as the result of diffusion of the pathogen's toxins host reaction products (e.g., phytoalexins), or both (Sparapano *et al*., 1998).

Symptoms on leaves

Symptoms on leaves consist of light green or chlorotic, rounded or irregular spots between the veins or along the leaf margins that usually spread outward to the distal parts of the shoots. The spots, initially small and scattered over the lamina, gradually expand and coalesce, become partly necrotic, and ultimately leave only a narrow strip of unaffected green tissue

along the main veins. As the chlorotic tissue turns yellow-brown or red-brown (or, in the case of certain cultivars, develops rust-colored necrotic areas with dark red margins), the diseased leaves assume a “tiger-stripes” pattern (Fig. 3). Sometimes the necrotic areas of the lamina dry out and become detached, leaving irregular leaf margins.

Fig. 3. Chlorosis and necrosis on the leaves showing typical “tiger-stripes” pattern.

The appearance of wilt symptoms in the crown could be explained in terms of xylem dysfunction. However, for various reasons, it seems unlikely that leaf necrosis and wilting of shoots and fruits are due to water stress caused by vascular occlusion (Van Alfen, 1989; Calamai *et al.*, 2008). It seems more likely that the foliar symptoms of esca are mainly caused by substances that originate in the discolored woody tissues of the trunk and branches and are then translocated to the leaves in the transpiration stream. These substances can be reaction products of the wood, phytotoxic metabolites excreted by esca fungi, or a combination of both.

Symptoms on shoots and branches

Symptoms can start in spring with delayed and weak growth and end in autumn with reduced lignifications of the canes. In late spring and summer, an irreversible wilt of some shoots or of an entire branch may occur.

Symptoms on berries and clusters

Spotting of berries, sometimes without accompanying foliar symptoms, is common in California and southern Italy and has also been reported from France (Alsace) on adult as well as young vines, especially white grape cultivars (Chiarappa, 1959a; Graniti 1960, Grasso, 1969; Dubos, 1996). Minute dark brown, violet, or purple spots develop more or less abundantly on the skin of the berry, hence the name “black measles” was given to this condition in California (Fig. 4).

Fig. 4. Symptoms associated with esca of grapevine: black measles on the berries: Minute dark brown or purple spots on the berry skin.

Spots are due to browning and necrosis of groups of epidermal and hypodermal cells near the terminal part of a xylem vessel and do not extend to the underlying tissues (Graniti, 1996). Within a grape cluster, the severity of spotting may differ from berry to berry. Even with only light spotting, table grapes are not easily sold on the market. Berries with heavy spotting often show skin cracks and even longitudinal or transverse splits. Such fruits shrivel and dry up or become prey to soft rotting fungi or bacteria. Sometimes clusters on diseased vines show late ripening with low sugar content and altered berry flavor, or a slow wilt of the distal parts, whose berries lose turgor and fail to ripen. The dark spotting of grape berries could result from diffusion through the vascular system of enzymes that oxidize or polymerize phenolic substances in the epicarp, in conditions of high irradiation, but more probably it is caused by toxins translocated to the fruits by the same route. Symptoms can affect only some berries in a single cluster, all clusters on a branch, or a number of branches (Mugnai *et al*., 1999).

White rot

White rot occurs when the vine trunks, and often the main branches are invaded by *Fomitiporia mediterranea*. The rot caused by this fungus is a typical white decay, where the wood is transformed into a soft, friable, spongy mass. Rotted tissues appear creamy yellow or whitish, and in cross section are often bordered by a thick black or dark brown line separating rotted from non decayed wood. The decay usually starts from a large pruning wound on the trunk extending into the woody tissue and either remains restricted to the older part of the wood or spreads along a sector in the woody cylinder.

Esca proper

As mentioned, the esca proper syndrome is found when a vine shows the superimposed effects of all three main fungi associated with esca. The wood symptoms of esca proper are therefore simply those of tracheomycosis, plus those of white rot (Fig. 5).

Fig. 5. Typical wood symptoms in a vine affected by "esca proper" : brown-red wood, black spots, and central white decay.

Fungi that have been associated with "esca proper" symptoms have essentially been for many years only wood-rotting basidiomycetes. In Europe, the more common agent of wood decay on grapevine is *Fomitiporia mediterranea*, and, to a much lesser extent, *Stereum hirsutum* or *Trametes hirsuta* (Mugnai *et al*., 1999; Cortesi *et al*., 2002; Fischer, 2002). On the other hand *Phaeomoniella chlamydospora* and *Phaeoacremonium aleophilum* are the principal hyphomycetes associated with the full range of wood and foliar esca symptoms (Larignon & Dubos, 1997; Mugnai *et al*., 1999). It is the contemporary presence on the same vine of these fungi, and of the two diseases they cause – the white decay and the vascular disease - that is called "Esca proper" to honor the old traditional description of the disease (Surico, 2001), affecting mostly vines older than 15 yr. Another plant pathogenic group of ascomycetes is commonly isolated from the trunks and branches of esca-affected grapevines which is Botryosphaeriacea species. The most commonly isolated are *Diplodia seriata* (= "*Sphaeropsis malorum'*, anamorph of '*Botryosphaeria obtusa*' and *Botryosphaeria dothidea*' (Mugnai *et al*., 1999). Some of these species are claimed by some authors to cause also Black Dead Arm disease, which in different periods and countries, has been identified by different authors and its syndromes described differently (Lehockzky, 1974; Cristinzio, 1978; Rovesti and Montermini, 1987; Larignon *et al*., 2001). Botryospheriaceous fungi may cooperate, when

present, in causing the esca foliar symptoms but are considered agents of different specific diseases, mainly as dieback and canker pathogens of woody host plants (Niekerk *et al.*, 2006).

Apoplexy

"Vine apoplexy" was formerly considered as the acute from of esca. It appears suddenly in the middle of summer as a rapid basipetal wilt of entire vines, including the clusters, or often of single branches. Green, healthy-looking leaves turn pale green, then gray-green, and quickly wither, drying up completely in a few days (Fig. 6).

Fig. 6. A grapevine plant showing "vine apoplexy" with typical foliar symptoms of esca.

Apoplexy is thought to be favored by hot summers, in particular when rainfall is followed by dry, hot weather. It may be connected with a rapid rise in the concentration and activity of toxic metabolites in the crown when the rate of transpiration is high.

Affected vines with such a syndrome do not show any sign of decline before the sudden collapse. Often they die after the apoplectic stroke, but some vines start a very stunted growth the following spring or even in that year. In every case, they all show quite extended decayed tissues. It has been ascertained that apoplexy mostly strikes older vines that already exhibit very extensive rot. And since moreover tracheomycosis can also affect vines of only two years and without any rot, it seems reasonable to assume that apoplexy is above all a condition associated with white rot, and that it is mainly caused by a dysfunction of the conducting system of the plant. However, since apoplexy almost occurs in plants that are also affected by tracheomycosis, it cannot be excluded that the disease is also favoured by the activity of the two tracheomycotic fungi, *Pa. chlamydospora* and *Pm. aleophilum*, most likely by an accumulation of phytotoxines in the leaves.

In this thesis, special attention has been focused on *Phaeoacremonium* and *Phaeomoniella* genera, the principal hyphomycetes associated with esca complex of grapevine.

The genus *Phaeoacremonium* and *Phaeomoniella*: STATE OF THE ART

Hosts

Phaeoacremonium species have been isolated from grapevines and from a range of woody hosts, either as endophytes or associated with wilting, die-back and death of woody hosts. The species more commonly associated with esca of grapevine is *Pm. aleophilum* (teleomorph of *Togninia minima*). *Phaeoacremonium* spp. have in recent years also been isolated from human patients and have been also reported from various countries (Table 1).

Pm. parasiticum, was the first species of *Phaeoacremonium* reported to cause phaeohyphomycosis in humans (Ajello *et al*., 1974). *Phaeoacremonium* species associated with human infections cause phaeohyphomycosis (defined as tissue invasion by fungi with melanised cell walls), usually seen as phaeohyphomycotic cyst, a closed, painless, pus-filled cavity under the skin, seen in biopsy to have a boreder of fungal growth into the surrounding dermis (Fig. 7) (Ajello *et al*., 1974; Crous *et al*., 1996; Padhye *et al*., 1998; Guarro *et al*., 2003). The species of *Phaeoacremonium* most commonly causing human infections are *Pm. parasiticum* and *Pm. krajdenii* (Mostert et al., 2005).

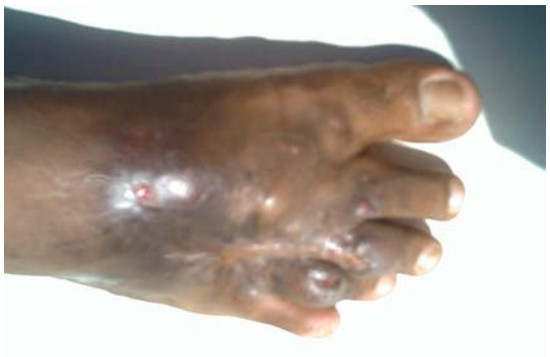

Fig. 7. A foot with white grain eumycetoma caused by *Pm. krajdenii* (photograph by A.A Padhye)

Regarding the *Phaeomoniella* genera, it includes include four species, "*chlamydospora*" which has been reported from different grapevine growing areas all over the world, "*pinifoliorum*" and "*zymoides*," which have been newly reported from the needle surface of *Pinus densiflora* in Korea (Hyang Burn *et al*., 2006) and more recently, Crous *et al*., (2008) described new species named "*capensis*" isolated from living leaves of *Encephalartos altensteinii* in South Africa.

Table 1. List of known *Phaeoacremonium* species, their host / substrate range and world-wide distribution[a].

***Phaeoacremonium* species**	**Host**	**Countries**
Pm. aleophilum	*Actinidia chinensis*, ***Vitis vinifera***, *Olea europaea*, *Prunus pennsylvanica*, *Prunus* sp., *Salix* sp.	Argentina[b], Australia[b], Austria[b], Canada, Chile[b], Iran[b], Israel[b] Italy[b], France[b], South Africa[b,d], Spain[b], Turkey[b], U.S.A[b] and Yugoslavia[b]
Pm. alvesii	*Dodoneae viscosa*, Human, ***Vitis vinifera***	Australia, Brazil[c], Turkey[b], U.S.A[c]
Pm. angustius	***Vitis vinifera***	Portugal[b], U.S.A[b]
Pm. australiense	***Vitis vinifera***, *Prunus salicina*	Australia[b], South Africa[d]
Pm. austroafricanum	***Vitis vinifera***	South Africa[b]
Pm. croatiense **sp. nov.**	***Vitis vinifera***	Croatia[b]
Pm. fuscum	*Prunus salicina*	South Africa[d]
Pm. griseorubrum	Human, ***Vitis vinifera***, *Prunus salicina*	Japan[c], Italy[b], South Africa[d], U.S.A[c]
Pm. hungaricum **sp. nov.**	**Vitis vinifera**	Hungary[b]
Pm. inflatipes	Hypoxylon truncatum, Nectandra sp., Quercus virginiana, ***Vitis vinifera***	Chile[b], Costa Rica, U.S.A
Pm. iranianum	*Actinidia chinensis*, ***Vitis vinifera***, *Prunus armeniaca*	Iran[b], Italy[b], South Africa[d]
Pm. krajdenii	Human, ***Vitis vinifera***	Canada[c], India[c], Japan[c], Norway[c], South Africa[b], U.S.A.[c], Zaire[c]
Pm. mortoniae	*Fraxinus excelsior*, *Fraxinus latifolia*, *Fraxinus pennsylvanica*, ***Vitis vinifera***	Croatia[b], Hungary[b], Sweden, U.S.A[b].
Pm. pallidum	*Prunus armeniaca*	South Africa[d]
Pm. parasiticum	*Actinidia chinensis*, *Aquilaria agallocha*, *Cupressus* sp., Human, *Nectandra* sp., *Phoenix dactylifera*, *Prunus armeniaca*, *Quercus virginiana*, ***Vitis vinifera***	Argentina[b], Australia[b], Brazil[c], Canada[c], Chile[b], Costa Rica, Finland[c], Greece, Iran[b], Iraq, Italy[b], South Africa[b,d], Tunisia, U.S.A[bc]
Pm. prunicolum	*Prunus salicina*	South Africa[d]
Pm. rubrigenum	Human, ***Vitis vinifera***	Croatia[b,] U.S.A[c]
Pm. scolyti	***Vitis vinifera***, larvae of *Scolytus intricatus*, *Prunus* sp.,	Czech Republic, France[b], Italy[b], South Africa[b,d]
Pm. sicilianum **sp. nov**	***Vitis vinifera***	Italy[b]
Pm. sphinctrophorum	Human	U.S.A. [c]
Pm. subulatum	***Vitis vinifera***, *Prunus armeniaca*	South Africa[b,d]
Pm. tardicrescens	Human	U.S.A.[c]
Pm. theobromatis	*Theobroma grandiflorum*	Equador
Pm. tuscanum **sp. nov.**	***Vitis vinifera***	Italy[b]
Pm. Venezuelense	***Human, Vitis vinifera***	Canada[c], South Africa[b], Venezuela[c]
Pm. viticola	*Sorbus intermedia*, ***Vitis vinifera***, *Prunus salicina*	Iran[b], France[b], Germany, Italy[b], South Africa[b,d], U.S.A[b].

[a](Hawksworth and Gibson, 1976; Hausner *et al.*, 1992; Crous *et al.*, 1996; Dupont *et al.*, 1998; Larignon and Dubos, 1997; Ari, 2000; Chicau *et al.*, 2000; Crous and Gams, 2000; Dupont *et al.*, 2000; Pascoe and Cottral, 2000; Péros *et al.*, 2000; Reisenzein *et al.*, 2000, Armengol *et al.*, 2001; Groenewald *et al.*, 2001; Rooney *et al*. 2001, Rumbos & Rumbo 2001, Dupont *et al.*, 2002; Auger *et al.*, 2005; Damm *et al.*, 2005; Eskalen *et al*., 2005; Mostert *et al.*, 2005; Overton *et al.*, 2005; Damm *et al.*, 2008; Essakhi *et al*., 2008).
[b]Countries where *Phaeoacremonium* strains were isolated from *Vitis vinifera*.
[c] Countries where *Phaeoacremonium* strains were isolated from human infections.
[d] Countries where *Phaeoacremonium* strains were isolated from *Prunus* spp.

Epidemiology

The main sources of inoculums of *Pa. chlamydospora* and *Phaeoacremonium* species in vineyards include infected propagation material and aerial spores. The role of soli infection has been hypothesized but has never been demonstrated. Infected mother vines have proven to be a source of infected propagation material (Mugnai *et al*., 1999; Pascoe & Cottral 2000; Rego *et al*. 2000; Fourie & Hallen, 2002; Halleen *et al*., 2003; Edwards *et al*., 2004 & Retief *et al*. 2005a). Propagation material can also get infected during the grafting process (Bertelli *et al*., 1998). Zanzotto *et al.,* (2001; 2007) found infection in rootstock and scion cuttings made from mother plants, and they did find *Pa. chlamydospora* and *Phaeoacremonium* species in certified, grafted plants and 1-year-old plants. These results support the fact that infections by both *Pa. chlamydospora* and *Phaeoacremonium* species take place during nursery operations, as widely shown by other authors and in recent reports also in Italy (Ridgway *et al*., 2002; Halleen *et al*., 2003; Fourie & Halleen, 2004; Retief *et al*., 2006).

The infection of field grapevines can be through the roots or pruning wounds. *Phaeomoniella chlamydospora* has been detected in soil from mother vines with PCR (Damm, 2005; Whiteman, 2003) but it has not been shown the presence of living and actively infecting propagules. *Phaeoacremonium aleophilum* has also been detected in soil and puddles of water under grapevines, by using a filtering system and Rose Bengal Chloramphenicol selective medium (Rooney, 2001). Pathogenicity studies have shown that *Pa. chlamydospora* and *Pm. aleophilum* can infect and colonise grapevine roots (Adalat, 2000), but pruning wounds are the most obvious port of entry for aerial inoculum. Various pathogenicity studies have shown that *Pa. chlamydospora* and *Pm. aleophilum* can readily infect pruning wounds following inoculation with conidia (Adalat, 2000; Larignon, 2000; Sparapano *et al*., 2000, 2001; Feliciano *et al*., 2004). Inoculation of grapevine spurs (cv. 'Chardonnay' and 'Pinot Noir') revealed that *Pa. chlamydospora* is much more aggressive than *Pm. aleophilum* as a pruning wound invader (Adalat *et al*., 2000; Sparapano *et al*. 2001).

Conidia of *Pa. chlamydospora* and *Phaeoacremonium* species can be aerially dispersed. The presence of aerial inoculum of *Pa. chlamydospora*, *Pm. aleophilum* and *Pm. mortoniae* has been detected in the field with Vaseline-covered glass slides (Larignon, 1997; Larignon & Dubos 2000; Eskalen, 2005). *Pm. aleophilum* was also found in symptomatic berries (Eskalen & Gubler, 2001), indicating that berries can become infected during the time when aerial conidia are present. The correlation of rainfall with the presence of aerial conidia showed that conidia of *Pa. chlamydospora* are released during and following rainfall in late winter and early spring in Californian vineyards (Eskalen, 2001). Van Niekerk *et al.,* (2005) correlated

the occurrence of *Pa. chlamydospora* and *Phaeoacremonium* spp. in cordons of mature grapevines with rainfall patterns and found that *Pa. chlamydospora* predominantly occurred in winter rainfall regions, whereas *Phaeacremonium* spp. had a similar distribution pattern, albeit higher incidences in summer rainfall regions.

Aerial spores tend to come from the production of anamorphic or teleomorphic structures on the grapevine surface. Perithecia of *Togninia* (*T.*) *minima* (Tul. and C. Tul) Berl., the teleomorph of *Phaeoacremonium aleophilum* were formed *in vitro* after 2–3 weeks of incubation on grapevine canes on water agar at 22 °C under continuous white light (Mostert, 2003). Rooney-Latham *et al.*, 2005 used however, a 12-h photoperiod with fluorescent light and grapevine shavings to induce perithecial formation after 4–5 weeks. Perithecia of *T. minima* (Rooney-Latham *et al.*, 2005), *T. fraxinopennsylvanica* (Eskalen *et al*., 2005) and *T. viticola* (Eskalen *et al*., 2005) were observed on grapevines in the field. Moreover, perithecia of *T. minima* were also found on dead vascular tissue in deep cracks along the trunks and cordons or on the surface of decayed pruning wounds (Rooney-Latham, 2005). The presence of perithecia in the field indicates that, under the right environmental conditions ensuring enough moisture, ascospore dispersal could also be a source of inoculum. *In vitro* studies showed that forcible discharge of ascospores can take place in rehydrated perithecia, and led Rooney-Latham *et al*., (2004) to conclude that ascospores of *T. minima* may be an important inoculum source in the field. Aerial spore catch data also showed that spores of *Pm. aleophilum* / *T. minima* were indeed present in the air after rainfall (Rooney-Latham, 2004). Diseased vines could release aerial inoculum from freshly cut pruning wounds or across the vine in places that favour anamorph or teleomorph sporulation.

Regarding *Pa. chlamydospora*; although it is known to have a coelomycete synanamorph, no teleomorph has thus far been reported for this fungus. *Pa. chlamydospora* has been detected in wound sap and bark at soil level (Rooney, 2001). The sporulation of the hyphomycete and the pycnidial synanamorphs of *Pa. chlamydospora* have been observed on protected wood surfaces inside deep cracks, 2-to 4-year-old pruning wounds and beneath the bark where injury resulted in exposed vascular tissue of grapevines (Edwards, 2001).

Insects such as fungus-feeding arthropods could also disperse conidia when coming into contact with the phialidic conidial heads and pycnidia of *Pa. chlamydospora* (Edwards *et al.*, 2001a). *Pm. scolyti* was isolated from insect larvae as well as *Pm. parasiticum* and *Pm. mortoniae* from larval galleries inside tree bark (Kubátová *et al*., 2004). Nevertheless, the role of insects in the spread of inoculums on grapevines remain uncertain.

Pathogenesis

Several pathogenicity studies have been conducted to understand the role that the different fungi play in esca diseases complex, especially the pathogenicity of *Pa. chlamydospora* and *Pm. aleophilum* that has been extensively tested on root, shoot and pruning wound with artificial inoculation studies. These studies showed that *Pa. chlamydospora* and *Pm. aleophilum* can cause black streaking and necrosis of xylem tissue, reduced plant growth, cause leaf symptoms and black lesions on grape berries. In their early studies, Larignon & Dubos (1997) concluded that *Pm. aleophilum* and *Pm. chlamydosporum* (= *Pa. chlamydospora*) were pioneering fungi that colonised living wood, whereas the basidiomycete fungi *Fomitiporia mediterranea* were responsible for the typical decay associated with esca.

Inoculation with *Fomitiporia punctata* (later reclassified in the new species *F. mediterranea*) on cultivars Italia and Matilde caused wood deterioration and spongy wood decay after six months, thereby showing its ability to act as a primary pathogen. However, after 3 years from inoculation, some symptoms appeared on leaves of inoculated plants (Sparapano *et al*., 2001), but the vines were not proved to be free from infections by the vascular pathogens. Bruno and Sparapano, (2005), also showed that colonies of *Pa. chlamydospora, Pm. angustius*, *Pm. inflatipes, Pm. parasiticum* and *Pm. viticola* had an antagonistic effect on the colonies of *F. mediterranea* with *in vitro* malt extract assays.

Sparapano *et al*., (2001) observed black measles (spotting on berries) on cv. ‘Italia’ after *Pa. chlamydospora* was inoculated through wounds on spurs and trunks of standing vines and on cv. ‘Matilde’ after inoculation of branches and spurs with *Pm. aleophilum*. The same authors also reproduced foliar symptoms on cv. Matilde and cv. Italia, but in a low percentage, inoculating both *Pa. chlamydospora* and *Pm. aleophilum* (Sparapano *et al.,* 2001). Furthermore, inoculation of pruning wounds with *Pa. chlamydospora* or *Pm. aleophilum* caused esca symptoms on leaves and berries on cv. ‘Thompson Seedless’, on one of the ‘Grenache’ vines and no symptoms developed on cv. ‘Cabernet Sauvignon’ (Feliciano, 2004). Significantly reduced shoot growth were also observed in shoots from infected spurs (Gubler, 2001).

Gubler *et al.,* (2004) carried out another study in order to reproduce esca symptoms by inoculating grape berries with *Pa. chlamydospora* and *Pm. aleophilum,* suggesting that lesions could be caused by airborne inoculum. Lesions on berries were larger when inoculated earlier in the season indicating that young, immature berries were more susceptible to infection than mature berries.

In grapevine nurseries, the grafting of the scion and rootstock canes becomes successful when adequate callus tissue is formed at the grafting wound. Wallace *et al*., (2004) inoculated the bases of seven rootstock and five scion varieties with *Pm. aleophilum* and *Pa. chlamydospora*. As result, *Phaeomoniella chlamydospora* inhibited callus formation on all cultivars, but *Pm. aleophilum* did not, in contrast with previous findings of Adalat *et al*., (2000) who tested the influence of *Pm. aleophilum* on callus formation and proved that this species inhibit callus formation in the cultivar "Chardonnay" more than *Pa. chlamydospora.*

Pathogenicity studies have shown that *Pm. aleophilum* and *Pa. chlamydospora* can infect and colonise grapevines roots (Adalat *et al.,* 2000). However, root symptoms are not typical of diseased vines and can only be rarely found in Petri disease affected vines (Morton, 2000). Root inoculations with these fungi significantly reduced number of roots, plant height, number of internodes, extent of root elongation and accumulation of dry weight in above-ground parts (Scheck *et al*., 1998; Adalat *et al.,* 2000).

Recently, Hallen *et al*., (2007), inoculated grapevine spurs and trunks of cv. *Periquita* with *Pm. krajdenii, Pm. venezuelense, Pm. subulatum, Pm. parasiticum, Pm. viticola, Pm. aleophilum* and *Pa. chlamydospora*. Results obtained from this field trial (evaluated after 14 months) confirmed *Pa. chlamydospora* as the most aggressive pathogen since it produced the largest lesions in the trunks, as well as from the pruning wound inoculation. Furthermore, it was re-isolated more frequently than any of the other fungi, especially from the pruning wounds. However, all the *Phaeoacremonium* species were able to infect, colonise and produce lesions statistically different to those caused by the water control and the non-pathogen confirming their status as possible decline pathogens.

Several substances involved in pathogenesis have been identified from fungi causing esca diseases complex, these include phytotoxic compounds (for *Pa. chlamydospora, Pm. aleophilum, Pm. angustius, Pm. inflatipes, Pm. parasiticum*, and *Pm. viticola*), pectic enzymes (for *Pa. chlamydospora, Pm. aleophilum*) and enzymes involved in lignin degradation (for *Pa. chlamydospora* and *Pm. aleophilum*).

Phytotoxic metabolites extracted from culture filtrates of *Pm. aleophilum* were identified as α-glucans (tentatively identified as pullulans) and two naphthalenone pentaketides (scytalone and isosclerone) (Sparapano *et al*., 2000). These metabolites caused foliar symptoms similar to those shown by esca-affected vines when absorbed by detached leaves or injected into woody tissue of shoots and branches of standing grapevines (Sparapano, 2000). Evidente, (2000) also isolated scytalone and isosclerone from culture filtrates of *Pm. aleophilum* and

showed that these substances cause leaf symptoms on detached leaves; the isosclerone caused pale green to chlorotic, rounded to irregular, interveinal or marginal spots when assayed on detached leaves of cv. 'Italia', while the isosclerone caused large, coalescent chlorotic and necrotic spots followed by distortion of the lamina and withering. Tabacchi *et al*., (2000) isolated p-hydroxybenzaldehyde and scytalone from culture filtrates of *Pm. aleophilum, Pa. chlamydospora* and *F. mediterranea*. These metabolites showed marked toxicity towards grapevine callus growth. Culture filtrates of *Pm. angustius, Pm. inflatipes, Pm. parasiticum* and *Pm. viticola* were found to cause phytotoxic reactions on detached leaves of 'Italia' or 'Sangiovese' grapevines, which was reported to be linked to the production of isosclerone, scytalone and pullulan by these fungi (Bruno & Sparapano, 2005). Pullulan is toxic to plants in general, and in particular causes severe symptoms on grapevine leaves (Sparapano *et al*., 2000). According to Bruno & Sparapano, (2005), pullulan is considered to be the principal phytotoxic element associated with *Phaeoacremonium*.

The production of the pectic enzymes polygalacturonase and polymethylgalacturonase was detected in *Pm. aleophilum* (Marchi *et al*., 2001). Pectic enzymes greatly aid to the spread of a fungus inside its host by killing plant cells and macerating tissue.

Analyses of the enzymes involved in lignin degradation showed that *Pm. aleophilum* showed low specific activity for the manganese peroxidase and laccase. This finding indicated that *Pm. aleophilum* has a greater capacity for degrading xylem walls than *Pa. chlamydospora* which showed no activity in tests for ligning degrading enzymes (Del Rio *et al*., 2004).

Molecular identification and detection

Different primers have been used to detect the fungi associated with esca diseases complex in soils, vines and in the different media used during the grafting process; to shed more light on the epidemiology of these fungi. In fact, the molecular identification of *Pa. chlamydospora* and *Phaeoacremonium* species has been done with phylogenetic analysis of the internal transcribed spacers (ITS 1 and 2) and 5.8 S rRNA gene, β-tubulin, actin and calmodulin gene regions. RFLP patterns of the ITS region were used to distinguish *Pa. chlamydospora*, *Pm. aleophilum,* and *Pm. inflatipes* (Tegli *et al*., 2000). Fourie & Halleen, (2002) found *Pa. chlamydospora* and *Phaeoacremonium* species in symptomless canes sampled from rootstock mother vines, although the incidence was very low (< 0.2%). Dupont *et al*., (2002) distinguished five species of *Phaeoacremonium*, namely *Pm. aleophilum, Pm. inflatipes, Pm. parasiticum, Pm. rubrigenum* and *Pm. viticola* using PCR-RFLP markers from the ITS regions and partial β-tubulin gene. Moreover, DNA phylogenies based on the internal

transcribed spacers (ITS 1 and 2) and 5.8 S rRNA gene, and β-tubulin, actin and calmodulin gene regions have been used in various studies to aid in the determination of new species of *Phaeoacremonium* (Dupont *et al*., 2000; Groenewald *et al*., 2001; Mostert *et al*., 2003, 2005; Essakhi *et al*., 2008). In addition, Mostert *et al*., 2005 developed a polyphasic identification tool including morphological and cultural characters as well as β-tubulin sequences generated with primers T1 (O'Donnell & Cigelnik, 1997) and Bt2b (Glass, 1995). A nested PCR assay has also been developed for the detection of *Pa. chlamydospora* and *Phaeoacremonium* spp. from soil and host tissue using universal primers ITS4 and ITS5 as external primers, and two sets of internal primers (Eskalen, 2001).

The presence of *Pa. chlamydospora* in naturally infected rootstock mother vines has also been confirmed by means of polymerase chain reaction (PCR) detection (Ridgway, 2003; Retief, 2005). *Phaeomoniella chlamydospora* DNA has also been detected in nursery and vineyard soils with a PCR protocol developed by Damm & Fourie, (2005) using the primers Pch1 and Pch2 (Tegli *et al*., 2000). Moreover, Overton *et al*., (2005) detected *Pa. chlamydospora* in roots, shoots and young trunks of drill-inoculated vines and *Pm. aleophilum* from trunks of naturally infected vines by making use of real-time PCR.

PCR detection is more reliable, sensitive and faster than traditional plating methods. As little as 1 pg of DNA could be detected from wood material (Retief *et al*., 2005b; Ridgway *et al*.; 2002) and 50 fg of DNA with a nested PCR approach (Whiteman *et al*., 2002). By comparing molecular detection and traditional plating from hot water treated and non-treated dormant nursery vines, Retief *et al.,* (2005b) demonstrated the inability of PCR detection to distinguish between dead material and viable cells. They suggested that further work is necessary on the detection of ribonucleic acids (RNA), that would have a relatively short life span following pathogen death, and would be more reliable to indicate the presence of viable fungal material.

Disease management

Published research on the management of esca diseases complex focuses either on *Pa. chlamydospora*, or the effect of treatments on symptom expression in naturally infected grapevines or its effect on the complex of Petri disease pathogens (i.e. *Pa. chlamydospra* + *Phaeoacremonium* spp.). Aspects to be considered in disease management are *in vitro* studies, host resistance, curative and preventive management in nurseries, and preventative, ameliorative and curative management strategies in vineyards. Numerous studies have been conducted to test for host resistance in scion and rootstock cultivars (Eskalen *et al.,* 2001; Marchi, 2001; Sparapano *et al*., 2001; Feliciano *et al*., 2004; Santos *et al*., 2005). None of

these studies has shown complete or high levels of resistance in any rootstock or scion cultivar tested. However, these studies show that different cultivars had a wide range of susceptibility.

Various control measures can be applied to ensure clean grapevine planting material. The presence of *Pa. chlamydospora*, *Pm. aleophilum*, Botryospheriaceae and *Phomopsis* spp. in pruning wound of rootstock mother blocks led Fourie & Hallen, (2004) to recommend that sanitation and pruning wound protection must be practiced. Hot water treatment of rootstock cuttings prior to grafting for 30 min at 50°C proved to be the most effective means of reducing the levels of infections of propagation material (Crous *et al*., 2001; Edwards *et al*., 2004; Fourie & Hallen, 2004).

In order to protect wounds from infection during the grafting processes, it is recommended the addition to hydration and drench water of quaternary ammonium disinfectants (Sporekill®), fungicides (benomyl) or biological control agents (*Trichoderma harzianum*) (Fourie & Hallen, 2004; 2005). *Trichoderma* treatments carried out during grafting (Messina, 1999; Di Marco *et al*., 2004) and soil amendments in field nurseries (Fourie, 2001) resulted in nursery grapevines with stronger graft unions, root systems and with lower levels of pathogen infection. In addition, the ability of *Trichoderma harzianum* and *T. longibrachiatum* against artificial infection by *Pa. chlamydospora* to colonise pruning wounds and reduce its infection by *Pa. chlamydospora* was also demonstrated by Di Marco *et al.*, (2004).

In Europe, sodium arsenite applications to the trunk and cordons of diseased grapevines in the period between pruning and bud burst have been successfully applied to combat esca since the beginning of the 20th century (Mugnai *et al*., 1999; Di Marco *et al*., 2000). Its high toxicity, however, has caused it to be banned from various countries. Later studies demonstrated the efficacy of fosetyl-Al used as trunk injections of mature grapevines, or as foliar sprays of potted grapevines and esca-diseased vineyards (Di Marco, 2000; 2005). Trunk injections resulted in moderate disease incidence and a preservation of vine productivity, whereas the foliar sprays resulted in significant reductions in the extent of necrotic areas following inoculation with *Pm. aleophilum* or *Pa. chlamydospora* in potted plants and a reduction in esca disease incidence in vineyards. Root zone application with triazoles and trunk injections with triazoles or fosetyl-Al in esca diseased vineyards resulted in significant reductions in foliar symptom development, whilst treatments were made in vineyards with a low disease incidence and with plants at an early stage of infection (Di Marco *et al*., 2000).

Preventive measures should be used through the limitation or prevention of stress factors in young vines that might predispose it to these diseases and by avoiding stress factors such as: nutrient deficiencies, water stress and poor soil preparation (Ferreira *et al*., 1999; Gubler *et al*., 2004; Surico *et al*., 2004; Edwards & Pascoe, 2005). Moreover, sanitation practices, such as the removal of infected plants, plant parts and/or pruning debris, will lead to lower inoculum loads in vineyards. Pruning wound protection in vineyards with benomyl and flusilazole reduced natural *Pa. chlamydospora* infections of pruning wounds by *ca* 80% (Fourie & Hallen, 2004; 2005).

Finally, disease prevention remains the most effective means in managing esca diseases complex by combining all the methods cited above, starting by ensuring that the propagation material is disease free.

OUTLINE AND SCOPE OF THE THESIS

Recently, vineyards have experienced a dramatic increase in grapevine trunk diseases, that cause a drastic decline in the health of grapevines, and in many cases kill mature vines and consequently lead to important losses in the majority of the grapevine producing countries of the world.

In this thesis, special interest will be given to esca disease, the most destructive trunk disease of grapevine worldwide and specific attention will be focused on *Phaeoacremonium* and *Phaeomoniella* genera, the principal hyphomycetes associated with esca diseases complex of grapevine.

Accordingly, the main objective of this study was to improve our knowledge on esca diseases complex of grapevine, with particular attention to the study of the level of genetic diversity that exist within and among populations of a global collection of *Phaeoacremonium* and *Phaeomoniella*. Findings obtained by this population genetics study should shed light on pathogen dispersal, host-pathogen co-evolution, and will improve our understanding of the biology of these important pathogens. Furthermore, it is planned to develop a molecular approach for the simultaneous detection of fungi associated with esca diseases complex of grapevine.

Chapter 1 gives an introduction to esca diseases complex, and the problems associated with its detection and management are summarised. Moreover, a general overview of the state of the art of *Pa. chlamydospora* and *Phaeoacremonium* is provided.

Chapter 2 describes the first part of a research to set up a multiplex PCR approach that can permit to detect *Phaeomoniella chlamydospora*, *Phaeoacremonium aleophilum* and five species of Botryopshaeriaceae using one reaction, in order to offer nursery owners and growers a very sensitive method for the detection and early identification of fungi associated with esca and related diseases of grapevine, bypassing the difficult and time consuming step of pure culture isolation from woody tissue of grapevine. In fact, the possibility of *taxon* specific primers to be applied simultaneously in one reaction is very useful to increase the diagnostic capacity of PCR, since this method allows saving time and material without compromising the specificity and sensitivity of the analysis. Moreover, this detection system could be a quick and reliable tool for fungal detection and disease diagnosis, especially for testing plant material designated for nursery production of plant propagation material.

Chapter 3 investigated the genetic variation among isolates of *Phaeomoniella chlamydospora* on grapevines, which is still insufficiently understood. A global collection of *Pa. chlamydospora* strains collected mainly from very old vines, in isolated locations in Italy and other countries, will be established as part of this study. The aim of this part of the study is to elucidate the probable presence of novel species of *Phaeomoniella* from grapevine and to gain a better understanding of the genotype geographic distribution of this important pathogen.

Chapter 4 analyses the phylogeny of a global collection of 118 *Phaeoacremonium* isolates from grapevines, in order to gain a better understanding of their involvement in esca diseases complex. Knowledge pertaining to the involvement of *Phaeoacremonium* species in esca diseases complex should shed light on the epidemiology of these destructive diseases of grapevine. Several species of *Phaeoacremonium* have already been attributed to the grapevine diseases within the esca complex worldwide. However, the identity, distribution and frequency of the *Phaeoacremonium* species involved in many grapevine growing areas, especially the area where *Vitis vinifera* evolved, and several isolated regions have not yet been studied, and many elusive aspects remain to be clarified. Therefore, isolates used in this study were collected mainly from very old vines, in isolated locations in Italy like Sardinia and Sicily where farmers grow local grape varieties. We also included a diverse set of isolates from other countries to get an as wide as possible variability within the population of the two fungi.

In **Chapter 5** the trial set up to investigate the pathogenicity of the newly described *Phaeoacremonium* species reported from grapevine (this work) is illustrated. In fact, the relative importance of the different *Phaeoacremonium* species in esca diseases complex is still insufficiently well understood, since several new species have only recently been described and their status as pathogens still unknown. Hence, pathogenicity tests were established to determine their potential as vascular pathogens, to test their effective capability to colonise pruning wounds and to cause vascular discoloration similar to that seen in esca diseased grapevines, with the final aim to understand their effective involvement in esca diseases complex of grapevine.

In **Chapter 6,** our aim was to isolate and characterise the mating type loci of *Pm. aleophilum* and to ascertain which mating type (s) are present in the different countries where diseases caused by these fungi occur. The identification of the mating type gene of *Pm. aleophilum* would be very useful and beneficial for population studies, especially in geographic regions where only the anamorph of *Togninia minima* has been identified, in order to prevent introduction events of isolates carrying the other type of mating type and inducing sexual reproduction, and to understand which are the modes of reproduction in a geographical area and the type of inoculum source. The mating type specific PCR is faster than traditional methods and could be implemented as a control method to test for the presence of the mating types for the species *Pm. aleophilum*, which would provide new insight into the distribution of mating types of *Pm. aleophilum* that should enhance the quality of quarantine regulations in the future.

Chapter 7 summarises the research presented in this thesis, discusses the important findings of this dissertation and lead to several propositions for future research initiatives.

Chapter 2

Development of a semi nested-multiplex PCR approach for the simultaneous detection of fungi associated with esca and related diseases of grapevine

ABSTRACT

Esca is the most destructive trunk disease of grapevine worldwide, which develop as a complex of syndromes (at least five) showing a large range of wood and leaf symptoms. Its origin is attributed to different fungi; the wood-rotting basidiomycete *Fomitiporia mediterranea* (casual agent of white rot), the two hyphomycetes *Phaeomoniella chlamydospora* and *Phaeoacremonium aleophilum* (casual agents of: brown wood streaking of rootstock; Petri disease and young esca). Another plant pathogenic group of ascomycetes commonly isolated from the trunks and branches of esca-affected grapevines is in the Botryosphaeriaceae family. Since no effective curative control measures are known, proactive measures must be taken in grapevine nurseries to manage this disease. Molecular tools are becoming very important for the early and sensitive detection of the fungi involved in esca complex on propagative materials. In this study, a semi nested multiplex PCR protocol was developed and used to successfully detect *Phaeomoniella chlamydospora***,** *Phaeoacremonium* spp. and *Botryosphaeria* spp. in apparently healthy samples and others obtained from esca diseased grapevines, in order to offer nursery owners and growers a very sensitive method for detecting the presence of pathogens associated with esca and related diseases of grapevine, especially, for testing plant material designated for nursery production of plant propagation material.

INTRODUCTION

Esca is a worldwide trunk disease, which develop as a complex syndrome showing a large range of wood, leaf and berries symptoms. It can be typically identified by internal wood decay and various types of wood deterioration that can be observed when a transverse cut is made on the trunk and main shoots, like black spots, but also pink-brown or dark red-brown areas and a central white rot of soft consistency surrounded by a dark borderline (Larignon and Dubos, 1997; Mugnai *et al*., 1999). Symptoms on the leaves consist of interveinal regions of chlorotic and yellowish tissue that turns yellow-brown or red-brown; leaves with such symptoms are also known as “tiger leaves”. In the USA, esca has been referred to as “black measles” because of the small, dark brown to purple spots that can develop on the berries. Foliar and fruit symptoms do not necessarily appear on the same diseased plant every year (Mugnai *et al*., 1999). Occasionally “apoplexy” can occur when vines or vine-parts suddenly wilt during hot, dry conditions in the summer. The causes of this last syndrome are still uncertain.

Fungi that have been associated with esca symptoms have essentially been for many years only wood-rotting basidiomycetes. In Europe the more common agent of wood decay on grapevine is *Fomitiporia mediterranea*, and, to a much lesser extent, *Stereum hirsutum* or *Trametes hirsuta* (Mugnai *et al*., 1999; Cortesi *et al*., 2002; Fischer, 2002). On the other hand *Phaeomoniella chlamydospora* and *Phaeoacremonium aleophilum* are the principal hyphomycetes associated with the full range (except white rot) of wood and foliar esca symptoms (Larignon and Dubos, 1997; Mugnai *et al*., 1999). It is the contemporary presence on the same vine of all the above fungi, and of the two diseases they cause – the white decay and the vascular disease - that is called "Esca proper" to honor the old traditional description of the disease (Surico, 2001), affecting mostly vines older than 15 yr. Another plant pathogenic group of ascomycetes which is commonly isolated from the trunks and branches of esca-affected grapevines include Botryosphaeriaceae species (Mugnai *et al*., 1999). The most commonly isolated are *Diplodia seriata* (anamorph of '*Botryosphaeria obtusa*') and *Botryosphaeria dothidea*'. Some of these species are claimed by some authors to cause also Black Dead Arm disease, which in different periods and countries, has been identified by different authors and its syndromes described differently (Lehockzky, 1974; Cristinzio, 1978; Rovesti & Montermini, 1987; Larignon *et al*., 2001). Botryosphaeriaceous fungi may cooperate, when present, in causing the esca foliar symptoms but are considered agents of different specific diseases, mainly as dieback and canker pathogens of woody host plants (Niekerk *et al*., 2006).

Conventional detection of *Phaeomoniella chlamydospora*, *Phaeoacremonium* spp., *Fomitiporia mediterranea* and *Botryosphaeria* spp. involves fungal isolation and morphological examination. However, these methods are time consuming and require well trained personnel in fungal taxonomy. Moreover, some of these fungi are slow growing, which usually take up 25 days to grow on enriched medium and they are easily overgrown by other microorganisms, then subculturing is required which makes the identification process longer. Hence, a molecular approach that can permit to detect these fungi using one reaction, becomes very useful for their detection and early identification, bypassing the difficult and time consuming step of pure culture isolation from woody tissue of grapevine. The possibility of the specific primers to be applied simultaneously in one reaction is very useful to increase the diagnostic capacity of PCR, since this method allows saving time and material without compromising the specificity and sensitivity of the analysis.

Preventive measures are the most effective means in managing esca diseases complex, starting by ensuring that the plant propagation material is disease free. This detection system

could be a quick and reliable tool for detection and disease diagnosis of fungi associated with esca and related diseases of grapevine, especially for plant propagation material which is suspected to be infected as having a bad performance or atypical wood discolorations, or in nurseries within clean certification program.

MATERIALS AND METHODS

Isolates

Isolates used in the current study were obtained from symptomatic material of diseased grapevines, from very old vines and young asymptomatic nursery plants (plant propagation material) (Fig.1a & Fig.1b).

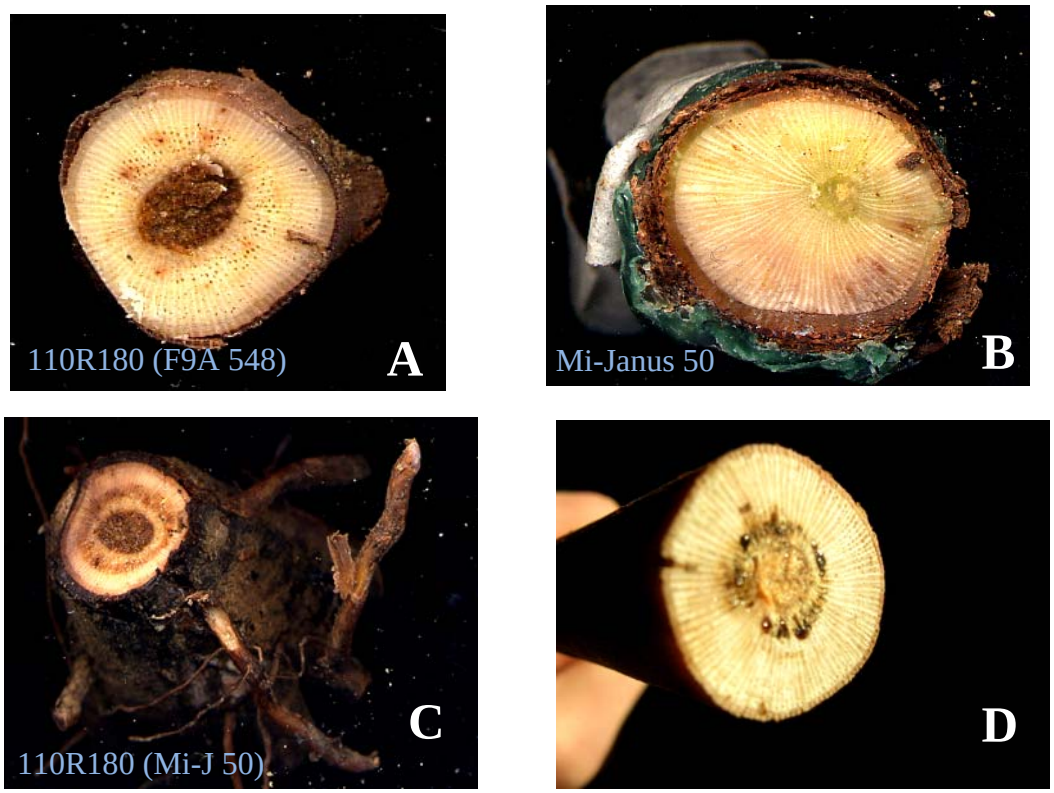

Fig. 1a. Plant propagation material used in this study: A-B: apparently healthy propagation material; C-D: symptomatic plant propagation material

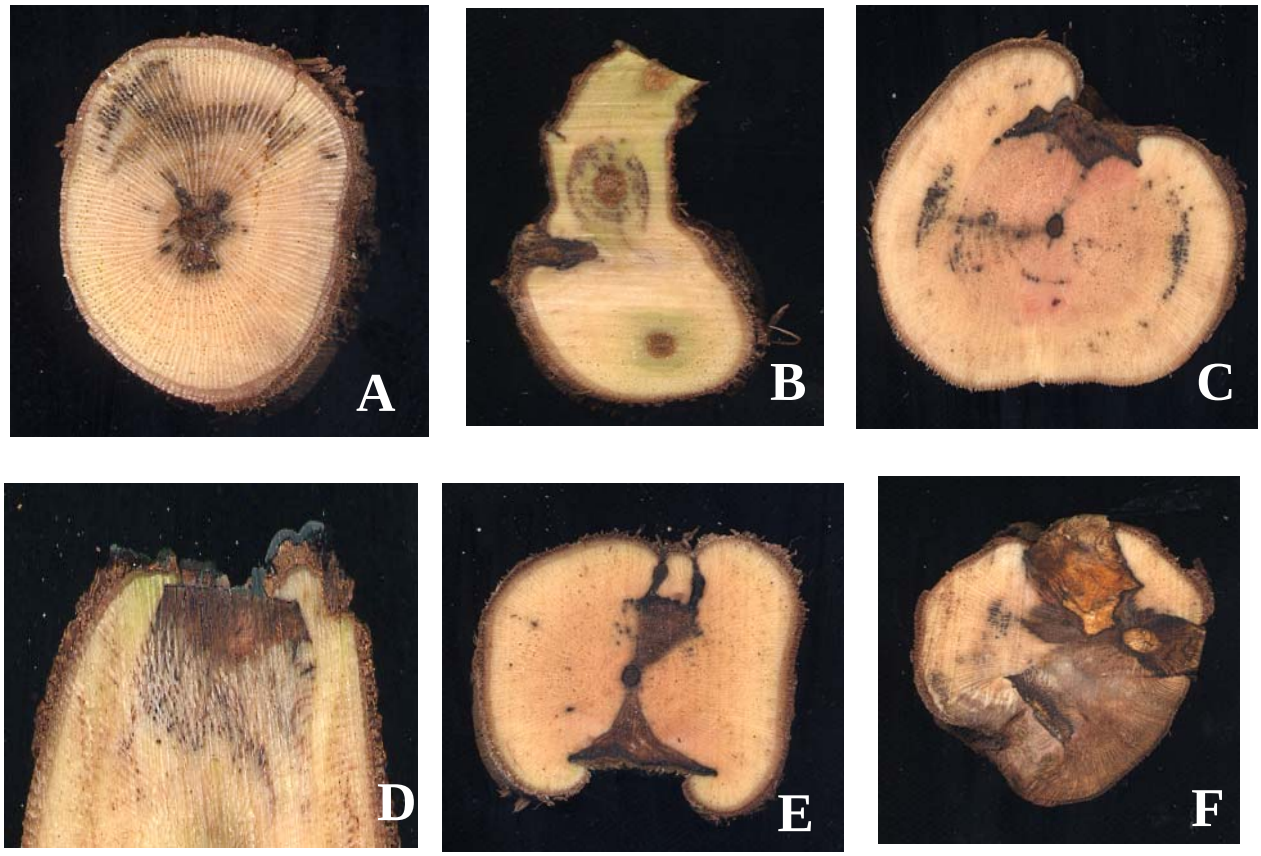

Fig. 1b. Symptomatic plant material used in this study obtained from a plant of 11 years old cv. Sauvignon blanc collected in Montepaldi Farm (San Cascinao, Florence). Black spots in transversal cut (A. B. C), Black streakings in longitudinal cut (D), and cross section showing sectorial necrosis (E) and white rot (F),

Trunk and shoots were surface sterilized by placing in 70% ethanol for 15 s, air dried under sterile conditions, then cut into disks and the surface was sterilized. Small pieces (1-2 mm thick) were cut from just below the surface, around and in the darkened vascular tissues and others from the margin between necrotic and apparently healthy tissue, then plated onto malt extract agar (MEA; 2 % malt extract, Oxoid Ltd., Basingstoke, Hampshire, England; 1.5 % agar, Difco, Detroit, Michigan, USA) and incubated at 25 °C in the dark for 2–3 weeks until cultures sporulated. Single conidial isolations were established from emerging colonies identified as *Pa. chlamydospora, Phaeoacremonium* sp., *Botryosphaeria* sp., *Fomitiporia mediterranea* (Table. 1).

Table 1. Names, origin and details of the collection of isolates used in this study.

Plant material	Samples	Origin	Internal symptoms
110R 151	1	Nursery "A", Tuscany	asymptomatic
	2	Nursery "A", Tuscany	asymptomatic
110R 151	3	Nursery "A", Tuscany	asymptomatic
	4	Nursery "A", Tuscany	asymptomatic
F9A 548/110R 180	5	Nursery "A", Tuscany	Browning
	6	Nursery "A", Tuscany	Browning
F9A 548/110R 180	7	Nursery "A", Tuscany	asymptomatic
	8	Nursery "A", Tuscany	Browning
MI-JANUS 50/110R 180	9	Nursery "A", Tuscany	Browning
	10	Nursery "A", Tuscany	Browning
MI-JANUS 50/110R 180	11	Nursery "A", Tuscany	Browning
	12	Nursery "A", Tuscany	asymptomatic
	3A	Nursery "B", Tuscany	asymptomatic
	3B	Nursery "B", Tuscany	asymptomatic
	3C	Nursery "B", Tuscany	asymptomatic
	3D	Nursery "B", Tuscany	asymptomatic
	3E	Nursery "B", Tuscany	asymptomatic
	4A	Nursery "B", Tuscany	asymptomatic
	4B	Nursery "B", Tuscany	asymptomatic
	4C	Nursery "B", Tuscany	asymptomatic
	4D	Nursery "B", Tuscany	asymptomatic
	4E	Nursery "B", Tuscany	asymptomatic
	5LA	Nursery "B", Tuscany	asymptomatic
	5LB	Nursery "B", Tuscany	asymptomatic
	6PA	Nursery "B", Tuscany	asymptomatic
	6PB	Nursery "B", Tuscany	asymptomatic
cv. Sauvignon blanc	S1	Montepaldi, Tuscany	Sectorial necrosis
	S2	Montepaldi, Tuscany	necrosis & black spots
	S3	Montepaldi, Tuscany	Sectorial necrosis
	S4	Montepaldi, Tuscany	Sectorial necrosis
	D1	Montepaldi, Tuscany	Necrosis & White rot
	D2	Montepaldi, Tuscany	Necrosis & White rot
	D3	Montepaldi, Tuscany	Necrosis & White rot
	D4	Montepaldi, Tuscany	Necrosis & White rot
	T1	Montepaldi, Tuscany	Necrosis & White rot
	T2	Montepaldi, Tuscany	Sectorial necrosis & White rot
	T3	Montepaldi, Tuscany	Necrosis & White rot

DNA Extraction

Genomic DNA was extracted from 100 mg fungal mycelium, or directly from disinfected pieces of plant tissue ground with liquid nitrogen, using the commercially available DNeasy Plant mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

As positive control for PCR amplification, we used DNA extracted from a pure culture of the respective target fungus (Table 2). The DNA extracted was kept at -20°C until it was used for PCR amplifications.

Table 2. Details of isolates used in this study as positive control for PCR assays.

Species name	**Isolate number**	**Oigin**
Phaeomoniella aleophilum	Pal 77	Umbria - Italy -
Phaeomoniella aleophilum	Pal 78	Abruzzo - Italy -
Phaeomoniella aleophilum	Pal 86	Tuscany - Italy -
Phaeomoniella chlamydospora	Pch 51	Tuscany - Italy -
Phaeomoniella chlamydospora	Pch 56 (ex Pch 52)	Tuscany - Italy -
Botryosphaeria dothidea	BO 2	Umbria - Italy -
Botryosphaeria dothidea	BO 209.03	New Zealand
***Botryosphaeria* sp.**	BO 210.03	New Zealand
'Botryosphaeria' obtusa	BO 211.03	Umbria - Italy -
Botryosphaeria sp.	BO 4	Umbria - Italy -
Fomitiporia mediterranea	Fop 200.03	Sicily - Italy -
Fomitiporia mediterranea	Fop 201.03	Sicily - Italy -

Primer design

The specific primers developed in the current study, were designed based on the ITS region-sequences (Internal Transcribed Spacer region (ITS1-5.8S-ITS2) of the nuclear ribosomal DNA. The ITS regions are most commonly used to design PCR primers for fungi. This region of ribosomal DNA comprise two variable non coding regions (ITS1 and ITS2) that separate the highly conserved 18S (small subunit), 5.8S and 28S (Large subunit) ribosomal RNA genes.

Botryosphaeriaceae species. ITS sequences of five species of Botryosphaeriaceae fungi were used to design specific primer pair Boits 1 and Boits 2 (Table 3).

Table 3. Isolates used for primers design of Boits 1and Boits 2 (this study).

Teleomorph name	**Anamorph name**	**Isolate name**	**Origin**	**GenBank accessions**
Botryosphaeria dothidea	*Fusicoccum aesculi*	STE-U 5045, CBS 110484	Argentina	AY 343414
		STE-U 5149	Portugal	AY 343415
Botryosphaeria lutea	*Fusicoccum luteum*	STE-U 4592, CBS 110862	Portugal	AY 343416
		STE-U 4594	Portugal	AY 343418
Botryosphaeria obtusa	*Diplodia seriata*	STE-U 4587	France	AY 343441
		STE-U 5037	Portugal	AY 343446
		STE-U 5164	R.S.A	AY 343460
Botryosphaeria parva	*Neofusicoccum parvum*	STE-U 4439, CBS 113032	R.S.A	AY 343468
		STE-U 4584, CBS 110952	France	AY 343471
		STE-U 5253, CBS 110888	Portugal	AY 343477
Botryosphaeria rhodina	*Lasiodiplodia theobromae*	STE-U 4583	R.S.A	AY 343482
		STE-U 5051, CBS 110495	Argentina	AY 343483

ITS sequences were obtained from the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA). Nucleotide sequences were aligned using Clustal X version 1.81 (Thompson *et al*. 1997) (Fig. 2). Based on these sequences, specific primers were developed that targeted the internal transcribed spacer region (ITS1-5.8S-ITS2) of the nuclear ribosomal DNA of *Diplodia seriata* (*Botryosphaeria obtusa*); *Fusicoccum aesculi* (*Botryosphaeria dothidea*); *Neofusicoccum parvum* (*Botryosphaeria parva*); *Fusicoccum luteum* (*Botryosphaeria lutea*); *Lasiodiplodia theobromae* (*Botryosphaeria rhodina*) (following the recent classification of Botryosphaeriaceous fungi in Crous *et al*., (2006).

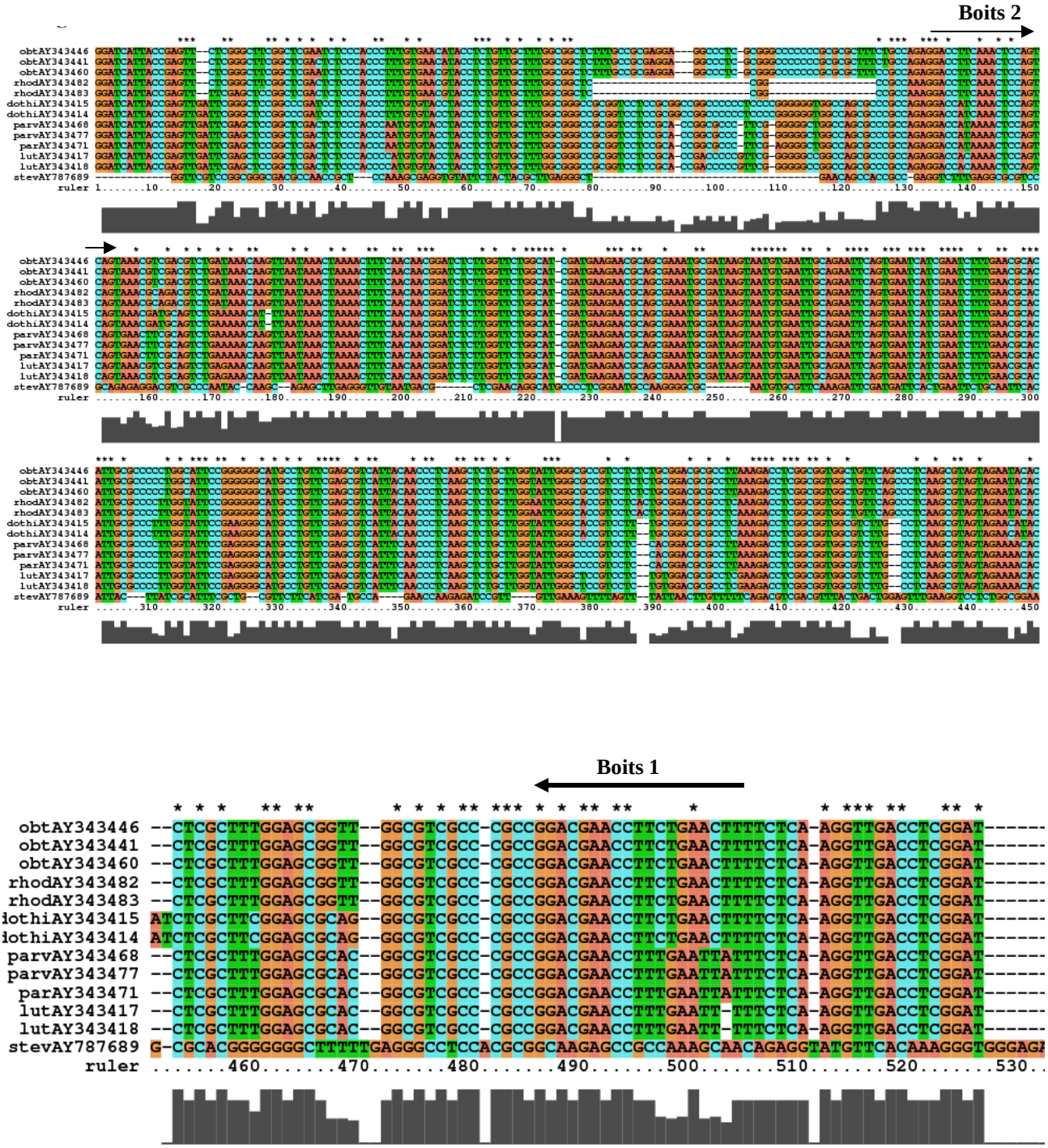

Fig. 2. Clustal X alignment of five species of Botryosphaeriaceae used for the design of primers Boits2 and Boits1 and their respective position.

Phaeoacremonium aleophilum. Primers Pal1N forward and Pal2 reverse primers (Tegli *et al*., 2000) for the detection of *Phaeacremonium aleophilum* are available but they amplified a fragment of 300 bp of the ITS region of *Phomopsis* sp. as well. Hence, due to the non-specificity of the previously published primers Pal1N and Pal2, a new primer has been designed; Phaeoits 3rev based on the alignment of different sequences of *Phaeacremonium* of the internal transcribed spacer region ITS of the nuclear ribosomal DNA (Table 4).

Table 4. *Phaeoacremonium* isolates used for the design of the primer Phaeoits3 rev.

Species of *Phaeoacremonium*	**Isolates**	**GenBank accessions**	**Origin**
Ph. aleophilum	CBS 100397	AF197981	Italy
Pm. aleophilum	CBS 100401	AF197982	Italy
Pm. aleophilum	CBS 100548	AF197983	Italy
Pm. aleophilum	CBS 100568	AF197984	U.S.A
Pm. aleophilum	STE-U 3093	AF197985	South Africa
Pm. aleophilum	CBS 100399	AF197991	Italy
Pm. aleophilum	CBS 100400	AF197992	Italy
Pm. aleophilum	CBS 100358	AF197993	Italy
Pm. aleophilum	CBS 631.94	AF266647	_
Pm. aleophilum	405.J.95	AF266648	Italy
Pm. aleophilum	444.J.95	AF266649	Italy
Pm. aleophilum	445.J.95	AF266650	Italy
Pm. aleophilum	157	AF266654	Italy
Pm. angustius	CBS 101737	AF197976	France
Pm. angustius	CBS 777.83	AF266651	Argentina
Pm. inflatipes	_	AF118140	France
Pm. inflatipes	CBS 101585	AF295328	U.S.A
Pm. inflatipes	CBS 166.75	U31843	Costa Rica
Pm. mortoniae	CBS 211.97	AF295329	Sweden

ITS sequences of the isolates detailed in Table 4 were aligned using Clustal X version 1.81 as shown in Fig 3.

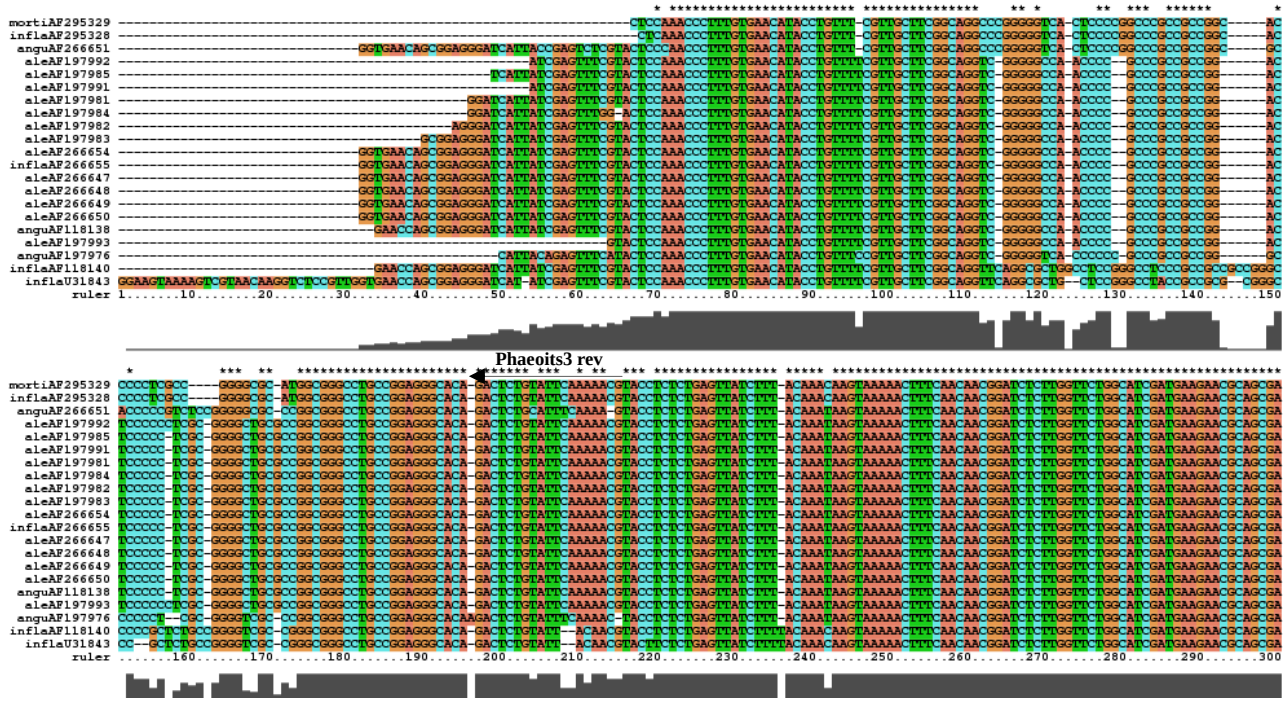

Fig. 3. Clustal X alignment of *Phaeoacremonium* species used for the design of the primer Phaeoits3 rev and its position.

Specificity of primers designed in the current study was assessed by PCR using DNA from other pathogenic, saprophytic and endophytic fungi, from fungal isolates known to be less significant esca agents but commonly isolated from grapevine, like: *Eutypa lata*, *Phomopsis* sp., *Botrytis cinerea*, *Alternaria* sp. , *Penicillum* sp., *Rhizopus* sp. and *Aspergillus* sp.

To summarize, 11 primers were tested in the current study as illustrated in Table 4.

Table 4. Details of primers used in this study.

Primer	**Sense**	**Sequence**	**Author**
ITS5	Forward	5'-GGA AGT AAA AGT CGT AAC AAG G-3'	White *et al*.,1990
ITS4	Reverse	5'-TCC TCC GCT TAT TGA TAT GC-3'	White *et al*.,1990
Pch1	Forward	5'-CTC CAA CCC TTT GTT TAT C-3'	Tegli *et al*., 2000
Pch2	Reverse	5'-TGA AAG TTG ATA TGG ACC C-3'	Tegli *et al*., 2000
Pal1N	Forward	5'-AGG TCG GGG GCC AAC-3'	Tegli *et al*., 2000
Pal2	Reverse	5'-AGG TGT AAA CTA CTG CGC-3'	Tegli *et al*., 2000
Fmed1	Forward	5'-GCA GTA GTA ATA ACA ATC- 3'	Fischer, personal communication
Fmed2	Reverse	5'-GGT CAA AGG AGT CAA ATG GT-3'	Fischer, personal communication
Boits2	Forward	5'-GAC CATC AAA CTC CAG TCA G-3'	This study
Boits1	Reverse	5'-AAA GTT CAG AAG GTT CGT CC- 3'	This study
Phaeoits3 rev	Reverse	5'-CGT TTT TGA ATA CAG AGT C-3'	This study

Non-nested species-specific PCR

Amplification of the DNA extracted from the previously described samples was performed according to Ridgway *et al*. (2002). Universal primers ITS5 and ITS4 (White *et al*., 1990), were used to verify that DNA extracts were suitable for amplification, to increase yield of the target template and to reduce the effects of inhibition associated with the DNA extraction, by using the cDNA of the first PCR as template for the second PCR. The primers used in the second round of nested PCR are internal to the primers used in the first PCR assay. Primers ITS5 and ITS4 are known to amplify the ITS regions and 5.8S gene of the nuclear ribosomal RNA operon (ITS 1 - 5.8S - ITS 2).

For specific detection of *Pa. chlamydospora*: A fragment of 360 bp of the 5' end of the ITS gene was amplified using primers Pch1 and Pch2 (Tegli *et al*., 2000). Whereas, the new primers Boits1 and Boits2 were used to amplify a fragment of 367 bp of the 5' end of the ITS gene of five species of Botryosphaeriaceae. Moreover, a fragment of 200 bp of the 5' end of the ITS gene was amplified using the universal primers ITS5 combined with the species specific primer Phaeoits 3 rev (this study), in order to assess the presence of *Phaeoacremonium* sp. DNA.

Finally, a fragment of 534 bp of the the 5' end of the ITS gene of *F. mediterranea* was amplified using primers Fmed1 and Fmed2.

Each 25 µl amplification reaction contained 1x PCR buffer, 1.5 mM $MgCl_2$, 20 µM dNTP's, 0.2 µM of each primer, 1.25 U Taq DNA polymerase (Fermentas, International INC, Ontario, Canada) and 2 µl of DNA. The reactions were performed in an Eppendorf® Mastercycler® Gradient PCR machine and consisted of 3 min at 94°C followed by 30 cycles of 30 s at 94°C, 30 s at 59°C and 30 s at 72°C, with a final extension of 72°C for 7 min.

Positive controls consisted of fungal DNA from a pure culture of the respective target fungus, while negative control consisted on the use of sterile water instead of fungal DNA. Positive and negative controls were used in all PCR reactions.

PCR products were separated by electrophoresis in 1% agarose gel, containing 0.1 µg/ml ethidium bromide, with 1x TBE buffer (Sambrook *et al*., 1989) and visualized under ultra violet light. The amplified products of interest have been analyzed, purified and supplied to be sequenced, then Blast search analysis in the GenBank (NCBI, Bethesda, MD, USA) was carried out.

Nested species-specific PCR

This method is based on two-step approach. In the first round of amplification, a primary PCR was done on the previously described DNA using the universal primers ITS5 and ITS4, in order to enrich the amount of fungal DNA present in the sample. The reaction conditions were as described for the species specific PCR with the exception of the concentration of $MgCl_2$ which is 2.5 mM and the thermal cycle which consisted of 3 min at 94°C followed by 35 cycles of 30 s at 94°C, 30 s at 50°C and 30 s at 72°C, with a final extension of 72°C for 7 min. The resulting PCR product was diluted 1:10 in sterile water and 2 µl of this dilution was then amplified in a secondary PCR using the species specific PCR protocol outlined above (using Pch1 / Pch2 or primer pair Boits1 / Boits2) However, this reaction contained only 1.5 mM MgCl2.

The amplified products of interest have been analyzed, purified and supplied to be sequenced.

Semi - nested species-specific PCR

In the first round of amplification, a primary PCR was done on the previously described DNA using the universal primers ITS5 and ITS4. The reaction conditions were as described above. The resulting PCR product was diluted 1:10 in sterile water and 2 µl of this dilution was then amplified in a secondary PCR using the species specific PCR protocol outlined above and by combining the universal Forward primer ITS5 and the specific reverse primers described previously. The amplified products of interest have been analyzed, purified and supplied to be sequenced.

Semi Nested Multiplex PCR using DNA extracted from fungal mycelium

Initially, a primary PCR was carried out using primers ITS5 and ITS4 on a mixture of DNA of *Phaeomoniella chlamydospora, Phaeoacremonium aleophilum, Botryosphaeria obtusa* and *F. mediterranea,* then a multiplex PCR was performed on the resulting PCR product of the universal PCR diluted 1:10 in sterile water. 2 µl of this dilution has been amplified in a secondary PCR using the universal primer ITS5 combined with the species specific reverse primers previously described. The multiplex PCR was carried out, testing different concentrations of $MgCl_2$ (1.5 mM and 2.5 mM) and the primer mix (0.2 µM and 2 µM), using 1x PCR buffer, 20 µM dNTP's, 1.25 U Taq DNA polymerase (Fermentas). Primer mix used in the reaction contains in total five primers (ITS5, Pch2, Phaeoits3 rev, Boits1 and Fmed2) with equal concentration of 2 µM. The reactions were performed in an Eppendorf®

Mastercycler® Gradient PCR machine and consisted of 3 min at 94°C followed by 30 cycles of 30 s at 94°C, 30 s at 57°C and 30 s at 72°C, with a final extension of 72°C for 7 min.
The expected products from each amplification are: 539 bp for the combination ITS5 / Boits1, 433 bp for the combination ITS5 / Pch2, 753 bp for the combination ITS5 / Fmed2 and 200 bp for the combination ITS 5 / Phaeoits 3 rev.

Semi Nested Multiplex PCR using DNA extracted from plant tissue

An initial PCR was done on the DNA extracted directly from pieces of asymptomatic plant propagation material tissue and infected plant material, using the universal primers ITS4 and ITS5 as previously described, then, multiplex PCR reaction was conducted on the resulting PCR product diluted 1:10 in sterile water and 2 µl of this dilution has been amplified in a secondary PCR using the universal primer ITS5 combined with the species specific reverse primers as previously described.

Sequencing

The amplified products of interest have been purified using Qiagen Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, then supplied to be sequenced.
Sequences obtained were edited, compared with the homologous sequences available in the GenBank database (National Center for Biotechnology Information, Bethesda, MD) using the similarity search program Blast (Altschul *et al*., 1990; 1997).

RESULTS

PCR amplification for primer specificity

The primers designed in the current study, specifically amplified DNA of only their respective targets. They did not react positively with DNA of any other fungal species non target. Primers pair Boits1/Boits2 and ITS5 / Phaeoits3 produced a single and specific expected amplicon of 367 bp and 200 bp respectively.

Nested / semi nested - PCR assays

The first round of nested PCR resulted in visible products of the ITS amplicon in some samples, including the apparently healthy propagation material. The second round of PCR resulted in visible specific products as well in some samples analysed. A fragment of 360 bp of the 5'end of the ITS gene was amplified using primers Pch1 and Pch2 (Fig. 4). In addition, the new primers designed for the detection of Botryosphaeriaceae (Boits1 / Boits2) have successfully amplified a specific fragment of 367 bp of the 5'end of the ITS gene (Fig. 5). Moreover, a fragment of 200 bp of the 5' end of the ITS gene was amplified using the universal primers ITS5 combined with the species specific primer Phaeoits 3 (Fig.6). Sequencing of the amplified products revealed the presence of *Pa. chlamydospora*, *Botryosphaeria dothidea* / *obtusa* and *Pm. aleophilum*. DNA of *Botryosphaeria* was also successfully amplified using Boits1 combined with ITS5, resulting in a fragment of 539 bp (Fig. 6). However, using conventional traditional methods, none of the fungi listed above has been isolated.

Nested species-specific PCR

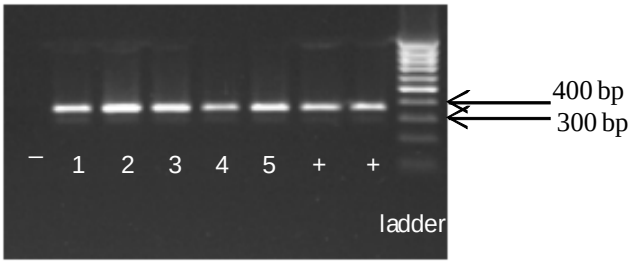

Fig. 4. PCR amplicons generated in a nested PCR using primers Pch1 and Pch2 on DNA extracted from apparently healthy propagation material. Arrow on left indicates the absence of amplification in the negative control, (lane 1-5): Amplified PCR products showing a fragment of 360 bp, 1: 4E; 2: 5LA; 3: 5LB, 4: 6PA; 5: 6PB, Lane named + : positive control showing a fragment of 360 bp obtained for *Phaeomoniella chlamydospora*, isolate Pch 52. Far right lane: 1- Kb MassRuler™ DNA Ladder, Low Range.

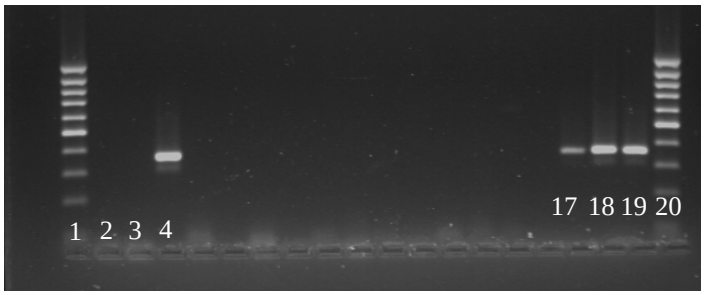

Fig. 5. PCR amplicons generated in a nested PCR using primers Boits1 and Boits2 on DNA extracted from apparently healthy propagation material. Lane 1, 20: 1- Kb MassRuler™ DNA Ladder, Low Range, Lane 2: negative control, Lane 4: 3A, 17: 6PB: Amplified PCR products showing a fragment of 367 bp, Lane 18 and 19: positive control (+) showing a fragment of 367 bp obtained for '*Botryosphaeria' obtusa*, isolate BO 211.03.

Semi - nested species-specific PCR

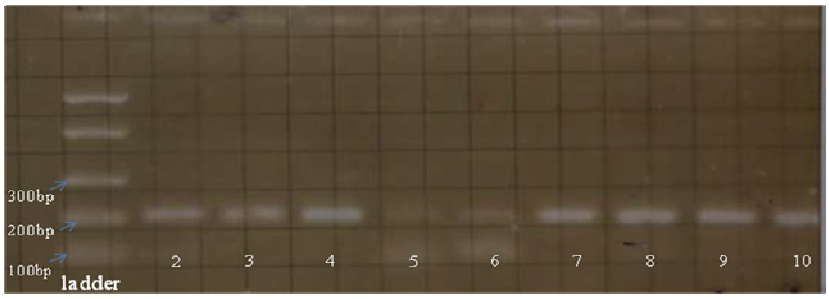

Fig. 6. PCR amplicons generated in a semi- nested PCR using primers ITS5 and Phaeoits 3. Lane 1: 1-Kb MassRuler™ DNA Ladder, Low Range, Lane 2-4, 7-9: Amplified PCR products using different concentrations of the primer Phaeoits3 (amplification band was more visible using a concentration of 0.4 μM, showing a fragment of 200 bp of DNA of isolate number 7 (Nursery "A"- Tuscany), Lane 10: positive control (+) showing a fragment of 200 bp obtained for *Phaeoacremonium aleophilum*, isolate Pal 86.

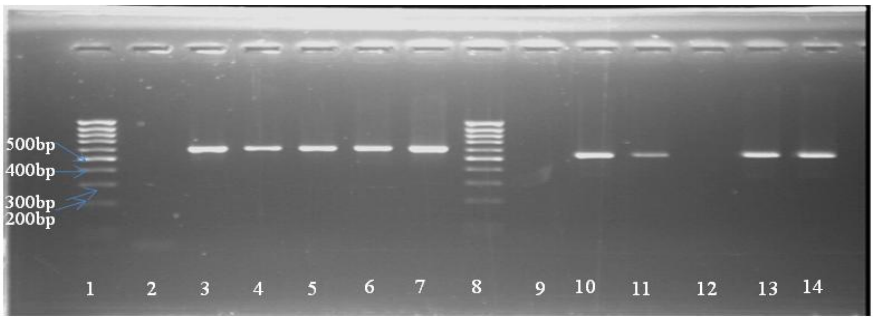

Fig. 7. PCR amplicons generated in a semi-nested PCR using primers ITS5 and Boits1 from esca diseased plant material. Lane 1,8: 1- Kb MassRuler™ DNA Ladder Low Range; Lane 2: Negative control; Lane 3-7, 10-11, 13: Amplified PCR products showing a fragment of 539 bp, Lane 14: positive control (+) showing a fragment of 539 bp obtained for *Botryosphaeria obtusa*, isolate BO 211.03.

Semi nested Multiplex PCR using DNA extracted from fungal mycelium

The Semi Nested multiplex PCR assay carried out using the universal primer ITS5 combined with Pch2 and Boits1, on DNA extracted from fungal mycelium of the isolate BO 211.03 and the isolate Pch 52 was successful and amplifications products were visible with equal intensity when we used the same concentrations of reverse primers Pch2 and Boits1.

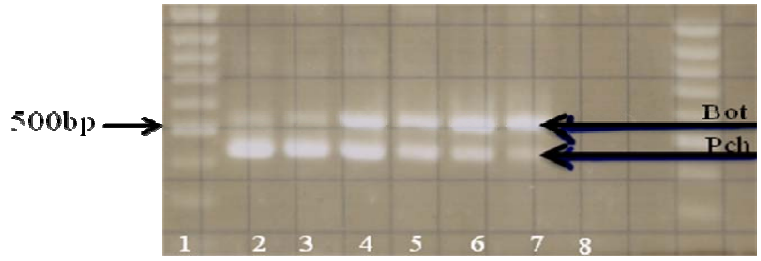

Fig. 8. Semi Nested multiplex PCR assay using the universal primer ITS5 combined with Pch2 and Boits 1, on DNA extracted from fungal mycelium of the isolate BO 211.03 and the isolate Pch 52 using different primers concentrations. Lane 8: PCR negative control (water control).

Semi nested Multiplex PCR using DNA extracted from plant material

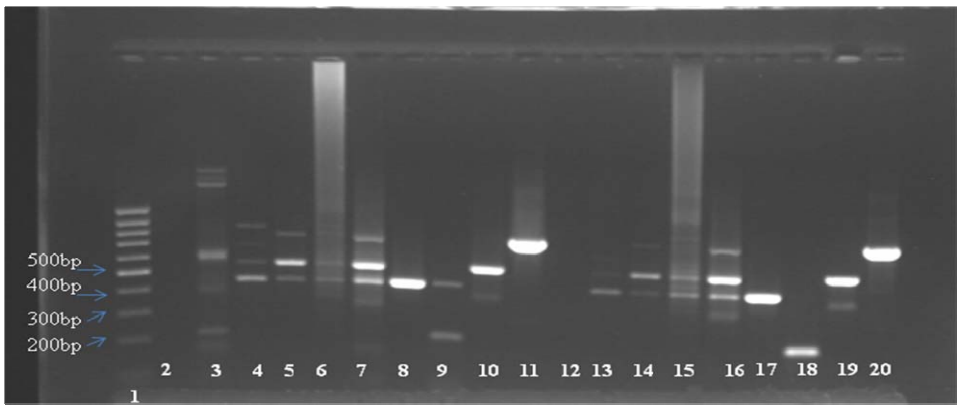

Fig. 9. Semi Nested multiplex PCR assay using the universal primer ITS5 combined with Pch2, Phaeoits3, Fmed 2 and Boits 1, on selected isolates that showed previously to have multiple infections according to the results obtained with nested PCR. Lane 1: Molecular weight marker, Lane 2 and 12: PCR negative control (water control). Lane 3-4, 6,13, 9, 15: PCR product from apparently healthy propagation material. Lane 5, 7, 14, 16: PCR product from esca diseased plant material. Lane 8, 17: *Pa. chlamydospora* DNA (positive control). Lane 18: *Pm. aleophilum* DNA (positive control). Lane 10, 19: *Botryosphaeria obtusa* DNA (positive control). Lane 11,20: *F. mediterranea* DNA (positive control).

The products amplified and having the size expected; 539 bp for the Botryosphaeriaceae, 433 bp for *Phaeomoniella chlamydospora*, 200 bp for *Phaeoacremonium aleophilum* and 753 bp for the combination *F. mediterranea* were extracted from the gel, purified and sequenced.

Sequence and blast search analysis confirmed the presence of DNA target, *Botryosphaeria obtusa* / *dothidea*, *Pa. chlamydospora* and *Phaeoacremonium*, which indicate that the multiplex PCR for the detection of these fungi was successful. However, we could not ascertain that the expected amplified product of 763 bp, correspond to *F. mediterranea*, due to some problems with sequencing of this product. As conclusion, the development of the multiplex PCR for the detection of five species of Botryosphaeriaceae, *Phaeomoniella chlamydospora*, and *Phaeoacremonium aleophilum* was successful, but still need to be optimized due to the presence of some aspecific amplified products. However, the specific primer for the detection of *F. mediterranea* could not be successfully combined in the multiplex reaction.

DISCUSSION

In this study, taxon specific priming multiplex PCR were developed, in order to achieve a detection method for the simultaneous detection of fungi associated with esca. In fact, the primers developed based on the internal transcribed spacer region (ITS1-5.8S-ITS2) of the nuclear ribosomal DNA of *Botryosphaeria* was used successfully in the semi-nested PCR and Nested PCR and Semi-nested multiplex PCR assays. The specific identity of the amplified DNA was determined by sequencing both the strands and pairwise comparisons with the homologous sequences available in the GenBank database. The trials indicated that by far the most common Botryosphaeriaceae species were *B. dothidea* and *B. obtusa*. These two species are not considered the most virulent of the *Botryosphaeria* species that attack grapevine, but the most widespread. On the other hand, the development of the multiplex PCR for the detection of five species of Botryosphaeriaceae, *Phaeomoniella chlamydospora*, and *Phaeoacremonium aleophilum* was successful, but still need to be optimized due to the presence of some aspecific amplified products. However, the specific primer for the detection of *F. mediterranea* could not be successfully combined in the multiplex reaction. In this study, with traditional methods of isolation and culturing, none of the fungi detected with PCR could have been isolated, while with PCR, DNA target has been amplified and we noticed the presence of DNA of fungi associated with esca in apparently healthy propagation material sampled from two nurseries in Tuscany (Table 5), suggesting that if *Ph. chlamydospora*, *Botryosphaeria* sp., *Phaeoacremonium* sp. was present in the grapevine samples, it was either on the outside of the cuttings and removed during surface sterilisation, or was at undetectable levels.

Table 5. Results of Nested and Semi- nested PCR assay studies carried out on plant propagation material coming from two different nurseries in Tuscany.

Propagation material	Name	Origin	Internal symptoms	Presence of specific PCR products (% of homology with sequences found inthe GenBank		
				Pa. chlamydospora	*Botryosphaeria* sp.	*Pm. aleophilum*
110R 151	1	Nursery "A"	asymptomatic	_	+ (98)	_
	2	Nursery "A"	asymptomatic	(99)	+ (100)	_
110R 151	3	Nursery "A"	asymptomatic	_	_	_
	4	Nursery "A"	asymptomatic	_	+ (99)	_
F9A 548/110R 180	5	Nursery "A"	Browning	_	_	_
	6	Nursery "A"	Browning	_	_	_
F9A 548/110R 180	7	Nursery "A"	asymptomatic	+ (99)	_	+ (98)
	8	Nursery "A"	Browning	_	_	_
MI-JANUS 50/110R 180	9	Nursery "A"	Browning	_	+ (99)	_
	10	Nursery "A"	Browning	_	+ (99)	_
MI-JANUS 50/110R 180	11	Nursery "A"	Browning	_	_	_
	12	Nursery "A"	asymptomatic	+ (99)	+ (99)	_
	3A	Nursery "B"	asymptomatic	_	+ (99)	_
	3B	Nursery "B"	asymptomatic	_	_	_
	3C	Nursery "B"	asymptomatic	_	_	_
	3D	Nursery "B"	asymptomatic	_	_	_
	3E	Nursery "B"	asymptomatic	_	_	_
	4A	Nursery "B"	asymptomatic	_	_	_
	4B	Nursery "B"	asymptomatic	_	_	_
	4C	Nursery "B"	asymptomatic	_	_	_
	4D	Nursery "B"	asymptomatic	_	_	_
	4E	Nursery "B"	asymptomatic	+ (100)	_	_
	5LA	Nursery "B"	asymptomatic	+ (100)	_	_
	5LB	Nursery "B"	asymptomatic	+ (100)	_	_
	6PA	Nursery "B"	asymptomatic	+ (100)	_	_
	6PB	Nursery "B"	asymptomatic	+ (100)	+ (99)	_

The molecular techniques used in this study proved to be highly sensitive; the nested PCR assay was more sensitive for detection than the single PCR, as little as 1 pg of DNA could be detected (Retief, 2005; Ridgway, 2002) from wood material. When traditional methods were compared with PCR detection, Retief *et al.*, (2005) found on average four times less positive detections with traditional methods in comparison with PCR detection in naturally infected grapevine material.

The results of this study showed that the potential exists for *Pa. chlamydospora* infection to be occuring during grapevine propagation operations in Italy. This is in accord with previous findings in New Zealand (Whiteman *et al.*, 2002) and South Africa (Retief *et al.*, 2006) nurseries.

Management of esca diseases complex relies on the use of pathogen-free plants for new planting. Infection may take place in nurseries during the propagation process and storage,

and may also happen because of the use of infected mother plants. In order to assess the use of healthy mother plant and propagation material, such a method is of the utmost necessary for testing of plant material designated for nursery production of plant propagation material. In addition, this detection system could also be used for ecological studies of esca on grapevine.

Chapter 3

Genetic diversity among isolates of *Phaeomoniella chlamydospora* obtained from esca diseased grapevines

ABSTRACT

Phaeomoniella chlamydospora has been implicated as the most important fungal organism associated with symptoms of Petri disease and believed to be its main causal agent worldwide. The aim of this study was to investigate the genetic diversity of a global collection of 250 isolates of *Pa. chlamydospora* collected from very old vines, in isolated locations in Italy like Sardinia and Sicily where farmers grow local grape varieties, and also collected in countries different from Italy, with the purpose of elucidating the possible existence of novel species of *Phaeomoniella* from grapevine and to gain a better understanding of the genotype geographic distribution of this important pathogen. Sequence data set analysis of the ß-Tubulin and EF 1-α genes of the isolates sampled from these geographic areas did not show important genetic diversity and did not reveal any subdivision in this population. The isolates used in the current study showed a high percentage of similarity and clustered together, indicating the absence of genotype- geographic structure. The occurrence of the same genotype in different countries and geographic areas is in agreement with previous studies and supports the fact that *Pa. chlamydospora* most likely reproduces asexually in the field. Till now, no sexual state has yet been identified for *Pa. chlamydospora*, which suggests it is either rare or nonexistent, and may account for the genetic uniformity in this species.

INTRODUCTION

Petri disease and esca are two vascular diseases associated with decline and dieback of young and older grapevines. Petri disease was formerly also known as black goo, young vine decline, slow dieback, *Phaeoacremonium* grapevine decline, and reported to cause decline in young vines (2-7 yr old), in many newly planted vineyards (Ferreira *et al.*, 1999; Morton, 2000; Mugnai *et al.*, 1999; Pascoe & Cottral, 2000; Scheck *et al.*, 1998). Field symptoms associated with Petri disease include stunted growth, vine decline, shoot dieback and gradual death of young grapevines. Other field symptoms include a high failure rate in the first year of planting; apparent normal growth the first and second year and then failure to reach normal size; weak plants at the row ends; abnormally low resistance to water stress, low soil fertility or low crop loads, and inconsistent disease expression of the same plant from year to year (Morton, 2000). Internal symptoms can normally be seen in the trunk and shoots. These include black spots when vines are cut transversely, and dark brown to black streaking when trunks or shoots are cut longitudinally. The damaged xylem vessels often ooze black sap and therefore, the popular name “black goo” was commonly used to describe this disease. The

black discolouration of the xylem tissue is caused by the formation of tylosis, gums and phenolic compounds by the host as a reaction to the presence of the fungus in the xylem tissue (Mugnai *et al.,* 1999). The blocking of the xylem tissue prohibits the normal uptake of water. During times of high water demand the host is predisposed by water stress (Ferreira *et al.*, 1999) leading to an increase in Petri disease symptoms. According to Petri, brown wood streaking predisposed the vine plant to wood decay or even represented the first signs of esca.

Petri disease has been recorded in grape-growing regions all over the world, for example in South Africa (Ferreira *et al*., 1994), France (Larignon & Dubos, 1997), the USA (Scheck *et al*., 1998), Australia (Pascoe, 1999), Italy (Mugnai *et al*., 1999), Argentina (Gatica *et al*., 2000), Austria (Reisenzein *et al*., 2000), Portugal (Chicau *et al*., 2000; Rego *et al*., 2000) and Turkey (Erkan Ari, 2000). It is caused by *Phaeomoniella* (*Pa*.) *chlamydospora* (W. Gams, Crous & M.J. Wingf. & L. Mugnai) Crous & W. Gams, but also several species of *Phaeoacremonium* (*Pm.*) W. Gams, Crous & M.J. Wingf. (Scheck *et al.,* 1998; Mugnai *et al,* ,1999; Groenewald *et al.,* 2001) are, less consistently, reported to be isolated from affected vines.

Esca is a disease typically reported on older vines – over 15-yrs-old, that since the end of the eighties affects more and more often also much younger vines. It is a vascular disease that also causes typical red-brown necrosis which, especially in older vines, are very often also colonized by a basidiomycetes fungus causing a typical white rot. The diseases causes a disfunction of leaves, that show chlorotic interveinal areas, necrosis, and finally leaf drop, leading to a loss in quality and quantity of harvest and slowly leading to death of the vines. A sudden death of the vines, apoplexy, is often common in vines, especially in older vines.

The *Phaeomoniella* genera include four species, "*chlamydospora*" which has been reported from different grapevine growing areas all over the world, "*pinifoliorum*" and "*zymoides*," which have been newly reported from the needle surface of *Pinus densiflora* in Korea (Hyang Burn *et al*., 2006) and more recently, Crous *et al.*, (2008) described new species named "*capensis*" isolated from living leaves of *Encephalartos altensteinii* in South Africa (Crous *et al*., 2008).

Phaeomoniella chlamydospora and *Phaeoacremonium* spp., mainly *Pm. aleophilum,* are the principal hyphomycetes, which are believed to be associated with Petri disease and esca disease symptoms (Mugnai *et al*., 1999; Chicau *et al*., 2000; Edwards & Pascoe, 2004), both producing a range of enzymes and phytotoxic metabolites. Although 20 species of *Phaeoacremonium* have been associated with grapevines, only the species *chlamydospora* is

known from this host. Moreover, the relative importance, frequency, geographic distribution and genetic diversity of populations of this species are still insufficiently understood.

Several studies have examined the genetic diversity of *Pa. chlamydospora* (Peros *et al*., 2000; Tegli *et al*., 2000b; Pottinger *et al*., 2002; Borie *et al*., 2002; Mostert *et al*., 2006; Cobos & Martin, 2008) using RAMS (Random Amplified Micro or Mini Satellites), RAPD (Random Amplified Polymorphic DNA) and UP-PCR (Universally Primed PCR) markers. They generally showed low levels of diversity in populations of *Pa. chlamydospora* from France, Italy and New Zealand and supported the presumed predominance of clonal reproduction (Tegli *et al*., 2000b; Borie *et al*., 2002; Pottinger *et al*., 2002).

Mostert *et al*., 2006 used AFLPs technique for the study of genetic variation among isolates of *Pa. chlamydospora* collected from several countries with grapevines exhibiting Petri disease or esca and noticed a high similarity among the *Pa. chlamydospora* isolates. However, these studies have not been applied on isolates coming from isolated regions like islands for example, from very old vineyards and local cultivars. Accordingly, a global collection of 250 isolates of *Pa. chlamydospora* strains collected mainly from very old vines, local cultivars, in isolated locations in Italy, like Sardinia and Sicily, and other countries was carried out. Old vines were included in this study as a source of esca tracheomycotic fungi with the specific objective of gathering a population of both genera, *Phaeomoniella* and *Phaeoacremonium*, showing an as wide as possible variability within the population of the two fungi, and with the purpose of elucidating the possible existence of novel species of *Phaeomoniella* from grapevine and to gain a better understanding of the genotype geographic distribution of this important pathogen.

Findings obtained by this population genetics study may also provide further information on the number and sources of the introductions of this fungus in Italy and other countries and should shed light on its dispersal, improving our understanding of the biology and epidemiology of these important pathogens.

MATERIALS & METHODS

Plant material

Branches and trunks of *Vitis vinifera* showing Petri disease and/or esca symptoms in wood, including brown and black streakings, brown-red wood, necrosis and white rot (Fig. 1) and in some cases also foliar symptoms of esca, were collected from different regions of Italy, primarily from isolated locations and very old vineyards (80–100 yr old), from different vineyards within the same region and from different positions on the same vine and from

spontaneous grapevine species growing in the wild (for example, in Italy: *Vitis vinifera*. subsp. *sylvestris*). The same type of plant material was also collected from different countries (Chile, Croatia, France, Germany, Greece, Hungary, Iran, New Zealand, Palestine, Portugal, Serbia, South Africa, Turkey, and the USA).

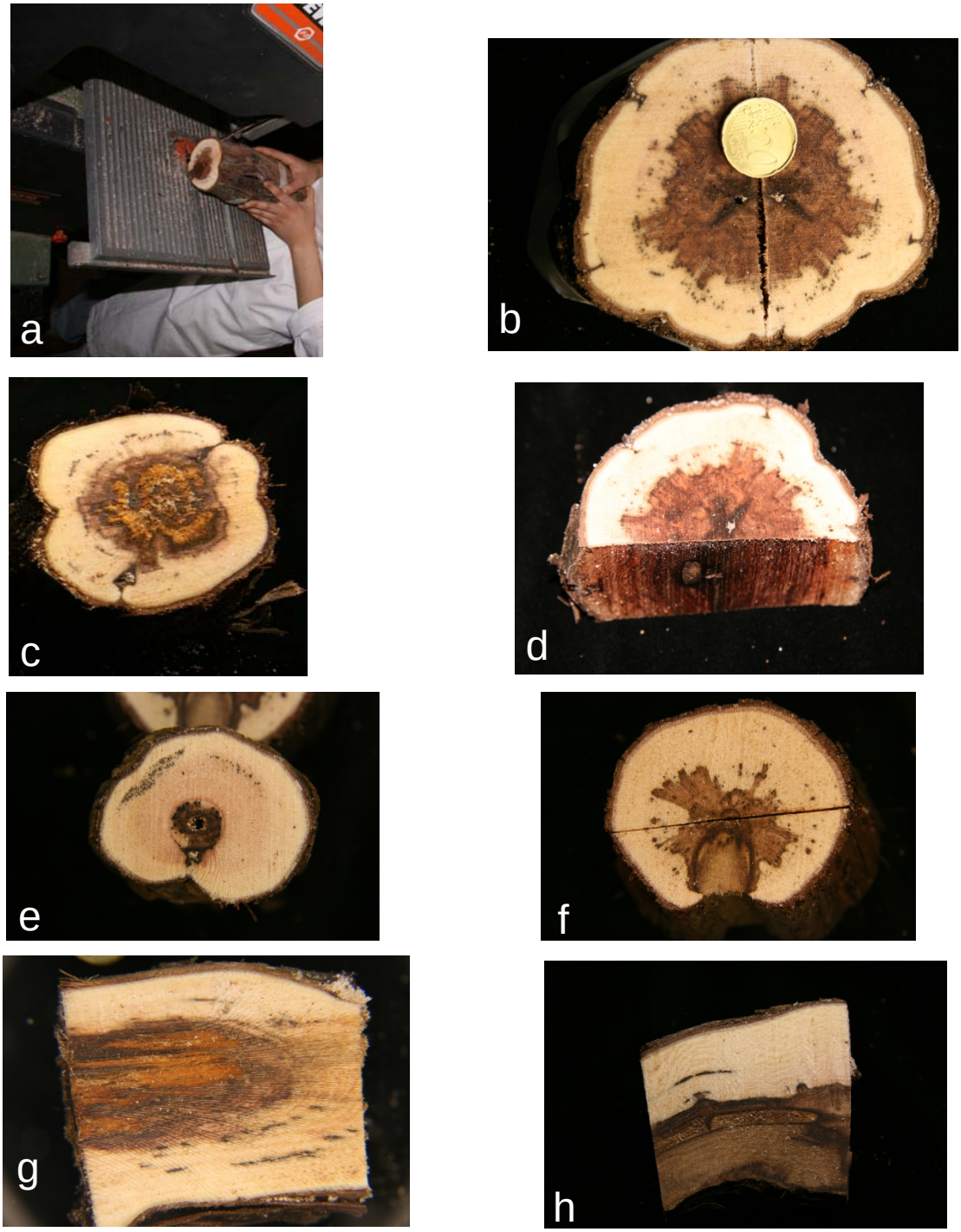

Fig. 1. Internal symptoms seen when transversal or longitudinal cuts were made in the trunk or cordon of vines used for fungal isolation. a. Trunks and shoots were cut into disks using a sawing machine.
b. Black spots and dark brown to black streaking of the xylem tissues; c. cross section showing a central white rot surrounded by brown-red wood; d. central brown-red necrosis; e. transversal cut showing blackspots, f. cross section showing sectorial necrosis; g. longitudinal section showing wood discoloration andcentral white rot; h. longitudinal section showing wood discoloration and black streaking.

Isolations

Trunk and shoots of diseased grapevines were cut into disks and the surface was sterilized. Small pieces of tissue were cut from just below the surface, around and in the darkened vascular tissues, and plated onto malt extract agar (MEA: 2 % malt extract, Oxoid Ltd., Basingstoke, Hampshire, England; 1.5 % agar, Difco, Detroit, Michigan, USA) and incubated at 25 °C in the dark for 2–3 wk until cultures sporulated. Single conidial isolations were established from emerging colonies identified as species of *Phaeomoniella chlamydospora*. Isolates were maintained at the Dipartimento di Biotecnologie Agrarie, Sezione di Patologia Vegetale, University of Florence, and representative strains lodged at the CBS Fungal Biodiversity Centre, Utrecht, Netherlands. Isolates used for morphological and sequence analysis are presented in Table 1. Samples were collected from different regions, and, in some of the locations, from different vineyards within the same region, from different vines within the same vineyard, from different positions of the vine and from different alterations observed.

Table 1. Collection details of *Pa. chlamydospora* isolates used in this study.

Continent	**Country**	**Region**	**Isolates number**	**Total number**
Europe	Italy	Tuscany – San Gimigniano	1-113	115
Europe	Italy	Tuscany -(*V. vinifera* subsp. *sylvestris*)	114-143	30
Europe	Italy	Tuscany -Arezzo	144-256	113
Europe	Italy	Trentino Alto Adige	257-269	13
Europe	Italy	Marche	270-272	3
Europe	Italy	Sardegna	273-297	25
Europe	Italy	Sicily (Montalbano, Milazzo-Barcellona)	298-388	91
Europe	Italy	Sicily (Monte Etna – Eolie- Montalbano)	389-544	156
Europe	Greece	_	545-566	22
Europe	Hungary	_	568-585	18
Europe	Germany	_	586-590	6
Europe	France	_	591-596	6
Europe	Portugal	_	597-601	5
Europe	Croatia	_	602-612	11
Eurasia	Turkey	_	613-617	5
Asia	Palestine	_	618-631	14
Asia	Iran	_	632-640	9
Oceania	New Zealand	_	641-677	37
South America	Chile	_	678-679	2
Europe	Serbia	_	680	1
Africa	South Africa	_	681-690	10
Europe	Austria	_	691	1
Total				**693**

Molecular analyses

DNA isolation and amplification

250 representative isolates of *Pa. chlamydospora* were selected among the 693 obtained, to be supplied for molecular analyses.

Genomic DNA was extracted, using the CTAB extraction method according to Möller *et al*., 1992. The concentration of all DNA was determined by separation on a 1% agarose gel containing 0.1 μg/ml ethidium bromide in 1x TAE buffer (0.4 M Tris, 0.05 M NaAc, and 0.01 M Ethylene Diamine Tertraacetic Acid [EDTA], pH 7.85) and visualized under ultra violet light.

Subsequently, a fragment of approximately 650 bp of the 5'end of the ß-Tubulin gene was amplified using primers T1 (O'Donnell & Cigelnik, 1997) and Bt2b (Glass & Donaldson, 1995) and a region of approximately 450bp of the Translation Elongation Factor EF 1-α gene was amplified using primers EF1-728F (Carbone & Kohn 1999) and EF-2 (O'Donnell *et al*., 1998) (Fig.2).

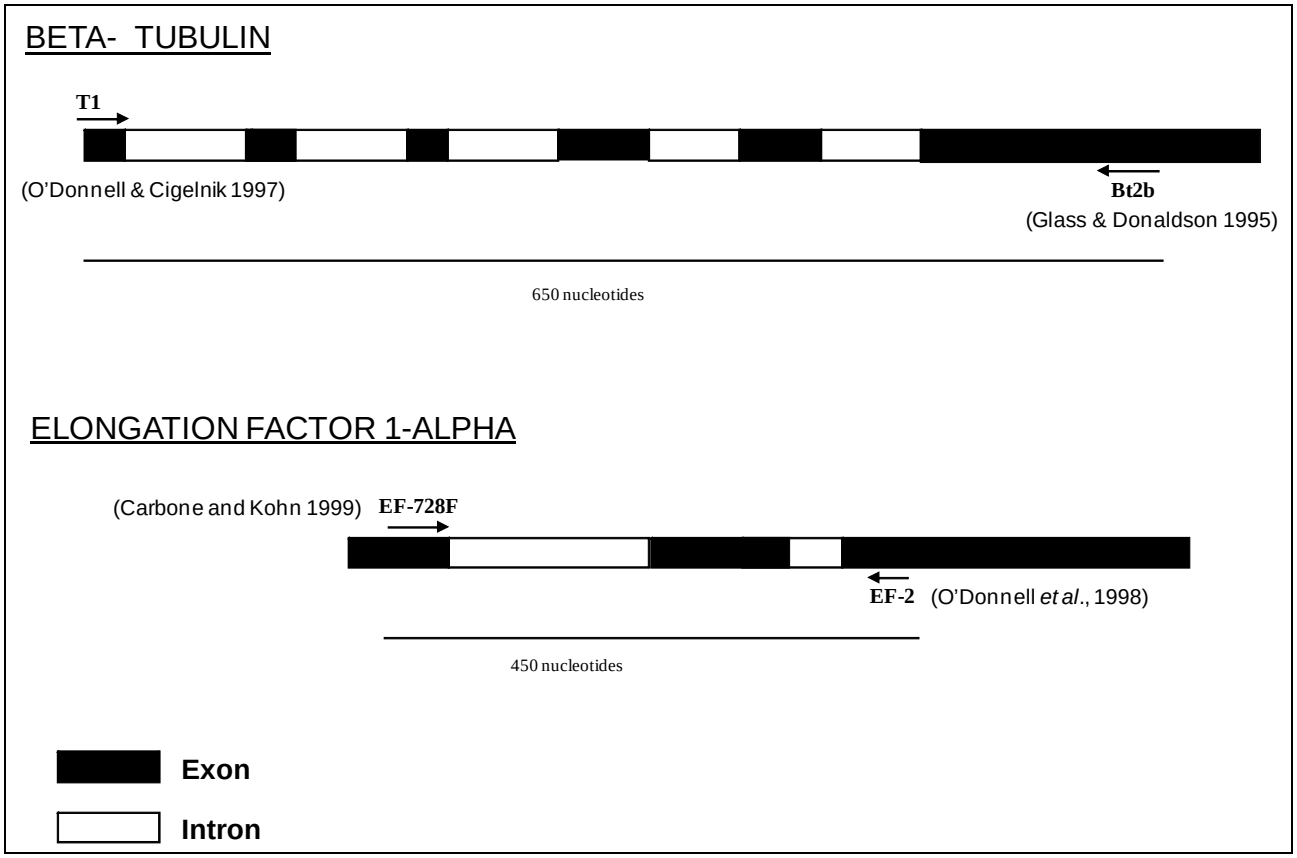

Fig. 2. Schematic representation indicating the introns and exons of the partial ß-Tubulin and EF 1-α genes. The ß-Tubulin gene include 1933 bp in total; 1344 coding for 448 amino acids, while the EF 1-α gene contain 1975 bp in total; 1383 coding for 461 amino acids.

Because Groenewald *et al.*, (2001) found the internal transcribed spacers (ITS1 and ITS2) and 5.8S rRNA insufficient to differentiate all taxa, this region was therefore excluded in the current study. The reaction mixture had a total volume of 12.5µl and contained 1µl of diluted DNA 1:10, 1x PCR buffer (Bioline, London, UK), 2.5 mM $MgCl_2$, 48 µM dNTP's, 0.2 µM of each primer, 0.7 units Taq DNA polymerase (Bioline).

The amplification reactions were done on a Gene Amp PCR System 9600 (Applied Biosystems, Foster City, California). The initial denaturation step was done at 94°C for 5 min, followed by 40 cycles of 45 s at 94°C, 30 s (at 58°C for ß-Tubulin and 55°C for EF-1 α), 90 s at 72°C, with a final extension of 72°C for 10 min. PCR products were separated by electrophoresis at 100 V for 1hr on a 1% (agarose gel containing 0.1 µg/ml ethidium bromide in 1x TAE buffer (0.4 M Tris, 0.05 M NaAc, and 0.01 M Ethylene Diamine Tertraacetic Acid (EDTA), pH 7.85) and visualized under ultra violet light.

The amplified products of interest have been analyzed, purified and sequenced.

Sequence and phylogenetic analysis

Amplicons were sequenced using both PCR primers with a BigDye Terminator Cycle Sequencing Kit v. 3.1 (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions, and sequences were analysed on an ABI Prism 3700 DNA Sequencer (Perkin-Elmer, Norwalk, Foster City, CA, USA). A consensus sequence was computed from the forward and reverse sequences with the SeqMan from the Lasergene package (DNA star, Madison, WI, USA).

The products were analysed on ABI Prism 3700 DNA Sequencer (Applied Biosystems). A consensus sequence was computed from the forward and reverse sequences with SeqMan from the Lasergene package (DNA star, Madison, WI).

Sequences were aligned using Clustal X version 2.0.9 Clustal (Larkin *et al*. 2007) and Sequence Alignment Editor v. 2.0a11 (Se-Al; Rambaut 2002) by inserting gaps, and additional reference sequences were obtained from GenBank and added to the alignment.

Phylogenetic analyses of the Elongation factor EF1- α gene was done using TREEVIEW software (Page 1996). *Pleurostomophora richardsiae* (CBS 270.33; GenBank TUB = AY579335, EF-1 α = AY179914) was used as outgroup in the phylogenetic analyses.

RESULTS

PCR results of the amplification of the Elongation factor EF 1-α gene

PCR conducted using primers EF1-728F and EF-2 revealed an amplification of a specific fragment of 450 bp of the 5'end of the EF1-α gene (Fig. 2).

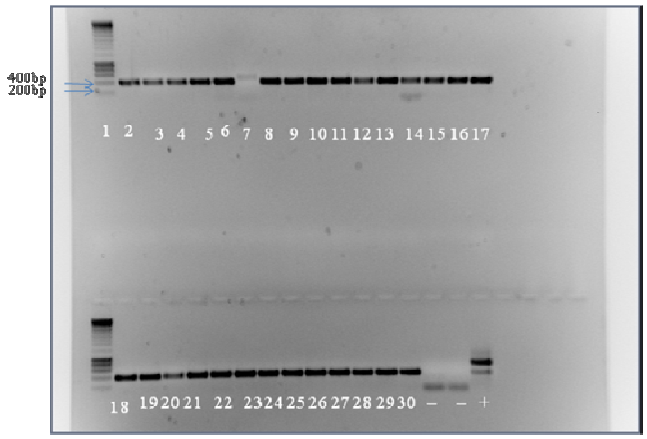

Fig. 2. PCR amplicons generated in a species specific PCR using primers EF1-728F and EF-2 on DNA extracted from some of *Pa. chlamydospora* isolates used in this study. Lane 1 contains the 1- Kb DNA marker (Hyperladder I, Bioline). The 450 bp fragment, is present for all the PCR amplifications of DNA of *Pa. chlamydospora* isolates (lane 2 to 30, except in lane7). Lane (-): PCR negative control (sterile distilled control). Lane (+): PCR positive control: DNA of *Ph. aleophilum* CBS 111014.

PCR results of the amplification of ß-Tubulin gene

PCR conducted using primers T1 and Bt2b revealed an amplification of a specific fragment of 650 bp of the 5'end of the ß-Tubulin gene (Fig. 3).

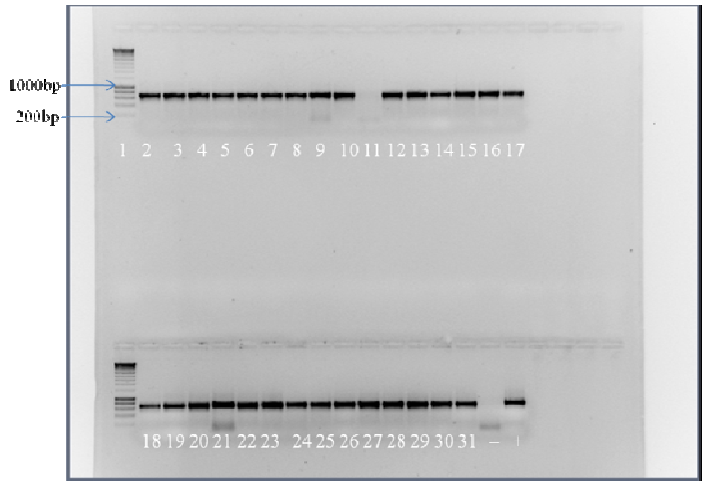

Fig. 3. PCR amplicons generated in a species specific PCR using primers T1 and Bt2b on DNA extracted from *Pa. chlamydospora* isolates used in this study. Lane 1 contains the 1- Kb DNA marker (Hyperladder I, Bioline), size is shown on the left of the gel. The 650-bp fragment, is present for all the PCR amplifications of DNA of *Pa. chlamydospora* isolates (lane 2 to 31, except in lane11). Lane (-): PCR negative control (water control). lane (+): PCR positive control: DNA of *Ph. aleophilum* CBS 111014.

The amplified products were subsequently sequenced and analyzed.

Sequence analysis

Sequences of the ß-Tubulin and the Elongation factor EF 1-α genes of the isolates studied revealed a low level of genetic diversity. In particular, all *Pa. chlamydospora* isolates were virtually indistinguishable one from the other.

Blast search analysis of the ß-Tubulin and the Elongation factor EF 1-α sequence data sets showed a high similarity with sequences of *Pa. chlamydospora* present in the GenBank data.

Phylogenetic analysis

Clustal X alignment of the sequences of the EF1-α and β-tubulin region obtained showed the amount of variation (in term of nucleotides differences). Minute nucleotide differences were observed when sequences of the EF1-α region were aligned, while with ß-Tubulin all the sequences were similar (Fig.2 & 3).

Fig. 2. Clustal X alignment of some of the ß-Tubulin sequences of representative isolates.

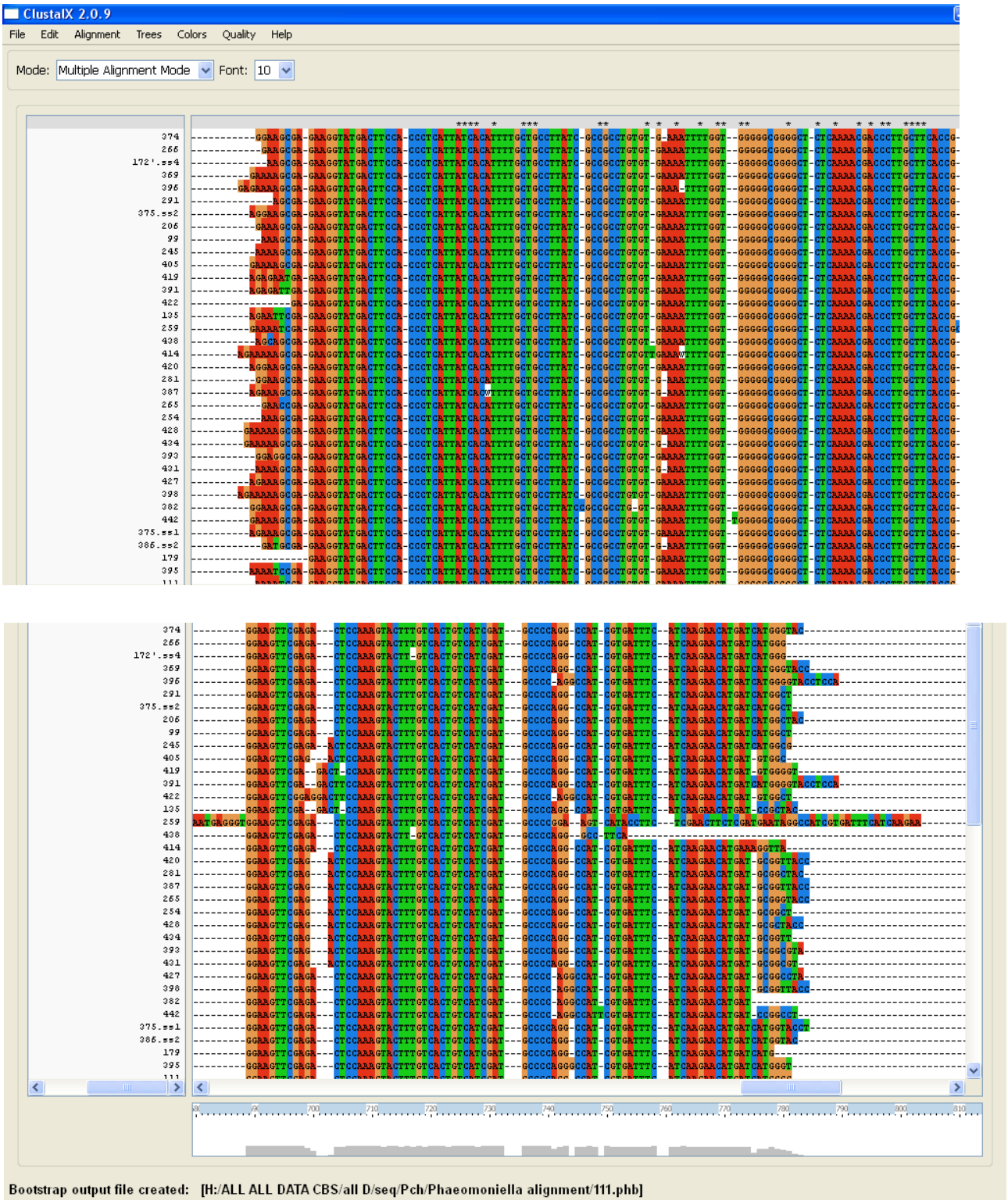

Fig. 3. Clustal X alignment of the Elongation factor EF-1 α sequences of representative isolates.

Nevertheless, the little variation noticed in clustal X alignment of sequences of the EF1-α gene was not supported by phylogenetic tree analysis. In fact, all the *Pa. chlamydospora* isolates clustered in a single clade, together with sequences of previously published species, available in GenBank. Isolates 6 & 382 which clustered in different clades showed to belong to another genus as they had been misidentified as *Pa. chlamydospora* (Fig. 4).

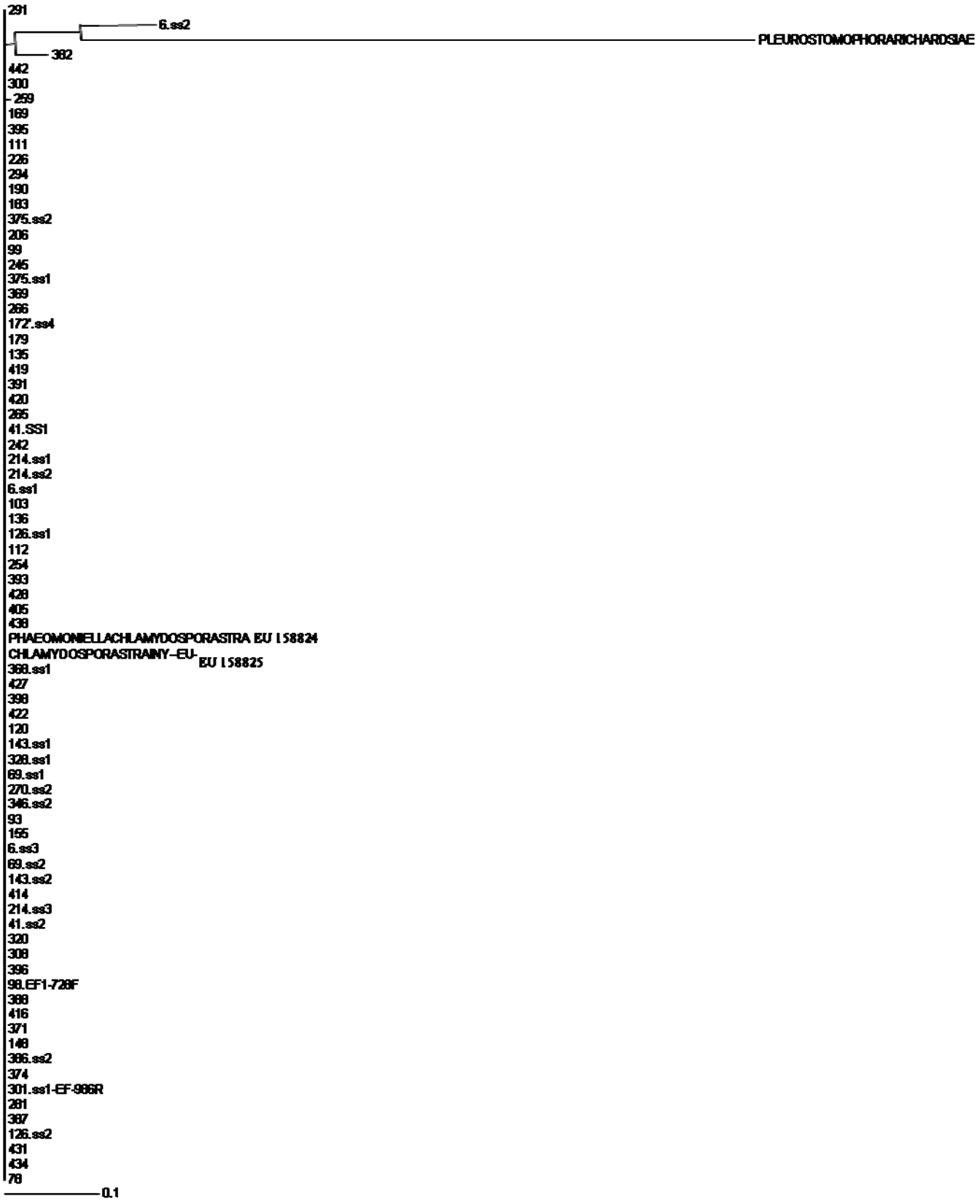

Fig. 4. Dendrogram generated using TreeView software from the Elongation factor EF 1-α sequence data stes. Accessions numbers of Sequences of *Ph. chlamydopora* used in the analysis are: Isolate Y121-20-1, EU 158825 and isolate Y116-18-3, EU 158824, available in the GenBank nucleotide database. *Pleurostomophora richardsiae* (CBS 270.33; EF-1 α = AY179914) was used as outgroup.

DISCUSSION

Contrary to our expectations, a high level of similarity was found among the isolates of *Pa. chlamydospora* used in this study, despite the fact that they were collected from areas not used in previous studies and despite the difference observed in cultural characteristics. The results of the present study are in general agreement with previous studies (Tegli *et al*., 2000; Borie *et al*., 2002; Pottinger *et al*., 2002; Mostert *et al*., 2006; Cobos & Martin, 2008) that noted also a low degree of genetic diversity among *Pa. chlamydospora* strains. However, is it common that a population of a pathogen like *Pa. chlamydospora* that is worldwide spread is so stable? Is it possible that a pathogen like *Pa. chlamydospora* that has been well adapted to so different climatic conditions, in such a wide variety of environments has practically no genetic variation?

The goal of this study was to answer these questions by carrying out an extensive sampling mainly from areas not included in previous research programmes. Actually, previous studies conducted on the genetic variability of *Pa. chlamydospora* focused mainly on variation within countries more than the variation of the origin of the plant material. However, isolates coming from different countries may have the same initial propagation material. As a mater of fact, similar clonal lineages of *Pa. chlamydopora* in different countries could be due to single introduction events from the same inoculum source (grapevine cuttings).

Moreover, these studies had not been previously applied on isolates coming from isolated regions like islands for example. Sampling from areas where cultivars used in viticulture originated, namely Eurasia and USA, from isolated locations in Italy and other countries, from vineyards using minor cultivars (different from the international ones usually sampled in previous papers as: cv. Savignon, cv. Chardonnay, cv. Sangiovese), from spontaneous grapevine growing areas (*Vitis vinifera* subsp. *sylvestris*) and from other countries selecting vines of local varieties (Chile, Croatia, France, Germany, Greece, Hungary, Iran, New Zealand, Palestine, Portugal, Serbia, South Africa, Turkey, and the USA) were therefore carried out. Furthermore old vines were, where available, were included in this study as a source of esca tracheomycotic fungi with the specific objective of gathering a population of both genera, *Phaeomoniella* and *Phaeoacremonium,* showing as wide as possible variability within the population of the two fungi. In this chapter, we report the results so far obtained in *Phaeomoniella.*

Sequence data analysis of the β- tubulin and EF 1-α genes of the isolates sampled from these geographic areas did not show important genetic diversity and did not reveal any subdivision in this population. These isolates showed a high percentage of similarity and clustered in a

single clade, together with sequences of previously published species, available in GenBank, indicating the absence of genotype- geographic structure.

The occurrence of the same genotype in different countries and geographic areas supports the fact that *Pa. chlamydospora* can occur in apparently healthy rooted grapevine cuttings (Bertelli *et al*., 1998), and apparently disease-free grapevines and could be distributed via exported grapevine material.

Finally, a high frequency of isolation of *Pa. chlamydospora* was noticed from the plant material collected in this study, since it has been isolated much more frequently than species of *Phaeoacremonium* and it was present in all the Petri disease/esca diseased plant material sampled, and a considerable differences was observed in term of cultural characteristics (different shape and colour of the grown colony of *Pa. chlamydospora*). Nevertheless, these differences were not support by molecular analyses and showed that the population of *Pa. chlamydospora* sampled in the present work is not subdivided, and revealed a high level of similarity. This finding might be explained by the fact that *Pa. chlamydospora* most likely reproduces asexually in the field. Till now, no sexual state has yet been identified for this species, which suggests it is either rare, may be only present in the area of origin, or nonexistent, and possibly accounts for the genetic uniformity in this species.

This high homogeneity could also suggests that the fungus has spread all around the world quite recently, probably via grafting, as if spores were the main way of spreading of the pathogen, some mutations could have been found. The hypothesis of an origin of the disease in North America, where the rootstock varieties resistant to *Phylloxera* were exported all over the grape growing countries, should be tested by a wider sampling in that area.

Chapter 4

Molecular and phenotypic characterisation of novel *Phaeoacremonium* species isolated from esca diseased grapevines

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ABSTRACT

Petri disease and esca are very destructive grapevine decline diseases that occur in most countries where grapevine (*Vitis vinifera*) is cultivated. *Phaeoacremonium* species are among the principal hyphomycetes associated with Petri disease and esca symptoms, producing a range of enzymes and phytotoxic metabolites. The present study compared the phylogeny of a global collection of 118 *Phaeoacremonium* isolates from grapevines, in order to gain a better understanding of their involvement in Petri disease and esca. Phylogenetic analyses of combined DNA sequence data sets of actin and β-tubulin genes revealed the presence of 13 species of *Phaeoacremonium* isolated from esca diseased grapevines. *Phaeoacremonium aleophilum* was the most frequently isolated species with an incidence up to 80 % of all isolates investigated. Previously described species, namely *Pm. alvesii*, *Pm. griseorubrum* and *Pm. rubrigenum* are newly reported on grapevine from Turkey, Italy and Croatia, respectively. *Phaeoacremonium viticola* and *Pm. scotyli* represent new records for Italy, as well as *Pm. mortoniae* for Hungary and Croatia. In addition, four new species of *Phaeoacremonium,* namely *Pm. croatiense*, *Pm. hungaricum, Pm. sicilianum* and *Pm. tuscanum* are newly described from grapevine based on morphology, cultural characteristics, as well as molecular phylogeny.

INTRODUCTION

Phaeoacremonium species are well-known vascular plant pathogens causing wilt and dieback of woody hosts. In grapevine, the two principal diseases in which they are involved are Petri disease and esca, the latter of which comprises young esca and esca proper according to the nomenclature of esca diseases proposed by Graniti *et al*., (2000). Petri disease causes stunted growth and dieback of young grapevines. It often occurs in 1–5 yr old grapevines and causes significant losses in newly planted vineyards (Mugnai *et al*., 1999; Morton, 2000; Pascoe & Cottral, 2000; Edwards & Pascoe, 2004; Surico *et al*., 2006). Internal symptoms can normally be seen when transverse or longitudinal cuts are made in the rootstock. These include black spots and dark brown to black streaking of the xylem tissues. Esca can be typically identified by various types of internal wood deterioration as well as symptoms on leaves and berries. Vines with typical symptoms on the leaves show interveinal areas of chlorotic tissue that turn yellow-brown or red-brown and finally necrotize, an appearance that can also be described as ‘tiger stripes’ (Larignon & Dubos, 1997; Mugnai *et al*., 1999; Edwards *et al*., 2001; Calzarano & Di Marco, 2007) (Fig. 1). In the USA, esca has been referred to as ‘black measles’ because

of the small, dark-brown to purple spots that can develop on the berries (Fig. 2) (Vasquez *et al*., 2007). When a transverse cut is made in the trunk and main shoots black spots (black streaking in longitudinal section) appear in the wood as in the case of Petri disease, but in young esca also pink-brown or dark red-brown areas can be found, occasionally with other wood discolorations (Mugnai *et al*., 1999). Esca proper (Surico, 2001) differs from young esca for the presence of wood decay (Mugnai *et al.* 1999; Fischer, 2002; Surico *et al*., 2006) (Fig. 3).

Foliar and fruit symptoms do not necessarily appear on the same diseased plant every year (Mugnai *et al.,* 1999; Marchi *et al.,* 2006), and often infected vines remain asymptomatic (Surico *et al.,* 2006). In severe cases 'apoplexy' can occur when vines or vine-parts suddenly wilt during hot, dry conditions in the summer.

Fungi that have been associated with esca symptoms in Europe include the wood-rotting basidiomycete *Fomitiporia mediterranea*, and occasionally *Stereum hirsutum* (Larignon & Dubos, 1997; Fischer, 2006), while *Phaeomoniella chlamydospora* and *Phaeoacremonium aleophilum* are the principal hyphomycetes associated with black streaking and brown-red wood (Larignon & Dubos, 1997; Mugnai *et al.,* 1999; Crous & Gams, 2000). It is the combination of these fungi that causes 'esca proper', affecting mostly vines older than 15 yr, while vines showing esca foliar symptoms, wood black streaking and necrosis show to be mainly infected with *Phaeomoniella chlamydospora* and/or *Phaeoacremonium* species.

Species of *Phaeoacremonium* mainly involved in Petri disease and esca are *Pm. aleophilum*, *Pm. angustius*, *Pm. parasiticum*, and *Pm. mortoniae* (Eskalen *et al.*, 2005a; Mostert *et al.*, 2006a; Martin & Cobos, 2007), but the degree of involvement of other *Phaeoacremonium* species remains uncertain. Furthermore, the identity of fungi associated with esca syndrome in many grapevine-growing areas, especially from the area where grapevine has originated, and several isolated regions have not yet been studied, and therefore many elusive aspects remain to be clarified.

Fig. 1. Symptoms associated with esca of grapevine: chlorosis and necrosis on the leaves showing typical "tiger-like" pattern

Fig. 2. Symptoms associated with esca of grapevine: black measles on the berries

Fig. 3. Typical wood symptoms in a vine affected by esca: brown-red wood, black streaking, and central white decay.

The present study investigates the identity of a group of 118 *Phaeoacremonium* isolates from grapevine, collected mainly from very old vines, in isolated locations in Italy and other countries. Knowledge pertaining to the involvement of *Phaeoacremonium* species in esca and Petri disease should shed light on the epidemiology of these destructive diseases of grapevine, with the final aim of helping in refining control strategies, since there are no effective curative chemicals for Petri disease and esca.

MATERIAL AND METHODS

Fungal isolates

Branches and trunks of *Vitis vinifera* showing wood esca symptoms, including brown and black streakings, brown-red wood, necrosis and white rot (Fig. 4) and in same cases also foliar symptoms of esca, were collected from different regions of Italy, primarily from isolated locations and very old vineyards (80-100 yr old), from different vineyards within the same region and from different positions on the same vine. Other strains, collected from different countries (Croatia, Greece, Hungary, Israel, Turkey and USA), were also used in this study.

Trunk and shoots of diseased grapevines were cut into disks and surface sterilised. Small pieces of tissue were cut from just below the surface, around and in the darkened vascular tissues, and plated onto malt extract agar (MEA; 2 % malt extract, Oxoid Ltd., Basingstoke, Hampshire, England; 1.5 % agar, Difco, Detroit, Michigan, USA) and incubated at 25 °C in the dark for 2–3 wk until cultures sporulated. Single conidial isolations were established from emerging colonies identified as species of *Phaeoacremonium*. Isolates were maintained at the Dipartimento di Biotecnologie agrarie, Sezione di Patologia vegetale, University of Florence, and representative strains lodged at the CBS Fungal Biodiversity Centre, Utrecht, the Netherlands. Isolates used for morphological and sequence analysis are presented in Table 1.

Fig. 4. Internal symptoms seen when transversal or longitudinal cuts were made in the trunk or cordon of vines used for fungal isolation. a, b. Black spots and dark brown to black streaking of the xylem tissues; c. cross section showing sectorial necrosis; d. longitudinal section showing wood discoloration; e. central brown-red necrosis; f. cross section showing a central white rot surrounded by brown-red wood.

DNA isolation and amplification

Genomic DNA was extracted from 118 strains identified as *Phaeoacremonium* using approximately 300 mg mycelium with the UltraClean™ Microbial DNA Kit (MO Bio, Carlsbad, CA, USA) according to the manufacturer's instructions. Approximately 600 bp of the 5' end of the β-tubulin (TUB) and approximately 300 bp of the 5' end of the actin (ACT) genes were amplified for the strains identified as *Phaeoacremonium* as described by Mostert *et al.,* (2006b) using primer sets T1 (O'Donnell & Cigelnik, 1997) and Bt2b (Glass & Donaldson, 1995) and ACT-512F and ACT-783R (Carbone & Kohn, 1999) respectively.

Amplicons were sequenced using both PCR primers with a BigDye Terminator Cycle Sequencing Kit v. 3.1 (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's recommendations, and sequences were analysed on an ABI Prism 3700 DNA Sequencer (Perkin-Elmer, Norwalk, Foster City, CA, USA). A consensus sequence was computed from the forward and reverse sequences with the SeqMan from the Lasergene package (DNA star, Madison, WI, USA).

Table 1. Names, GenBank accession numbers and collection details of *Phaeoacremonium* isolates studied.*Phaeoacremonium aleophilum* sequence types based on ACT and TUB respectively are indicated between round brackets (superscript in isolate number).

Species	Isolate number	Location	Gene bank accession numbers	
			ACT	**β-tubulin**
Phaeoacremonium aleophilum	4.ss2Pal $^{(1/1)}$	Tuscany, Italy	EU863496	EU863464
	146Pal $^{(1/1)}$	Abruzzo, Italy		
	32Pal $^{(1/1)}$	Marche, Italy		
	59Pal, 69Pal, 75Pal, 76.ss1Pal $^{(1/1)}$	Sicily, Italy		
	4ss1Pal, 23Pal $^{(1/1)}$	Tuscany, Italy		
	17Pal, 20Pal $^{(2/1)}$	Tuscany, Italy		
	30Pal $^{(2/1)}$	Trentino-Alto Adige, Italy		
	33Pal $^{(2/1)}$	Marche, Italy		
	38Pal, 39Pal $^{(2/1)}$	Sardinia, Italy		
	44ss1Pal, 51Pal, 52a.ss1Pal, 52ass2 Pal, 52-bPal,	Sicily, Italy		
	53Pal, 64Pal, 70Pal, 71Pal, 72Pal, 77Pal, 80Pal $^{(2/1)}$	Sicily, Italy		
	126Pal, 127Pal $^{(2/1)}$	Turkey		
	143ss2Pal $^{(2/1)}$	Umbria, Italy		
	124Pal, 125ss1Pal $^{(2/1)}$	Turkey		
	140Pal $^{(2/1)}$	Friuli-Venezia Giulia, Italy		
	60Pal, 61Pal, 62Pal, 73Pal, 74Pal, 78Pal, 79Pal $^{(3/1)}$	Sicily, Italy		
	142Pal $^{(3/1)}$	Tuscany, Italy		
	100Pal, 101Pal, 103Pal, 104Pal $^{(4/1)}$	Hungary		
	148Pal $^{(4/1)}$	Apulia, Italy		
	145Pal $^{(4/1)}$	Abruzzo, Italy		
	130Pal, 131Pal, 133Pal $^{(6/1)}$	Israel		

Table 1. (continued)

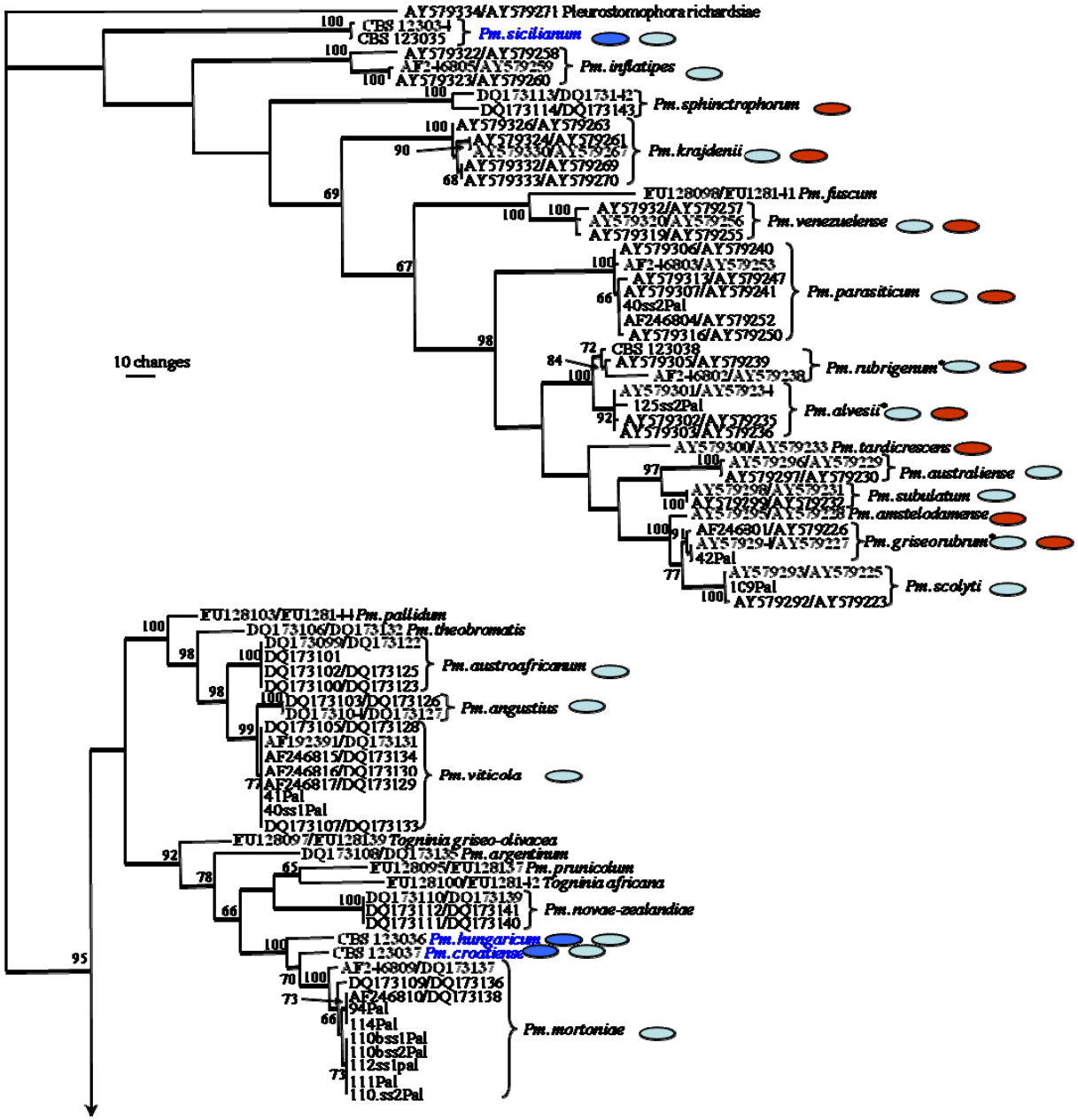
Species	Isolate number	Location	Gene bank accession numbers	
			ACT	**β-tubulin**
Phaeoacremonium aleophilum	81Pal (2/2)	Sicily, Italy	EU863497	EU863465
	168Pal (2/3)	Trentino-Alto Adige, Italy	EU863498	EU863466
	31Pal (1/3)	Trentino-Alto Adige, Italy		
	56Pal (1/3)	Sicily, Italy		
	158Pal (1/3)	Tuscany, Italy		
	13Pal, 14Pal, 28Pal (2/3)	Tuscany, Italy		
	167Pal, 171Pal (2/3)	Trentino-Alto Adige, Italy		
	139Pal (2/3)	Lombardy, Italy		
	116Pal (2/3)	Croatia		
	120Pal, 121Pal, 128Pal, 129Pal (2/3)	Turkey		
	22Pal, 24Pal, 25Pal, 152Pal, 153Pal (2/3)	Tuscany, Italy		
	58Pal, 68Pal (2/3)	Sicily, Italy		
	115Pal (2/3)	Croatia		
	65Pal, 66Pal (2/3)	Sicily, Italy		
	122Pal, 123Pal (2/3)	Turkey		
	159Pal, 161Pal (2/3)	U.S.A		
	47Pal, 57Pal, 67Pal (2/3)	Sicily, Italy		
	137Pal, 138ss1Pal (3/4)	Lombardy, Italy	EU863500	EU863468
	156Pal (5/4)	Tuscany, Italy	EU863499	EU863467
	84Pal, 85Pal (1/4)	Greece		
	117Pal, 118Pal (2/4)	Croatia		
	98Pal (3/5)	Hungary	EU863501	EU863469
	21Pal (4/6)	Tuscany, Italy	EU863502	EU863470

Table 1. (continued)

Species	Isolate number	Location	Gene bank	accession numbers
			ACT	**β-tubulin**
Phaeoacremonium aleophilum	144Pal [(1/7)]	Abruzzo, Italy	EU863503	EU863471
	132Pal [(6/8)]	Israel	EU863504	EU863472
Pm. alvesii	125ss2 Pal	Turkey		
***Pm. croatiense* sp. nov.**	CBS 123037	Croatia	EU863514	EU863482
***Pm. hungaricum* sp. nov.**	CBS 123036	Hungary	EU863515	EU863483
Pm. iranianum	2Pal	Tuscany, Italy	EU863491	EU863459
	3Pal	Tuscany, Italy	EU863492	EU863460
	6Pal	Tuscany, Italy	EU863493	EU863461
	7Pal	Tuscany, Italy	EU863494	EU863462
	163Pal	Piedmont, Italy	EU863495	EU863463
Pm. griseorubrum	42Pal	Sicily, Italy	EU863517	EU863485
Pm. mortoniae	110bss1Pal	Hungary	EU863507	EU863475
	110.ss2Pal	Hungary	EU863508	EU863476
	111Pal	Hungary	EU863509	EU863477
	112ss1Pal	Hungary	EU863510	EU863478
	110bss2Pal	Hungary	EU863511	EU863479
	114Pal	Hungary	EU863512	EU863480
	94Pal	Croatia	EU863513	EU863481
Pm. parasiticum	40ss2Pal	Sicily, Italy	EU863519	EU863487
Pm. rubigenum	CBS 123038	Croatia	EU863516	EU863484
Pm. scolyti	109Pal	Tuscany, Italy	EU863518	EU863486
***Pm. sicilianum* sp. nov**	CBS 123034	Sicily, Italy	EU863520	EU863488
	CBS 123035	Sicily, Italy	EU863521	EU863489
***Pm. tuscanum* sp. nov.**	CBS 123033	Tuscany, Italy	EU863490	EU863458
Pm. viticola	40ss1Pal	Sicily, Italy	EU863505	EU863473
	41Pal	Sicily, Italy	EU863506	EU863474

Phylogenetic analysis

Sequences were manually aligned using Sequence Alignment Editor v. 2.0a11 (Se-Al; Rambaut, 2002) by inserting gaps, and additional reference sequences were obtained from GenBank and added to the alignment. The TUB and ACT alignments were concatenated to make it possible to perform combined analyses. Phylogenetic analyses of the aligned sequence data were performed with PAUP (Phylogenetic Analysis Using Parsimony) v. 4.0b10 (Swofford, 2003) and consisted of neighbour-joining analyses with the uncorrected ('p'), the Kimura 2-parameter and the HKY85 substitution models. Alignment gaps were treated as missing data and all characters were unordered and of equal weight. Any ties were broken randomly when encountered. For parsimony analyses, alignment gaps were treated as a fifth character state and all characters were unordered and of equal weight. Maximum parsimony analysis was performed using the heuristic search option with 100 random simple taxa additions and tree bisection and reconstruction (TBR) as the branch-swapping algorithm. Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. The robustness of the trees obtained was evaluated by 1000 bootstrap replications (Hillis & Bull, 1993). Tree length (TL), consistency index (CI), retention index (RI) and rescaled consistency index (RC) were calculated and the resulting trees were printed with TreeView v. 1.6.6 (Page 1996). New sequences were lodged in GenBank and the alignment and phylogenetic tree in TreeBASE (www.treebase.org). *Pleurostomophora richardsiae* (CBS 270.33; GenBank ACT=AY579271, TUB=AY579334) was used as outgroup in the phylogenetic analyses.



(cont. on the next page)

Fig. 5. One of 317 most parsimonious trees obtained from heuristic searches of a combined alignment of the TUB and ACT gene sequences (length = 1304 steps, CI = 0.508, RI = 0.922, RC = 0.468, HI = 0.492). Bootstrap support values above 64 % are shown at the nodes. *Pleurostomophora richardsiae* was used as outgroup. Accessions numbers of sequences obtained from the GenBank nucleotide database are indicated on the tree in the format TUB/ACT. Ex-type strains are emphasised in **bold**. Names in blue are novel species described in this study.

New species described in this study; Human pathogenic species; Species isolated from grapevine; * Human pathogenic species newly reported from grapevine

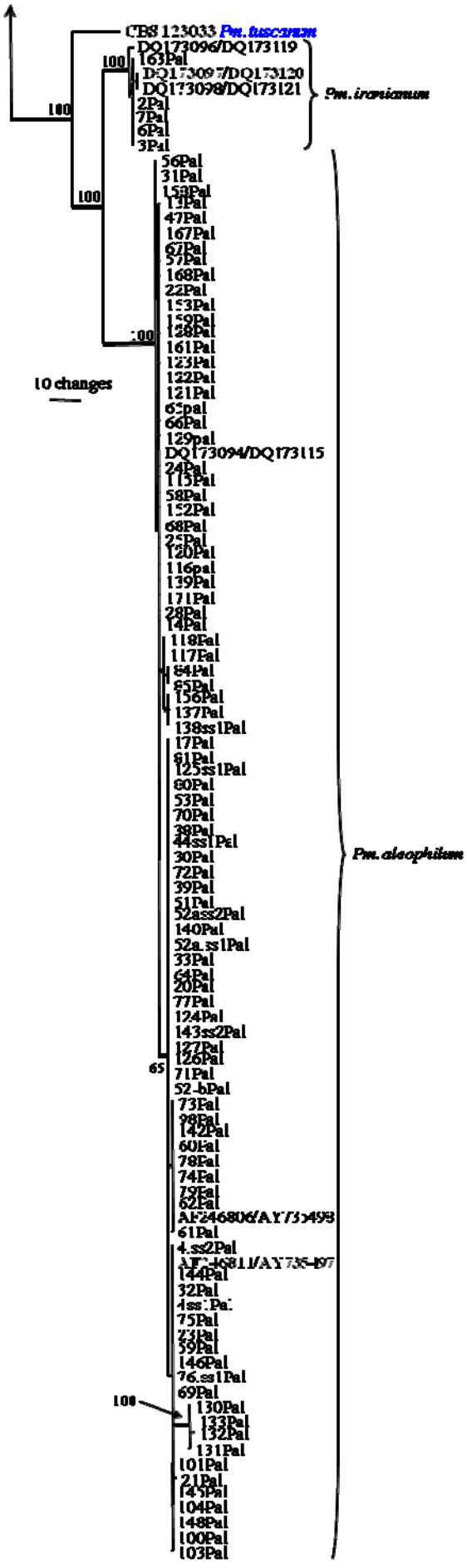


Fig. 5 (continued)

Morphology

Morphological characters used in distinguishing species included conidiophore morphology, phialide type and shape, size of hyphal warts, and to a lesser extent, conidial size and shape. Cultural characters that were investigated included the colour of colonies on MEA, the production of yellow pigment on potato-dextrose agar (PDA; 3.9 % potato-dextrose agar, Difco) and oat meal agar (OA, 30 g oats; 8 g Roko agar, La Coruña, Spain; 1000 mL water) (Gams *et al.*, 2007), the growth rate of colonies at 25 °C, and the maximum temperature for growth in vitro.

Microscopic observations were made from aerial mycelium of colonies cultivated on MEA or by using the transparent tape or slide culture technique, as respectively explained by Schubert *et al.*, (2007) and Arzanlou *et al.*, (2007). Photos were captured by means of a Nikon camera system (Digital Sight DS-5M, Nikon Corporation, Japan). Structures were mounted in lactic acid, and 30 measurements (× 1000 magnification) were determined. The 5^{th} and 95^{th} percentiles were defined for all measurements with the extremes given in parentheses.

Cardinal temperatures for growth were determined by incubating inoculated MEA plates in the dark at temperatures ranging from 6 to 40 °C. Radial growth was measured after 8 d at 25 °C. Colony colours were defined after 16 d from the same plates according to the colour charts of Rayner (1970).

RESULTS

Phylogenetic analyses

The combined alignment consisted of 184 sequences including the outgroup sequence and included 473 characters and alignment gaps (number of included characters: TUB = 266 and ACT = 207) that were subjected to the phylogenetic analyses. Of these, 275 were parsimony informative and 47 were variable and parsimony uninformative and 151 were constant. The small number of characters included for the TUB is due to the inclusion of GenBank accession AF192391, which represents the TUB sequence of the type strain of *Pm. viticola* and which is missing more than 250 characters on the 5' end when compared to the other sequences in the alignment. Parsimony analyses yielded 317 equally most parsimonious trees that mainly differed in the order of taxa at the terminal nodes; one of the trees is presented in Fig. 5 (TL = 1304; CI = 0.508; RI = 0.922; RC = 0.468; HI = 0.492). Neighbour-joining analysis using the three substitution models on the sequence data yielded trees with similar topology and bootstrap values. The phylogenetic tree clustered some isolates obtained in this study with previously published species and indicated that others did not match any sequences available in GenBank. The second group of sequences represents unknown species, which are described below.

Analyses of the individual loci did not reveal any significant deviation from the topology obtained from analyses of the combined alignment and 72 equally most parsimonious trees were obtained for both loci (data not shown). For the TUB data, the 266 characters including alignment gaps consisted of 136 parsimony informative, 31 variable and parsimony uninformative and 99 constant characters; for the ACT data the 207 characters including alignment gaps consisted of 139 parsimony informative, 16 variable and parsimony uninformative and 52 constant characters.

Taxonomy

According to DNA sequence analyses and morphological characters, the 118 strains isolated from wood of *Vitis vinifera* showing Petri disease and esca symptoms could be assigned to 13 different species of *Phaeoacremonium*. Four taxa proved to be distinct from known species and are described in this study. In addition to the novel species, the morphologically variant form seen in *Pm. rubrigenum* isolate CBS 123038 was discussed in contrast to the isolates described by Mostert *et al.*, (2006b).

Phaeoacremonium croatiense Essakhi, Mugnai, Surico & Crous, *sp. nov.* — MycoBank MB506947; Fig. 6

Phaeoacremonio mortoniae phylogenetice simile, sed coloniis olivaceo-griseis in agaro MEA, sine pigmento flavido in agaro OA.

Etymology. Named after Croatia, where this species was collected.

Aerial structures *in vitro* on MEA — *Mycelium* consisting of branched, septate hyphae that occur singly or in bundles of up to 4; tuberculate with warts up to 0.5 µm wide, subhyaline to pale brown, smooth to verruculose, 2–3 µm wide. *Conidiophores* mostly of medium length, usually unbranched, arising from aerial or submerged hyphae, erect to flexuous, up to 5-septate, often ending in a single terminal phialide, subhyaline to pale brown, paler towards the tip, smooth to verruculose, (10–)16–23(–48) (av. 19) µm long and (2–)2.5(–3) (av. 2.5) µm wide. *Phialides* terminal or lateral, mostly monophialidic, smooth to verruculose, hyaline to subhyaline, collarettes, 1.5–2 µm long, 1–1.5 µm wide; type I phialides predominant, mostly cylindrical to subcylindrical, or elongated ampulliform, attenuated at the base, (6–)11–13(–15) × (1.5–)2.5(–3) (av. 12 × 2.5) µm; type II phialides mostly subulate, some navicular, tapering towards the apex, (10–)15–19(–20) × (2–)2.5(–3) (av. 17 × 2.5) µm. Type III phialides subcylindrical, subulate, (20–)23–27(–28) × 2(–2.5) (av. 24 × 2) µm. *Conidia* hyaline, mostly subcylindrical or allantoid, some cylindrical or ellipsoidal, (2–)3–4.5(–7) × (1–)1.5(–2) (av. 4 ×1.5) µm, L/W = 2.6. On surface or submerged in the agar — *Phialides* hyaline, mostly cylindrical to subcylindrical, (2–)5–8(–12) × (1.5–)2(–3) (av. 7 × 2) µm. *Conidia* hyaline, subcylindrical, or allantoid, (4–)6–7(–9) × (1–)2 (av. 6.5 × 2) µm, L/W = 3.25.

Cultural characteristics — Colonies reaching a radius of 10 mm after 8 d at 25 °C. Minimum temperature for growth 12 °C, optimum 27 °C, maximum 33 °C. Colonies on MEA flat, cottony, with entire margin; after 16 d, pale olivaceous-grey to whitish above, orange to yellowish white in reverse. Colonies on PDA flat, short woolly to felty, with entire edge, after 16 d, colonies smoke-grey to pale grey-olivaceous above, white towards the margin above, brownish grey in reverse. Colonies on OA flat, felty, with entire margin, after 16 d, grey-olivaceous to whitish towards the edge above, pale olivaceous-grey, yellowish white towards the edge in reverse.

Substrate — *Vitis vinifera*.

Known distribution — Croatia.

Specimen examined. CROATIA, Moslavina, Voloder, isolated from *Vitis vinifera* (cv. Škrlet) cutting showing necrosis and black streakings, June 2007, *B. Cvetkovic*, holotype CBS H-20120, culture ex-type CBS 123037.

Notes — DNA sequence analyses revealed *Pm. croatiense* to be closely related to *Pm. mortoniae*. It can, however, be distinguished based on its olivaceous-grey colonies on MEA, as well as by the absence of yellow pigment production on OA.

Phaeoacremonium hungaricum Essakhi, Mugnai, Surico & Crous, *sp. nov.* — MycoBank MB506948; Fig. 7

Phaeoacremonio mortoniae phylogenetice simile, sed structuris coremioidibus fertilibus in agaro MEA et phialidibus plerumque typi II.

Etymology. Named after Hungary, where this species was collected.

Aerial structures *in vitro* on MEA — *Mycelium* composed of branched, septate hyphae that occur singly or in bundles of up to 14, subhyaline to medium brown, smooth, occasionally verruculose, 1–3.5 µm wide. *Conidiophores* mostly short, usually unbranched, arising from aerial or submerged hyphae, erect, simple, up to 2-septate, often ending in a single terminal phialide, subhyaline to pale brown, paler towards the tip, smooth to verruculose, (20–)26–30(–36) (av. 27) µm long and (2–)2.5(–3) (av. 2.5) µm wide. *Phialides* terminal or lateral, mostly monophialidic, smooth to verruculose, mostly subhyaline, sometimes pale brown, collarettes, 1 µm long, 1.5 µm wide; type I phialides most predominant, elongated ampulliform, attenuated at the base, or constricted, some cylindrical, (3–)7–12(–15) × (1–)2.5(–3) (av. 7 × 2.5) µm; type II phialides navicular or subulate, subcylindrical, tapering towards the apex, (9–)12–15(–20) × (1.5–)2.5(–3) (av. 13 × 2.5) µm. *Conidia* hyaline, mostly, subcylindrical or cylindrical, often allantoid, (3–)4–5(–6) ×(1.5–)2 (av. 4.5 × 2) µm, L/W = 2.25.

On surface or submerged in the agar — *Phialides* hyaline, cylindrical to subcylindrical, occasionally navicular, (2–)7–11(–15) × (1.5–)2(–3) (av. 9 × 2) µm. *Conidia* hyaline, cylindrical, subcylindrical or allantoid (3–)5–7.5(–12) × (1–)2.5(–3) (av. 6.5 × 2) µm, L/W = 3.75.

Cultural characteristics — Colonies reaching a radius of 10 mm after 8 d at 25 °C. Minimum temperature for growth 10 °C, optimum 27 °C, maximum 33 °C. Colonies on MEA flat, woolly, with entire margin; after 16 d, whitish yellow to whitish above, dark brown to

pale orange in reverse. Colonies on PDA flat, felty, with entire edge, after 16 d, colonies beige to whitish grey-olivaceous, white towards the margin above. Colonies on OA flat, felty, with entire margin, after 16 d, greenish olivaceous to white towards the edge; greenish olivaceous above, olivaceous-grey in reverse.

Substrate — *Vitis vinifera*.

Known distribution — Hungary.

Specimen examined. HUNGARY, Mád, Tokaj, isolated from *Vitis vinifera* (cv. Hárslevelű) showing external esca symptoms, wood necrosis and black streaking, February 2007, *B.T. Dula*, holotype CBS H-20119, culture ex-type CBS 123036.

Notes — Phylogenetically, this species clusters as a sister clade to *Pm. mortoniae*. However, it can be distinguished by its conidiophores which are mostly reduced to phialides. The aerial mycelium has an abundant number of phialides, which are elongated ampulliform in shape.

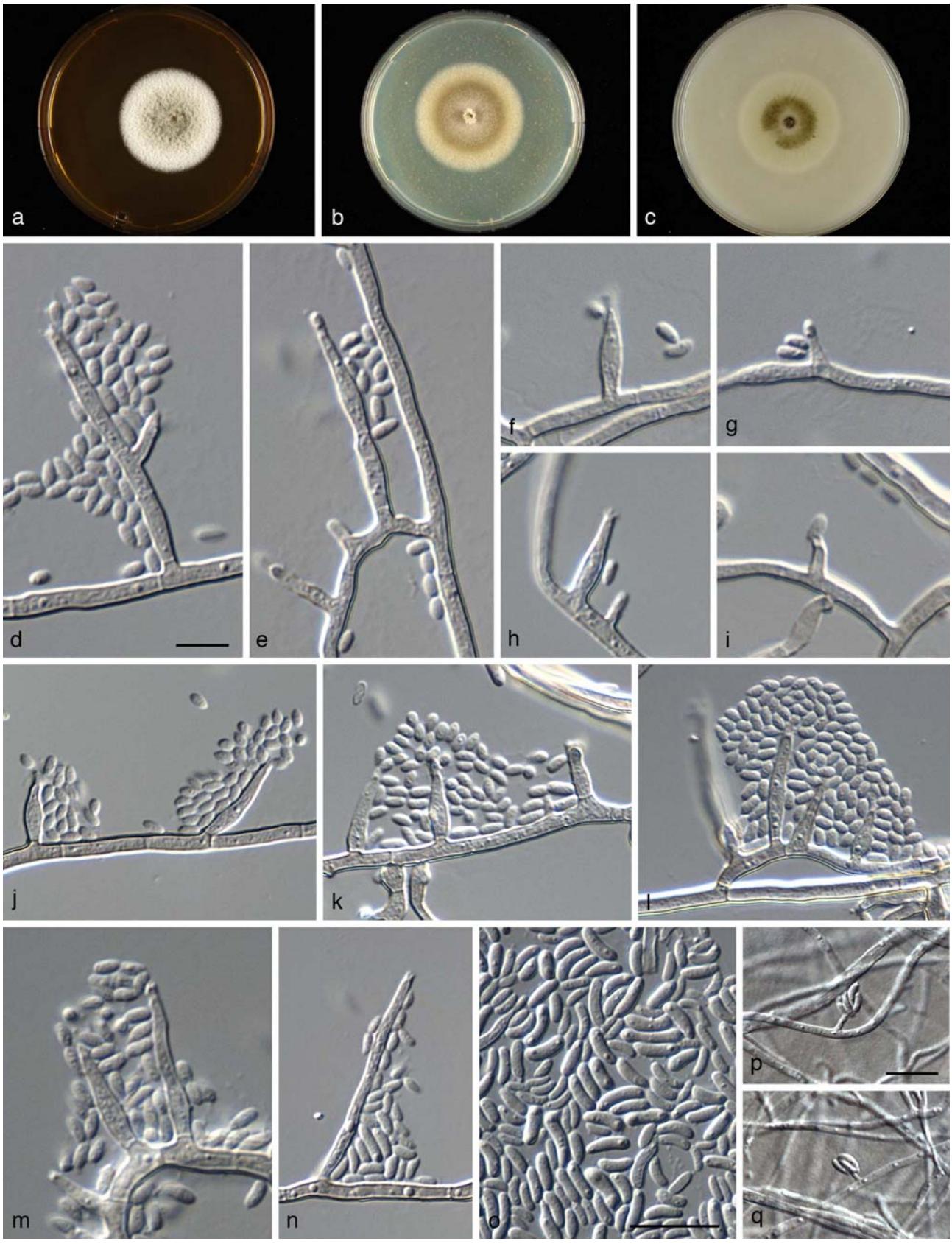


Fig. 6. *Phaeoacremonium croatiense*. a–c. Sixteen day old colonies on 2 % MEA (a), PDA (b) and OA (c). d–o. aerial structures on 2 % MEA; d–e. conidiophores; f–i. type I phialide; j–l. type II phialide; m–n. type III phialide; o. conidia; p–q. structures on the surface of and in 2 % MEA: adelophialides with conidia; all from CBS H- 20120 (holotype); d–q: DIC. — Scale bars: d–p = 10 μm; scale bar for d applies to e–n and q.

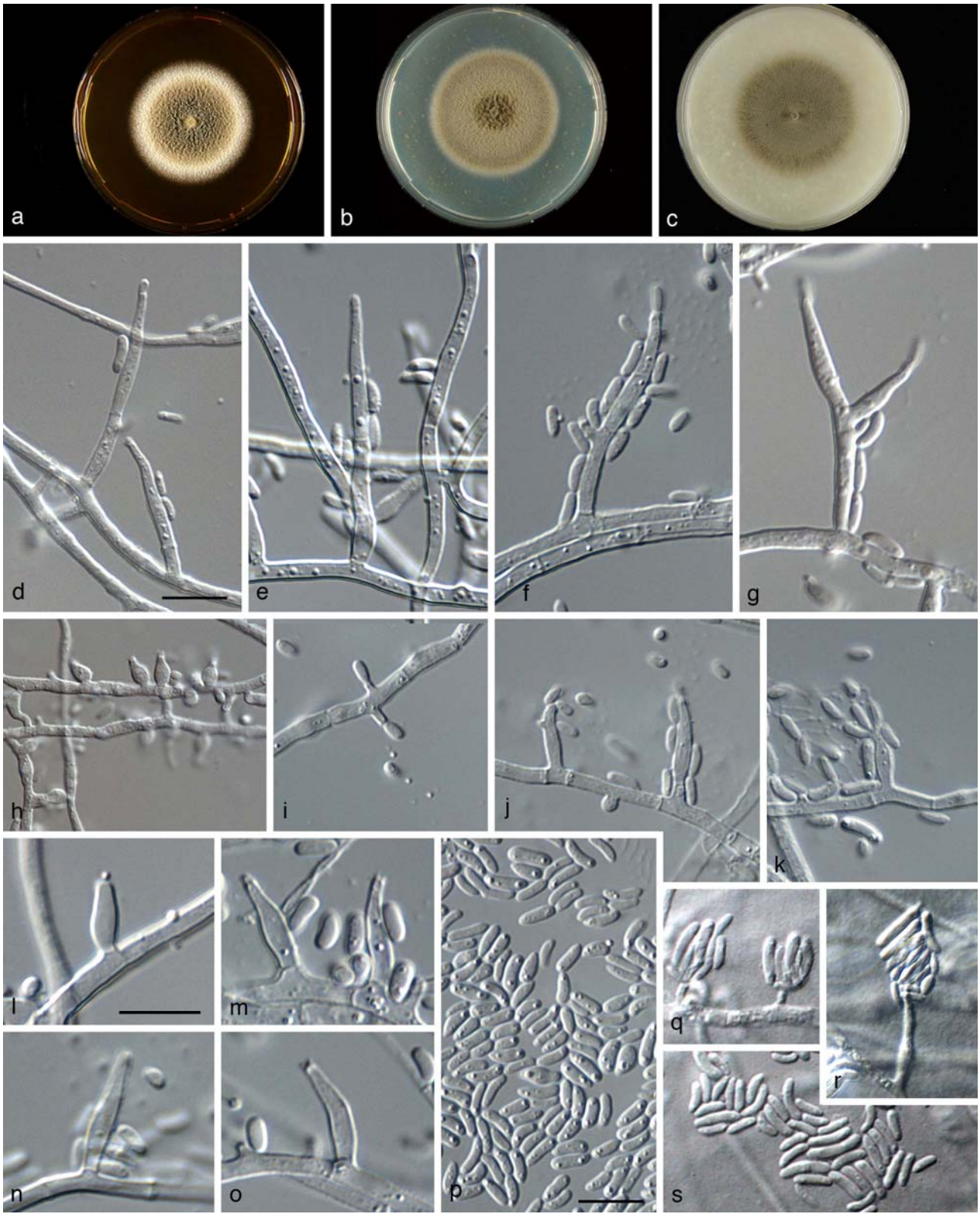


Fig. 7. *Phaeoacremonium hungaricum*. a–c. Sixteen day old colonies on 2 % MEA (a), PDA (b) and OA (c). d–p. aerial structures on 2 % MEA. d–g. conidiophores; h–k. type I phialide; i–o. type II phialide; p. conidia; q–s. structures on the surface of and in 2 % MEA. q–r. adelophialides with conidia; s. conidia; all from CBS H-20119 (holotype); d–s: DIC. — Scale bars: d-s = 10 μm; scale bar for d applies to e–k and q–s; bar for l applies to m–o.

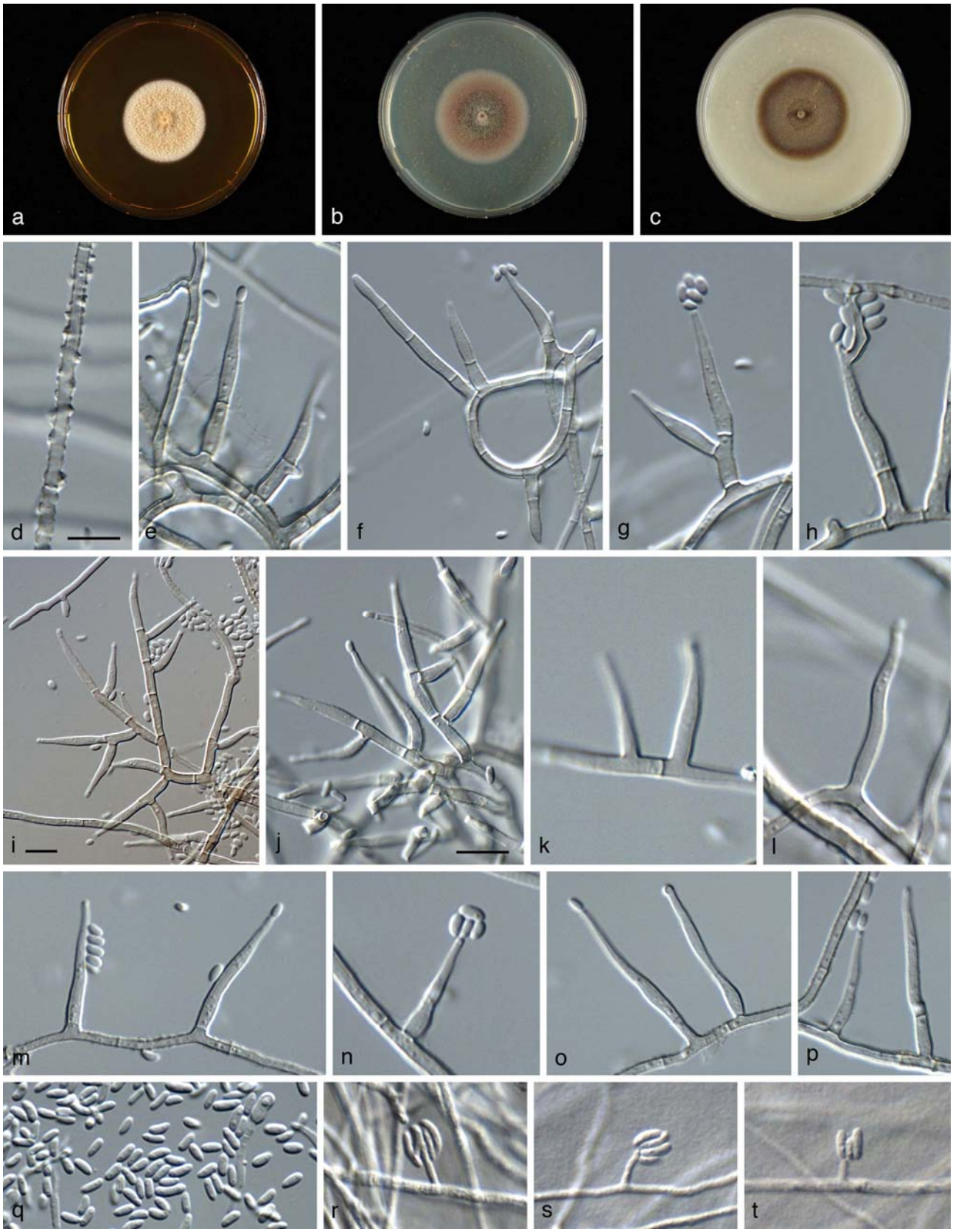


Fig. 8. *Phaeoacremonium rubrigenum*. a–c. Sixteen day old colonies on 2 % MEA (a), PDA (b) and OA (c). d–q. Aerial structures on 2 % MEA; d. mycelium showing prominent exudate droplets observed as warts; e–h. single conidiophores; i–j. branched conidiophores; k–i. type I phialide; m–n. type II phialide; o–p. type III phialide; q. conidia; r–t. Structures on the surface of and in 2 % MEA: adelophialides with conidia; all from H-20121 (holotype); d–t: DIC. — Scale bars: d = 10 μm; scale bar for d applies to i–k and k–t.

Phaeoacremonium rubrigenum W. Gams, Crous & M.J. Wingf., Mycologia 88: 795. 1996. Fig. 8.

Aerial structures *in vitro* on MEA — *Mycelium* consisting of branched, septate hyphae that occur singly or in bundles of up to 8; tuberculate with warts up to 1 µm wide, subhyaline to pale brown, smooth to verrucose, 1.5–2.5 µm wide. *Conidiophores* mostly of medium length, arising from aerial or submerged hyphae, branched, occasionally unbranched, each branched conidiophores often ending in a single terminal phialide, occasionally also with lateral phialides; erect, up to 6-septate, subhyaline to pale brown, paler towards the tip, smooth to verrucose, (18–)27–32(–48) (av. 29) µm long and (1.5–)2.5(–3) (av. 2.5) µm wide. *Phialides* terminal or lateral, mostly monophialidic, smooth to verruculose, pale brown to subhyaline; collarettes, 2–3 µm long, 1–1.5 µm wide; type II phialides most common, type I phialides subcylindrical, or elongated ampulliform, attenuated at the base, or constricted, (5–)8–11(–15) × (1–)2(–2.5) (av. 9 × 2) µm; type II phialides mostly subulate, some navicular, tapering towards the apex, (10–)15–18(–20) × (2–)2.5(–3) (av. 17 × 2.5) µm. Type III Phialides rarely present, subulate, tapering towards the apex, (25–)26(–28) × (2–)2.5(–3) (av. 26 × 2.5) µm. *Conidia* hyaline, mostly ellipsoidal, some cylindrical, (3–)3.5–4(–6.5) × (1–)1.5(–2) (av. 4 × 1.5) µm, L/W = 2.6. On surface or submerged in the agar — *Phialides* hyaline, mostly navicular, tapering towards the apex, some cylindrical, (2–)5–8(–12) × (1.5–)2(–3) (av. 9 × 2) µm. *Conidia* hyaline, allantoid or subcylindrical, (3–)4.5–5.5(–8) × 1.5(–2) (av. 5 × 1.5) µm, L/W = 3.3.

Cultural characteristics — Colonies reaching a radius of 9 mm after 8 d at 25 °C. Minimum temperature for growth 12 °C, optimum 27 °C, maximum 33 °C. Colonies on MEA flat, cottony to woolly, with entire margin; after 16 d, beige, whitish towards the margin above, brown to orange in reverse. Colonies on PDA flat, appressed, woolly to powdery, with entire edge, after 16 d, colonies brown to vinaceous-white towards the margin above, brown to violet in reverse. Colonies on OA flat, felty, with entire margin, after 16 d, brown to greyish sepia above, pale purplish grey in reverse.

Substrate — Human, *Vitis vinifera*.

Known distribution — USA, Croatia.

Specimen examined. CROATIA, Šibenik, isolated from *Vitis* vinifera (cv. Lasina), showing necrosis and black streakings, June 2007, *B. Cvetkovic*, CBS H-20121, culture ex-type CBS 123038.

Notes — Phylogenetically this isolate clusters with other strains of *Pm. rubrigenum*. It is morphologically different, however, in its predominance of branched conidiophores, and in its beige colonies on MEA. In contrast, Mostert *et al.*, (2006b) described colonies of *Pm. rubrigenum* as having usually unbranched conidiophores, and being pink to purplish on MEA.

Phaeoacremonium sicilianum Essakhi, Mugnai, Surico & Crous, *sp. nov.* — MycoBank MB506949; Fig. 9

Phaeoacremonio parasitico et *P. inflatipedi* simile. Differt a *P. parasitico* hyphis magnis sine verrucis, et a *P. inflatipedi* phialidibus plerumque typorum I et II.

Etymology. Named after the island of Sicily, from where the species was collected.

Aerial structures *in vitro* on MEA — *Mycelium* consisting of branched, septate hyphae that occur singly or in bundles of up to 5; tuberculate with warts up to 1 µm diam, smooth to verruculose, medium to pale brown, 1.5–3 µm wide. *Conidiophores* mostly short and branched, occasionally unbranched, arising from aerial or submerged hyphae, erect, up to 4-septate, often bearing a terminal phialide and an additional lateral one, pale brown to subhyaline, paler towards the tip, smooth to verruculose, (15–)22–47(–68) (av. 35) µm long and (1.5–)2.5(–3) (av. 2.5) µm wide. *Phialides* terminal or lateral, mostly monophialidic, smooth to verruculose, mostly subhyaline, occasionally pale brown, collarettes, 1.5–3 µm long, 1–2 µm wide; type I phialides, cylindrical to subcylindrical, tapering towards the apex and often widened at the base, (4–)9–12(–17) × (1–)2(–3) (av. 9 × 2) µm; type II phialides subulate, subcylindrical, tapering towards the apex, (9–)15–18(–23) × (1.5–)2.5(–3) (av. 18 × 2.5) µm. Type III phialides subcylindrical, navicular, (20–)23–27(–28) × 2(–2.5) (av. 25 × 2) µm. *Conidia* hyaline, mostly allantoid, subcylindrical (3–)4–6(–10) × 1.5–2(–2.5) (av. 5 × 2) µm, L/W = 2.5. On surface or submerged in the agar — *Phialides* hyaline, cylindrical to subcylindrical, (3–)4–13(–17–) × 2.5 (av. 6 × 2) µm. *Conidia* hyaline, mainly allantoid, some subcylindrical (–3.5)6–8(–11) x 1.5 (–2) (av. 7 × 2) µm, L/W = 3.5.

Cultural characteristics — Colonies attained a radius of 12 mm after 8 d at 25 °C. Minimum temperature for growth 15 °C, optimum 27 °C, maximum 33 °C. Colonies on MEA flat, cottony, with entire margin; after 16 d, pale greyish sepia to beige towards the edge above, brown to pale orange in reverse. Colonies on PDA flat, cottony to woolly, with entire edge, after 16 d, colonies pale brown to smoke-grey above, olivaceous-grey in reverse. Colonies on OA flat, felty to powdery, with entire margin; after 16 d, smoke-grey to pale olivaceous above, olivaceous-grey to pale mouse-grey in reverse.

Substrate — *Vitis vinifera*.

Known distribution — Italy.

Specimen examined. ITALY, Sicily, Messina, San Filippo del Mele, isolated from the necrotic margins and brown to black streakings of branches and trunk of very old *Vitis vinifera* vines showing wood esca symptoms, May 2007, *L. Mugnai*, holotype CBS H-20118, culture ex-type CBS 123034.

Notes — DNA sequence analysis revealed this species to be basal to other species of *Phaeoacremonium.* Nevertheless, in terms of morphological characters, *Pm. parasiticum* and *Pm. inflatipes* are similar to *Pm. sicilianum* in the predominance of branched conidiophores. *Pm. parasiticum* is distinct from *Pm. sicilianum* by virtue of its dark brown hyphae, and large hyphal warts of up to 3 μm diam, while *Pm. sicilianum* can be distinguished from *Pm. inflatipes* by the predominance of type I and II phialides, in comparison with the predominance of phialide type III in *Pm. inflatipes*. Differences in colony colour also distinguish *Pm. sicilianum* from *Pm. inflatipes.*

Phaeoacremonium tuscanum Essakhi, Mugnai, Surico & Crous, *sp. nov.* — MycoBank MB506950; Fig. 10

Phaeoacremonio iraniano phylogenetice simile, sed structuris coremioidibus fertilibus in agaro MEA et phialidibus plerumque typi II.

Etymology. Named after Tuscany, Italy, where this fungus was collected.

Aerial structures *in vitro* on MEA — *Mycelium* composed of branched, septate hyphae that occur singly or in bundles of up to 22; tuberculate with warts up to 1 μm diam, smooth to verruculose, medium brown to subhyaline and 1.5–2.5 μm wide. *Conidiophores* mostly short and usually unbranched, occasionally branched, arising from aerial or submerged hyphae, straight, simple, up to 3-septate, usually bearing one terminal phialide, pale brown to subhyaline, paler towards the tip, smooth to verruculose, (13–)25–30(–40) (av. 28) μm long and (1.5–)2(–2.5) (av. 2) μm wide. *Phialides* terminal or lateral, mostly monophialidic, smooth to verruculose, pale brown to hyaline, collarettes, 1.5–3 μm long, 1–1.5 μm wide; type II phialides predominant, type I phialides subcylindrical, occasionally widened at the base, (4–)9–11(–17) × (1.5–)2(–2.5) (av. 10.5 × 2) μm; type II phialides subulate, navicular, or subcylindrical, attenuated at the base and tapering towards the apex, (8–)13–15(–20) × 1.5–2(–3) (av. 14 × 2) μm. Type III phialides subcylindrical, subulate (20–)21–23(–25) × 2–(–2.5) (av. 22 × 2) μm. *Conidia* hyaline, mosltly allantoid, subcylindrical or cylindrical, ellipsoidal (2.5–)4(–5.5) × (1.5–)2 (av. 4 × 2) μm, L/W = 2. On surface or submerged in the agar —

Phialides hyaline, subcylindrical, 4–9(–14) × 1(–2) (av. 6 × 1) µm. *Conidia* hyaline, subcylindrical or allantoid, 2.5–5(–8.5) × (1–)2(–3) (av. 5 × 2) µm, L/W = 2.5.

Cultural characteristics — Colonies attaining a radius of 8 mm after 8 d at 25 °C. Minimum temperature for growth 12 °C, optimum 33 °C, maximum 37 °C. Colonies on MEA flat, cottony, with entire margin; after 16 d, colonies pale brown to beige towards the edge above, pale orange in reverse. Colonies on PDA flat, short, woolly to felty, with entire edge, after 16 d colonies brown to beige, pale greyish orange and whitish towards the margin above, pale greyish sepia in reverse, becoming whitish towards the edge. Colonies on OA flat, felty, with entire margin; after 16 d pale orange to yellow towards the margin above, same in reverse, producing yellow pigmentation in the agar.

Substrate — *Vitis vinifera*.

Known distribution — Italy.

Specimen examined. ITALY, Tuscany, San Gimignano, isolated from the margin of necrosis in the trunk of *Vitis vinifera* sampled from an about 100 yrs-old vineyard that showed wood esca symptoms, March. 2007, *L. Mugnai*, holotype CBS H- 20118, culture ex-type CBS 123033.

Notes — According to the phylogenetic analysis, *Pm. tuscanum* can be considered as a sister clade to *Pm. iranianum.* However, it can be distinguished from it by the production of coremium-like structures on MEA. These are fertile, erect hyphal bundles up to 2 mm tall and 1 mm wide, dark to pale brown, composed of conidiophores bearing conidia at the apex. Type II phialides are predominant in *Pm. tuscanum*. By comparison, *Pm. iranianum* lacks these structures, and has a predominance of type III phialides.

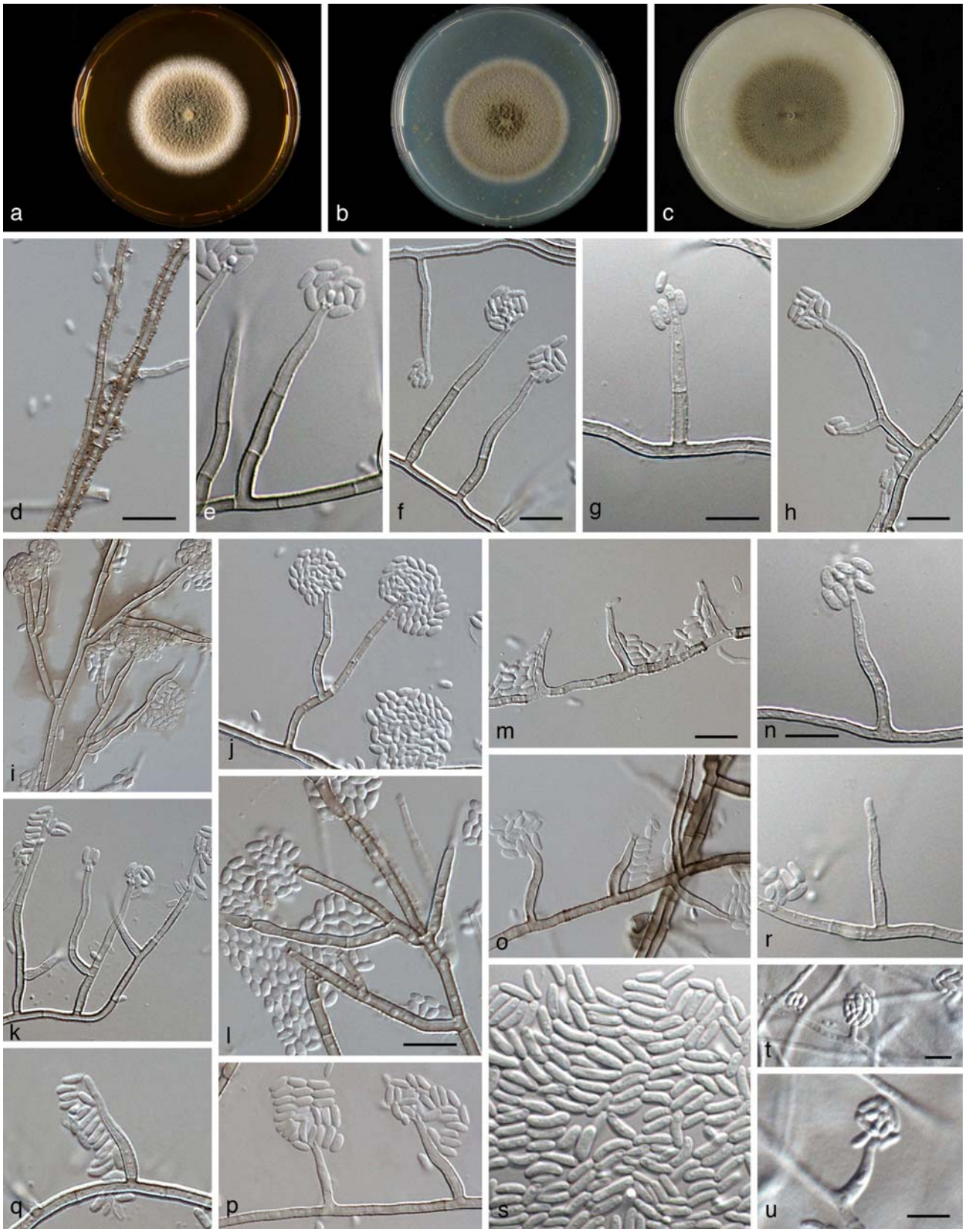


Fig. 9. *Phaeoacremonium sicilianum*. a–c. Sixteen day old colonies on 2 % MEA (a), PDA (b) and OA (c). d–s. aerial structures on 2 % MEA. d. mycelium showing prominent exudate droplets observed as warts; e–h. single conidiophores; i–l. branched conidiophores; m–p. type I phialide; q. gtype II phialide; r. type III phialide; s. conidia; t–u. structures on the surface of and in 2 % MEA: adelophialides with conidia; all from CBS H-20118 (holotype); d–u: DIC. — Scale bars: d–u = 10 μm; scale bar for d applies to i–k and e; bar for n applies to o–r.

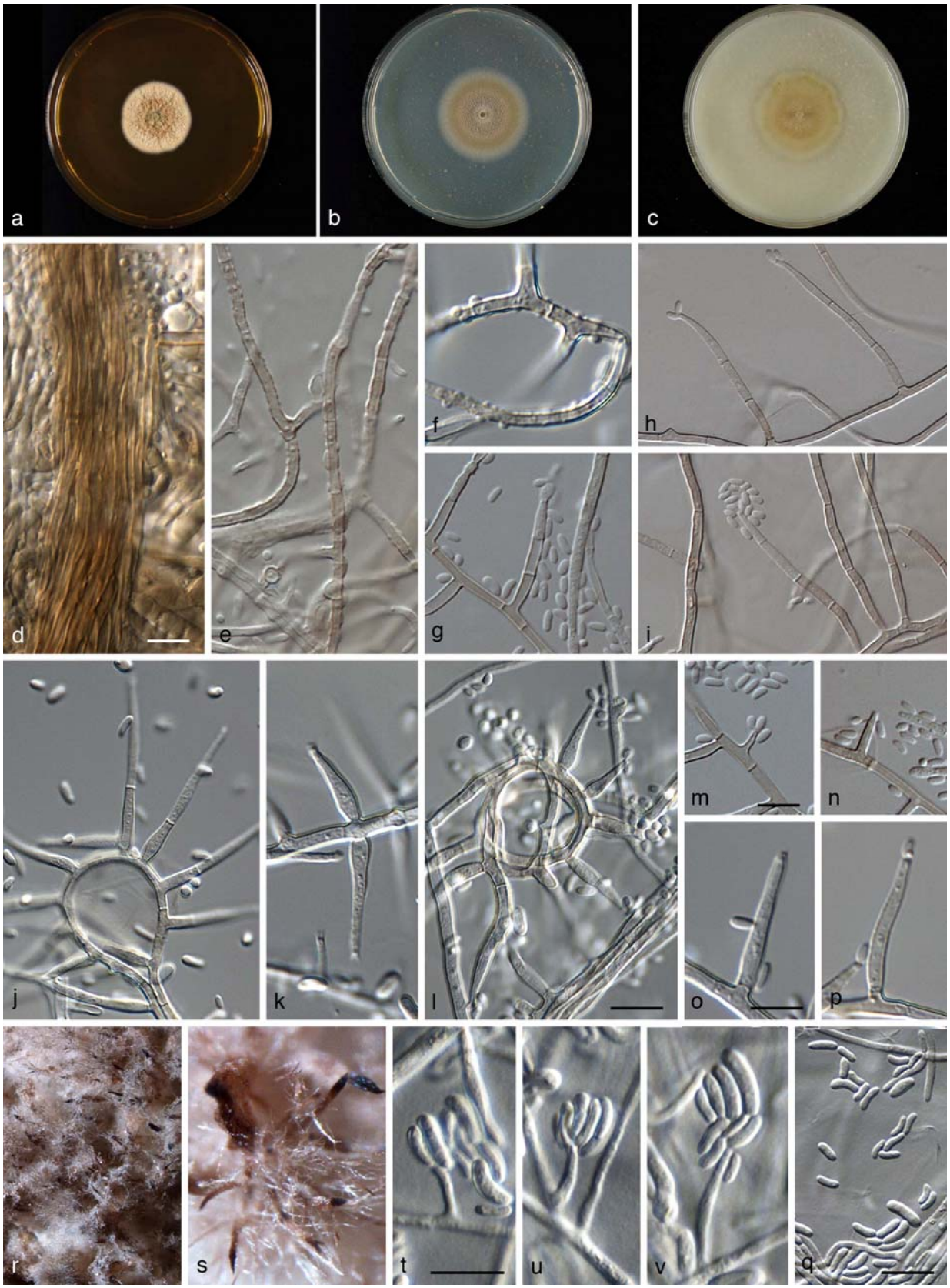


Fig. 10. *Phaeoacremonium tuscanum*. a–c. Sixteen day old colonies on 2 % MEA (a), PDA (b) and OA (c). d–q. Aerial structures on 2 % MEA; d. mycelium occurring in bundles of up to nine; e–f. mycelium showing prominent exudate droplets observed as warts; g–j. single conidiophores; k–l. type II phialide; m–n. type I phialide; o–p. type III phialide; q. conidia; r–v. structures on the surface of and in 2 % MEA; r–s. coremium-like structures; t–v adelophialides with conidia; all from CBS H-20118 (holotype); r–s: DM; d–q: DIC. — Scale bars: d–v = 10 µm; scale bar for d applies to e–k and p–s; bar for m applies to n; bar for t applies to u–v.

DISCUSSION

The correct identification of fungi involved in diseases within the esca complex is a crucial precondition for the conduct of meaningful studies on the epidemiology of these destructive diseases of grapevine. Epidemiological studies will be especially important in the design of control strategies, since no fully effective chemical or biological control measures exist for this disease complex.

Several species of *Phaeoacremonium* have already been attributed to the grapevine diseases within the esca complex worldwide. However, the identity, distribution, and frequency of the *Phaeoacremonium* species involved in many grapevine-growing areas, especially, the area where *Vitis vinifera* evolved, have not yet been studied. The present study has included a wide collection of *Phaeoacremonium* isolates from Italy, mainly from isolated locations like Sardinia and Sicily where farmers grow local grape varieties. It also included a diverse set of isolates from other countries.

Integration of morphology, cultural characters, and DNA sequence data revealed the presence of 13 *Phaeoacremonium* species in the areas sampled. Phylogenetic analyses of ACT and TUB sequences revealed that four of these species were novel. It is of interest to notice that the four new species here described were isolated from very old vines growing in Italy, Hungary and Croatia. Old vines were included in this study as a source of esca tracheomycotic fungi with the specific objective of gathering a population of both genera, *Phaeomoniella* and *Phaeoacremonium*, showing an as wide as possible variability within the population of the two fungi. Here are reported the results so far obtained in *Phaeoacremonium*.

Morphological traits such as presence or absence of hyphal warts, size of warts, conidiophore structure, and cultural characteristics have been shown to be useful in species identification. For example, *Pm. parasiticum* can easily be distinguished from other species based on the occurrence of hyphal warts that are up to 3 μm diam (Mostert *et al.*, 2006b). Some other species such as *Pm. inflatipes* and *Pm. sphinctrophorum* have frequently branched conidiophores, which can be used to distinguish them from species with short and infrequently branched or unbranched conidiophores. Nevertheless, species delimitation in this genus solely based on morphological and cultural characteristics has proven to be difficult. This difficulty is mainly inherent to the overlapping nature of morphological characters among the different species. Hence, DNA sequence data analysis remains of the utmost

importance for complete and reliable species delineation. Mostert *et al.*, (2006b) developed a multiple-entry polyphasic identification key for *Phaeoacremonium* spp. This tool combines DNA sequence data, with different morphological and cultural characters to identify up to 22 *Phaeoacremonium* spp. The four new species described in this study can be distinguished from the existing *Phaeoacremonium* spp. based on a combined cultural, morphological and DNA sequence data set. *Phaeoacremonium tuscanum* clusters as sister clade to *Pm. iranianum.* However, it can be distinguished from *Pm. iranianum* by the production of coremium-like structures on MEA, consisting of erect, fertile hyphal bundles up to 2 mm high and 1 mm wide.

Two of the other new species described in this study, *Pm. hungaricum* and *Pm. croatiense*, cluster as sister clade to *Pm. mortoniae*, and can only be distinguished by minute morphological differences. *Phaeoacremonium hungaricum* is distinct from *Pm. mortoniae* by the rare presence of conidiophores which are mostly reduced to conidiogenous cell or phialides. In fact, the aerial mycelium is composed of mostly elongated, ampulliform phialides, whereas *Pm. croatiense* can be differentiated from *Pm. mortoniae* based on cultural characters such as its olivaceous-grey colonies on MEA, and the absence of yellow pigment production on OA. It is not surprising that these three species have a similar morphology, as they have a close phylogenetic affinity, suggesting that they have evolved from a common ancestor.

The newly described *Pm. sicilianum* is markedly distinct from the other known *Phaeoacremonium* spp. DNA sequence analysis showed that this clade is basal to the others, even though the species overlaps morphologically with *Pm. parasiticum* and *Pm. inflatipes* in its predominance of branched conidiophores. *Pm. parasiticum* differs from *Pm. sicilianum* in its dark brown hyphae and large hyphal warts up to 3 µm diam. *Pm. sicilianum* differs from *Pm. inflatipes* in its predominance of type I and II phialides and in its colony colour.

Growth temperature studies carried out for new species described in this study showed that *Pm. tuscanum* has a maximum growth temperature at 37–40 °C, reaching a radius of 7 mm after 8 d. This finding is quite interesting in relation to the ecology of *Phaeoacremonium* spp., as several thermotolerant *Phaeoacremonium* spp. are associated with phaeohyphomycosis in humans (Mostert *et al.*, 2005). *Phaeoacremonium parasiticum*, *Pm. rubrigenum* and *Pm. inflatipes* (later identified as *Pm. alvesii*), were initially reported from human phaeohyphomycosis (Padhye *et al.*, 1998; Guarro *et al.*, 2003). *Pm. krajdenii*, *Pm.*

parasiticum and *Pm. venezuelense* cause phaeohyphomycosis and have also been isolated from grapevines showing esca symptoms (Mostert *et al.,* 2005). Four *Phaeoacremonium* species have heretofore been isolated only from human infections: *Pm. tardicrescens, Pm. amstelodamense, Pm. griseorubrum,* and *Pm. rubrigenum* (Mostert *et al.,* 2005). The present study revealed that *Pm. alvesii, Pm. griseorubrum* and *Pm. rubrigenum* are also associated with grapevine. This finding further supports the hypothesis that human pathogenic *Phaeoacremonium* spp. may have originated from woody host plants. The same hypothesis has been proposed for some other groups of human opportunistic pathogens such as the black yeasts (de Hoog *et al.,* 2007). To confirm this hypothesis, studies are needed inoculating strains from woody hosts into an animal model, and strains from human cases into woody plants.

Although several species of *Phaeoacremonium* are associated with necrosis and discolorations in grapevine wood, *Pm. aleophilum* is the most common species associated with foliar symptoms. It appears also to be the most widely distributed species (Crous *et al.,* 1996; Larignon & Dubos, 1997; Mugnai *et al.,* 1999; Tegli *et al.,* 2000; Mostert *et al.,* 2006a) in grapevine. Our data were consistent with this pattern. *Phaeoacremonium aleophilum* was the most frequently isolated species with an incidence up to 80 % in all the samples examined. Inoculation studies have proven this species to be pathogenic on grapevines, showing that *Pm. aleophilum* can cause brown wood streaking (Mugnai *et al.,* 1997; Adalat *et al.,* 2000; Sparapano *et al.,* 2000b; Feliciano *et al.,* 2004; Halleen *et al.,* 2005) and reduced shoot growth (Gubler *et al.* 2001), as well as esca symptoms on leaves and berries (Sparapano *et al.,* 2000a; Feliciano *et al.,* 2004).

A recent study has shown the occurrence of several novel *Phaeoacremonium* species. on other woody hosts such as species in the genus *Prunus* (Damm *et al.,* 2008). However, on grapevine the pathological relevance of the other *Phaeoacremonium* species. as well as of the novel species described in this study remains to be determined.

The prevalence of the other 12 *Phaeoacremonium* species differs among grapevine growing areas. *Pm. viticola, Pm. scolyti* and *Pm. griseorubrum* represent new records for Italy, *Pm. mortoniae* and *Pm. rubrigenum* for Croatia, *Pm. alvesii* for Turkey and *Pm. mortoniae* for Hungary. Other species that have also been isolated in relatively high frequencies from the different grapevine growing areas studied here include *Pm. iranianum* and *Pm. mortoniae*.

Where known, *Phaeoacremonium* species have teleomorphs in the genus *Togninia* (Diaporthales, Togniniaceae). Several researchers have induced the teleomorph of *Phaeoacremonium* species by crossing complementary strains in vitro (Mostert *et al.,* 2005; 2006b; Damm *et al.,* 2008). Our attempts to induce the teleomorph for the species described in this paper were unsuccessful.

Given the occurrence of various *Phaeoacremonium* species on grapevine and the involvement of some of those species in human infections, accurate identification is critical. Since, as mentioned, morphological identification is not reliable, robust molecular-based detection tools are of great help in species detection. As mentioned, an existing multiplex PCR assay based on use of species-specific TUB and ACT primers can identify 22 species of *Phaeoacremonium* (Mostert *et al.,* 2006b). It is necessary to test these primer sets on DNA from the new species described here to determine if target specificity remains intact. The ACT and TUB gene barcodes generated in the present study can be used to develop species-specific molecular detection tools that will facilitate ecological and epidemiological studies.

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Chapter 5

Pathogenicity testing of novel *Phaeoacremonium* species isolated from esca diseased grapevines

ABSTRACT

Species previously described mainly as human pathogenic species, namely *Phaeoacremonium* (*Pm) alvesii*, *Pm. griseorubrum* and *Pm. rubrigenum* were newly reported on grapevine from Turkey, Italy and Croatia, respectively. In addition, four new species of *Phaeoacremonium,* namely *Pm. croatiense*, *Pm. hungaricum*, *Pm. sicilianum* and *Pm. tuscanum* were newly described from vascular tissues of grapevines. However, their status as pathogens and their capability as colonisers of pruning wounds are still not illustrated. Hence, pathogenicity studies were conducted, to determine their potential as vascular pathogens and to test their effective capability to colonise pruning wounds and to cause vascular discoloration similar to that seen in esca diseased grapevines, with the final aim to understand their effective involvement in esca diseases complex. Pathogenicity tests, which are still underway, were conducted with grapevine nursery plants of 1yr.old (cv. R24 *Sangiovese*, rootstock 161/49) in a greenhouse using wound inoculations. This trial includes also the inoculation of *Phaeomoniella chlamydoposra* and *Pm. aleophilum* to verify the efficacy of this pathogenicity test.

INTRODUCTION

Species of *Phaeoacremonium* (*Pm.*) W. Gams, Crous & M.J. Wingf. are known vascular pathogens, causing brown wood streaking of rootstock, Petri disease and young esca (diseases included in the esca diseases complex). Several species of *Phaeoacremonium* have been isolated from esca diseased vines, including *Pm. aleophilum* W. Gams, Crous, M.J. Wingf. & L. Mugnai, *Pm. parasiticum* W. Gams, Crous & M.J. Wingf., W. Gams, Crous & M.J. Wingf., *Pm. inflatipes* W. Gams, Crous & M.J. Wingf., *Pm. angustius* W. Gams, *Pm. viticola* Crous & M.J. Wingf., *Pm. mortoniae* Crous & W. Gams, *Pm. australiense* L. Mostert, Summerb. & Crous, *Pm. krajdenii* L. Mostert, Summerb. & Crous, *Pm. scolyti* L. Mostert, Summerb. & Crous, *Pm. subulatum* L. Mostert, Summerb. & Crous and *Pm. venezuelense* L. Mostert, Summerb. & Crous (Crous *et al*. 1996, Dupont *et al*. 2000, Groenewald *et al*. 2001 and Mostert *et al.* 2005). More recently, four new species were described from esca diseased grapevines and three species were newly reported from this host (Essakhi *et al.*, 2008). Up to date, a total of 20 *Phaeoacremonium* species have been isolated from grapevine (Table 1).

Table 1. List of known *Phaeoacremonium* species reported from grapevine, their other hosts, substrate range and world-wide distribution [a].

***Phaeoacremonium* species**	**Host**	**Countries**
Pm. aleophilum	*Actinidia chinensis*, ***Vitis vinifera***, *Olea europaea*, *Prunus pennsylvanica*, *Prunus* sp., *Salix* sp.	Argentina[b], Australia[b], Austria[b], Canada, Chile[b], Iran[b], Israel[b] Italy[b], France[b], South Africa[b], Spain[b], Turkey[b], U.S.A[b] and Yugoslavia[b]
Pm. alvesii	*Dodoneae viscosa*, Human, ***Vitis vinifera***	Australia, Brazil[c], Turkey[b], U.S.A[c]
Pm. angustius	***Vitis vinifera***	Portugal[b], U.S.A[b]
Pm. australiense	***Vitis vinifera***, *Prunus salicina*	Australia[b], South Africa
Pm. austroafricanum	***Vitis vinifera***	South Africa[b]
Pm. croatiense **sp. nov.**	***Vitis vinifera***	Croatia[b]
Pm. griseorubrum	Human, ***Vitis vinifera***, *Prunus salicina*	Japan[c], Italy[b], South Africa, U.S.A[c]
Pm. hungaricum **sp. nov.**	**Vitis vinifera**	Hungary[b]
Pm. inflatipes	Hypoxylon truncatum, Nectandra sp., Quercus virginiana, ***Vitis vinifera***	Chile[b], Costa Rica, U.S.A
Pm. iranianum	*Actinidia chinensis*, ***Vitis vinifera***, *Prunus armeniaca*	Iran[b], Italy[b], South Africa
Pm. krajdenii	Human, ***Vitis vinifera***	Canada[c], India[c], Japan[c], Norway[c], South Africa[b], U.S.A.[c], Zaire[c]
Pm. mortoniae	*Fraxinus excelsior*, *Fraxinus latifolia*, *Fraxinus pennsylvanica*, ***Vitis vinifera***	Croatia[b], Hungary[b], Sweden, U.S.A[b].
Pm. parasiticum	*Actinidia chinensis*, *Aquilaria agallocha*, *Cupressus* sp., Human, *Nectandra* sp., *Phoenix dactylifera*, *Prunus armeniaca*, *Quercus virginiana*, ***Vitis vinifera***	Argentina[b], Australia[b], Brazil[c], Canada[c], Chile[b], Costa Rica, Finland[c], Greece, Iran[b], Iraq, Italy[b], South Africa[b], Tunisia, U.S.A[bc]
Pm. rubrigenum	Human, ***Vitis vinifera***	Croatia[b,] U.S.A[c]
Pm. scolyti	***Vitis vinifera***, larvae of *Scolytus intricatus*, *Prunus* sp.	Czech Republic, France[b], Italy[b], South Africa[b]
Pm. sicilianum **sp. nov.**	***Vitis vinifera***	Italy[b]
Pm. subulatum	***Vitis vinifera***, *Prunus armeniaca*	South Africa[b]
Pm. venezuelense	Human***, Vitis vinifera***	Canada[c], South Africa[b], Venezuela[c]
Pm. viticola	*Sorbus intermedia*, ***Vitis vinifera***, *Prunus salicina*	Iran[b], France[b], Germany, Italy[b], South Africa[b], U.S.A[b].

[a] (Hawksworth & Gibson, 1976; Hausner *et al.*, 1992; Crous *et al.*, 1996; Dupont *et al.*, 1998; Larignon & Dubos, 1997; Ari, 2000; Chicau *et al.*, 2000; Crous and Gams, 2000; Dupont *et al.*, 2000; Pascoe and Cottral, 2000; Péros *et al.*, 2000; Reisenzein *et al.*, 2000; Armengol *et al.*, 2001; Groenewald *et al.*, 2001; Rooney *et al.*, 2001; Rumbos & Rumbo 2001, Dupont *et al.*, 2002; Auger *et al.*, 2005; Damm *et al.*, 2005; Eskalen *et al.*, 2005; Mostert *et al.*, 2005; Overton *et al.*, 2005b; Essakhi *et al.*, 2008).

[b] Countries where *Phaeoacremonium* strains were isolated from *Vitis vinifera*.

[c] Countries where *Phaeoacremonium* strains were isolated from human infections.

Several pathogenicity studies have been conducted to understand the role that *Phaeoacremonium* species play in the complex of esca diseases, especially testing the pathogenicity of *Pm. aleophilum*, the most frequently isolated species from esca diseased grapevines, that has been extensively tested on root, shoot and pruning wound with artificial inoculation studies. These studies showed that *Pm. aleophilum*, together with *Pa. chlamydospora* can cause black streaking and necrosis of xylem tissue, reduced plant growth, leaf symptoms and black lesions on grape berries.

Recently, Hallen *et al*., 2007 tested the pathogenicity of *Pm. krajdenii*, *Pm. parasiticum*, *Pm. subulatum*, *Pm. venezuelense* and *Pm. viticola* isolates obtained from vascular tissues of grapevines, and showed that all the species were able to infect, colonise and produce lesions statistically different from those caused by the water control and the non-pathogen in the field trial.

However, vine pathogenicity tests have not been done for several of the *Phaeoacremonium* species recently described from grapevine. Moreover, their relative importance in esca diseases complex is still insufficiently well understood and their status as pathogens still unknown. This study aims to investigate the pathogenicity of the newly described *Phaeoacremonium* species previously known as human pathogenic, namely *Pm. alvesii*, *Pm. griseorubrum* and *Pm. rubrigenum*, and the newly reported from grapevine, namely *Pm. croatiense*, *Pm. hungaricum*, *Pm. sicilianum* and *Pm. tuscanum* (Essakhi *et al*., 2008) (Chapter 4, this thesis), in order to determine their potential as vascular pathogens, to test their effective capability to colonise pruning wounds and to cause vascular discoloration similar to that seen in esca diseased grapevines, with the final aim of understanding their effective involvement in esca diseases complex.

MATERIALS & METHODS

Greenhouse experiment

Pathogenicity studies were conducted in a greenhouse using a total of 100 grapevine nursery plants of 1 yr. (cv. R24 *Sangiovese*, rootstock 161/49). Plants were placed in 15 cm plastic pots, filled with a sterile new soil, then left in the greenhouse (23-26°C) for few days before inoculations were made.

A total of 9 fungal species were used for pathogenicty tests, including *Phaeomoniella (Pa) chlamydospora* and *Pm. aleophilum*, as positive controls for the inoculation test (Table 2).

Table 2. Names, GenBank accession numbers and collection details of isolates used for pathogenicity tests.

Species name	Isolate number	Location	GenBank accessions	
			ACT	**β-tubulin**
Pm. aleophilum	21Pal	Tuscany, Italy	EU863502	EU863470
Pm. alvesii	125ss2 Pal	Turkey	EU883991	EU883990
***Pm. croatiense* sp. nov**	CBS 123037 (113Pal)	Croatia	EU863514	EU863482
***Pm. hungaricum* sp. nov**	CBS 123036 (90Pal)	Hungary	EU863515	EU863483
Pm. griseorubrum	42Pal	Sicily, Italy	EU863517	EU863485
Pm. rubigenum	CBS 123038 (119Pal)	Croatia	EU863516	EU863484
***Pm. sicilianum* sp. nov**	CBS 123034 (48Pal)	Sicily, Italy	EU863520	EU863488
***Pm. tuscanum* sp. nov**	CBS 123033 (1Pal)	Tuscany, Italy	EU863490	EU863458
Pa. chlamydospora	31Pch	Tuscany, Italy		

Plant surface was sterilized with 70% ethanol, then wounds were made in the rootstock between the graft union and soil surface using a battery operated drill. After that, one colonised mycelium plug (5mm diam) obtained from the outer, young growth of fungal colonies maintained for 3 weeks on MEA was inserted as inoculum into the wound and sealed with Parafilm (Fig. 1). Uncolonised MEA plugs were used as negative control inoculations.

Fig. 1. Steps of inoculation; a. wound generated using a drill; b. inoculation of the mycelium plug into the wound; c. inoculated wound covered with parafilm.

Ten plants were inoculated with each of the nine fungal isolates and the same number of plants were inoculated with uncolonized mycelium as negative controls. The pots were watered twice weekly.

Fig. 2. Closer look to the inoculated plants.

Fig. 3. Overview of the greenhouse where inoculated plants were placed.

Plants will be evaluated each month and the length of lesions – where present – will be calculated. Lesion length data will be subjected to analyses of variance with SAS version 8.1 (SAS Institute, Cary, North Carolina) and Student's t-least significant difference will be calculated at the 5% significance level to compare treatment means for the different taxa. Subsequently, re-isolations will be made from the leading edges of lesions and the resulting cultures will be identified to determine whether the inoculated fungus could be re-isolated.

RESULTS

Some of the inoculated plants including positive controls, were collected from the greenhouse after two months and examined in the laboratory. Plants were split lengthwise through the inoculation hole to reveal the xylem and pith regions for observation and measurement of the lesions.

At the time being, the information obtained was difficult to interpret due to the small lesions observed both in control as in inoculated vines.

CONCLUSION

In this part of the thesis, the trial set up to investigate the pathogenicity of the newly described *Phaeoacremonium* species reported from grapevine (this work) was illustrated. pathogenicity tests were established to determine the potential of the novel species as vascular pathogens, to test their effective capability as colonisers of pruning wounds and to cause vascular discoloration similar to that seen in esca diseased grapevines, with the final aim to understand their effective involvement in esca diseases complex.

Pathogenicity tests, which are still underway, were conducted in a greenhouse using wound inoculations.

Ssome of the inoculated plants including positive controls, were collected from the greenhouse after two months and examined in the laboratory. Plants were split lengthwise through the inoculation hole to reveal the xylem and pith regions for observation and measurement of the lesions. At the time being, the information obtained was difficult to interpret due to the small lesions observed both in control as in inoculated vines, because of the short length of time between the starting of the inoculation experiment and the evaluation. Future surveys will be carried out up to the observation of significant lesions in the positive controls, and subsequently statistical analysis and re – isolation

Chapter 6

Molecular characterization of a portion of the mating type (MAT 1-2) gene in *Phaeoacremonium aleophilum*

ABSTRACT

In the present study, we present our first attempt to describe the mating type gene of *Phaeoacremonium aleophilum* (teleomorph *Togninia minima*), in order to understand the distribution of mating types and the mating behaviour of this important pathogen associated with esca and related diseases of grapevine. Degenerate primer sets (NcHMG1 and NcHMG2) were successfully used to amplify a portion of 300 bp of the MAT1-2 gene of *Pm. aleophilum*. The nucleotide sequence (~300bp) of this idiomorph showed homology to the HMG-box of other related ascomycetes. Based on this sequence, different primers were designed to be used in chromosome walking strategy to characterize the whole dimorph. Further work is in progress to obtain the whole idiomorph by means of chromosome walking and genomic analyses. Identification of mating types using PCR is faster than traditional methods and would be very useful and beneficial for population studies, especially in geographic regions where only the anamorph of *T. minima* has been identified, in order to prevent introduction events of isolates carrying the other type of mating type and inducing sexual reproduction. The mating type specific PCR could be implemented as a control method to test for the presence of the mating types for the species *Pm. aleophilum*, which would provide new insight into the distribution of mating types of *Pm. aleophilum* that should enhance the quality of quarantine regulations in the future.

INTRODUCTION

In fungi, sexual reproduction is controlled by mating type loci. Mating in fungi is a complex process governed by mating types. The term ‘mating type’ is used to define sexually compatible individuals. Blakeslee, (1904), who coined the terms homothallism and heterothallism, was the first to notice the existence of mating types in sexually reproducing fungi. Heterothallism (self-sterility) the most common reproduction strategy among fungi, only occurs between two fungal strains with a compatible mating system. However, even in the presence of both mating types, other genetic barriers may prevent real mating (Debuchy & Turgeon, 2006). Homothallism (self-fertility) represents the situation where a single isolate is capable of completing a successful sexual cycle. Mating type loci contain a number of genes which occupy a continuous region of the chromosome, but do not span an entire chromosome (Debuchy & Turgeon, 2006). In ascomycetes, sexual development is controlled by a single mating type locus (*MAT*). This mating type locus is structurally unusual, because it contains

one of two forms of dissimilar sequences occupying the same chromosomal position. The two non-allelic versions of the mating type locus in fungal species with heterothallic mating strategy were labelled 'idiomorph' (Metzenberg & Glass, 1990). By convention, mating type idiomorphs of complementary isolates are termed *Mat1-1* and *Mat1-2* (Turgeon & Yoder, 2000). The number of genes in each idiomorph varies among different groups of fungi. However, they all contain homeodomain-encoding genes, either alpha box or high mobility group (HMG) domain transcription factors (Turgeon, 1998; Debuchy & Turgeon; 2006). In all heterothallic filamentous ascomycetes studied to date, the mating type locus contains one of two forms of dissimilar sequences occupying the same chromosomal position in their genome. In contrast, homothallic species contain both mating type alleles in a single genome.

The fact that different mating types are necessary for sexual reproduction was first recognized for the genus *Rhizopus* by Blakeslee, (1904); and the first molecular characterization of the mating type idiomorphs was achieved for the yeast *Saccharomyces cerevisiae* (Astell *et al*., 1981). Since the molecular characterization of the mating types genes for *Neurospora crassa*, the first filamentous ascomycete for which mating type genes (*MAT 1-1* and *MAT 1-2*) were cloned and sequenced, the deployment of PCR-based techniques and the increasing availability of genome sequences have resulted in the isolation and characterisation of mating-type genes of a steadily increasing number of fungi (Astell *et al*., 1981; Arie *et al*., 1997; 2000; Waalwijk *et al*., 2002; Barve *et al*., 2003; Bennett *et al*., 2003; Goodwin *et al*., 2003; Inderbitzin *et al*., 2005; Debuchy & Turgeon, 2006; Groenewald *et al*., 2006, 2007; Conde-Ferráez *et al*., 2007; Stergiopoulos *et al*., 2007). These studies show that mating type genes are commonly present, even in presumed asexual species, indicating that lack of sexual recombination is not due to the absence of basal elements controlling the sexual reproductive machinery (Sharon *et al.*, 1996). The presence of mating type genes in asexual species further supports that asexual life style arises from sexual progenitors.

The direct target genes of the mating type proteins have not yet been described, although there is evidence for the control of some genes such as pheromone genes (Bobrowicz *et al*., 2002). The DNA and amino acid sequences of mating type genes show no obvious similarities, although the mating type locus is surrounded by common flanking regions (Turgeon *et al*., 1993). Except for the high mobility group (HMG)- and the alpha domains, the similarity of homologus mating type genes is usually very low between different species (Turgeon, 1998). Regions with similarities of up to 90% can be found in the HMG domain, and these homologus regions have been used to design degenerative primers for amplification and cloning of the MAT 1-2 gens (Arie *et al*., 1997). Analysis of mating type loci in closely

related species provides insights in the evolutionary plasticity of this unique region of the genome, as well as the evolutionary history of closely related species (Fraser *et al*., 2007).

The close co-occurrence of multiple species of *Phaeoacremonium* on a single vine can lead to close physical interactions and potential exchange of genetic material through interspecies mating, hybridisations or anastomosis. This could ultimately result in the origin of new species with altered virulence patterns or host specificity, and thus could be an example of microevolution. Analysing the structure of the mating type genes of the *Phaeoacremonium* species occurring on grapevine might help us to understand the evolutionary history of these species.Mostert *et al*., 2003 showed that *Togninia minima* (Tul. and C. Tul) Berl., the teleomorph of the asexual fungus *Phaeoacremonium aleophilum* W. Gams, Crous, M. J. Wingfield and L. Mugnai, one of the causal agents of esca and related diseases of grapevine, has a biallelic heterothallic mating system and that field isolates obtained from individual diseased vines could be induced to form the teleomorph in vitro on cane sections, indicating that compatible mating types co- occur in nature on the same vine. In addition, Rooney *et al*. 2004 found perithecia of *T. minima* on grapevines in the field in five California counties and suggest ascospores as an additional source of inoculum for new grapevine infections. Sexual reproduction in heterothallic species like in the case of *Togninia minima,* requires the presence of two genetically distinct fungal isolates carrying alternate forms of mating types, MAT1-1 and MAT1-2, at the same geographical locations. In most parts of the world where *Pm. aleophilum* has been introduced and causes serious disease, only the asexual state was observed. This could be attributed to the introduction of only one mating type into these new environments. With this study, we aim to isolate and characterise the mating type loci of *Pm. aleophilum* and to ascertain which mating type (s) are present in the different countries where diseases caused by these fungi occur. The identification of the mating type gene of *Pm. aleophilum* would be very useful and beneficial for population studies, especially in geographic regions where only the anamorph of *Togninia minima* has been identified, in order to prevent introduction events of isolates carrying the other type of mating type and inducing sexual reproduction, and anyway in order to understand which are the modes of reproduction in a geographical area and the type of inoculum source. Sexual reproduction contributes to the spread of the pathogen by producing airborne ascospores acting as new inoculum, it generates variation at the population level and might benefit the organism by purging the genome from deleterious mutations (Heitman 2006, Zhan *et al.* 2007). This knowledge has therefore clear implications in epidemiological studies and in determining control measures.

MATERIALS & METHODS

Isolates

The initial screening of the mating type genes was undertaken for six isolates of *Phaeoacremonium* (Table 1.). The identities of the six isolates used for the characterization of the mating type had previously been confirmed using comparisons of DNA sequence data for the Actin and β-tubulin regions.

Table 1. List of the isolates used in this study.

***Phaeoacremonium* species**	**Isolate name**	**Location**	**Gene bank**	**accessions**
			ACT	**ß-tubulin**
Pm. aleophilum	51Pal	Sicily, Italy		
	159Pal	U.S.A		
Pm. iranianum	6Pal	Tuscany, Italy	EU863493	EU863461
Pm. griseorubrum	42Pal	Sicily, Italy	EU863517	EU863485
Pm. mortoniae	94Pal	Croatia	EU863513	EU863481
Pm. parasiticum	40ss2Pal	Sicily, Italy	EU863519	EU863487

Isolation and characterization of mating type loci of *Phaeoacremonium* species

The previously degenerate primer pairs, ChHMG1 and ChHMG2, NcHMG1 and NcHMG2 described by Arie *et al*., 1997, for the screening *Mat1-1* and *Mat1-2* genes, respectively, were used in an attempt to amplify a fragment of 300 bp of the mating type genes of species of *Phaeoacremonium* (Table 2). The amplifications were done according to the author's instructions, and additional annealing temperatures were tested.

Table 2. Degenerate primers used in this study.

Primer name	**Sequence 5' —3'**	**Target**	**Direction**	**Reference**
NcHMG1	CCYCGYCCYCCYAAYGCNTAYAT	MAT1-2	F	Arie *et al.*, 1997
NcHMG2	CGNGGRTTRTARCGRTARTNRGG	MAT1-2	R	Arie *et al.*, 1997
ChHMG1	AAGGCNCCNCGYCCNATGAAC	MAT1-1	F	Arie *et al.*, 1997
ChHMG2	CTNGGNGTGTAYTTGTAATTNGG	MAT1-1	R	Arie *et al.*, 1997

Amplification reactions were performed in a total reaction volume of 12.5 µl, containing 1× PCR Buffer (Bioline, London, UK), 1.5 mM $MgCl_2$, 48 µM dNTPs, 0.6 µM of each degenerate primer, 0.7 U of *Taq* DNA polymerase (Bioline, London, UK) and 1–10 ng of genomic DNA. PCR amplifications were performed in a GeneAmp PCR System 9600 (Applied Biosystems, Foster City, CA). DNA was initially denatured for 5 min at 94 °C, followed by 15 cycles at 94 °C for 30 s, 52 °C for 30 s, 72 °C for 40 s, followed by 25 cycles at 94 °C for 30 s, 50 °C for 30 s, 72 °C for 40 s, and with a final elongation step at 72 °C for 7 min. The PCR products were separated by electrophoresis at 100 V for 1h on 1% (w/v)

agarose gel containing 0.1µg/ml ethidium bromide in 1 x TAE buffer (0.4M Tris, 0.05M NaAc, and 0.01 M EDTA, pH 7.85) and visualized under UV light.

Cloning, Bacterial transformation and PCR amplification of the recombinant DNA

Amplicons showing the expected product size were cloned into a pGEM -T Vector system (Promega Madison, WI, USA) by a ligation reaction; Five microlitre of 2x ligation buffer (Promega) were mixed with pGEM-T vector (50ng/µl), 1µl 3Weiss units/µl T4 DNA ligase (Promega) and 3µl PCR product. The mixture was incubated at room temperature overnight at 4°C. Then, plasmids were engineered into *Escherichia coli* strain JM109 (Promega), which was used for propagation of constructs as the following; 2ul of the ligated product was added to 30 ul of E. coli cells, then incubated on ice for 10 min. Cells were then shocked by heating at 42°C for 50 sec and immediately chilled in ice for 2 min, then incubated at 37 °C on shaker (250 rpm) for 1 hour.

An aliquot of 200µl of bacteria was plated on LB plates, containing 100-mg/ml ampicilline and 20 µl X-Gal (40 µg/µl) (for white / blue selection), with a sterile glass rod. Plates were incubated overnight at 37°C.

White colonies, carrying the insert, were selected, and picked up and put in 10 µl of LB medium. 1-2µl of the mixture were used for PCR amplification reaction, which were performed in a total reaction volume of 12.5 µl, containing 1× PCR Buffer (Bioline, London, UK), 1.5 mM $MgCl_2$, 48 µM dNTPs, 0.2 µM of the standard universal primers pair; M13F (5'-GTTTTCCCAGTCACGAC-3') and M13R (5'-CAGGAAACAGCTATGAC-3'), 0.7 U of *Taq* DNA polymerase (Bioline, London, UK) and 1-2µl of the recombinant DNA (white colonies in 10 µl of LB medium). DNA was initially denatured for 5 min at 94 °C, followed by for 40 cycles at 94 °C for 20 s, 55 °C for 20 s, 72 °C for 1 min, and with a final elongation step at 72 °C for 7 min. PCR amplifications were performed in a GeneAmp PCR System 9600 (Applied Biosystems, Foster City, CA).

The PCR products were separated by electrophoresis at 100 V for 1h on 1% (w/v) agarose gel containing 0.1µg/ml ethidium bromide in 1 x TAE buffer (0.4M Tris, 0.05M NaAc, and 0.01 M EDTA, pH 7.85) and visualized under UV light, in order to check whether the inserts are present and if the cloning operation was successful.

Sequencing

The identity of the cloned fragments was confirmed by sequencing. Amplicons were sequenced in both directions using a DYEnamic ET Terminator Cycle Sequencing kit

(Amersham Biosciences, Roosendal, The Netherlands) on an ABI Prism 3700 DNA Sequencer (Perkin-Elmer, Foster City, CA) using the same primers as used for PCR. Sequence fragments were assembled using SeqMan (Lasergene package, DNAstar, Madison, WI). Contigs obtained were then analysed using the basic local alignment search tools (BLAST) at NCBI (http://www.ncbi.nlm.nih.gov) (Altschul *et al*., 1997).

RESULTS

Isolation and characterization of *MAT1-2* in *Phaeoacremonium aleophilum*

The degenerate primers NcHMG1 and NcHMG2 successfully amplified a fragment of 300 bp for *Pm. aleophilum* (Fig 1.).

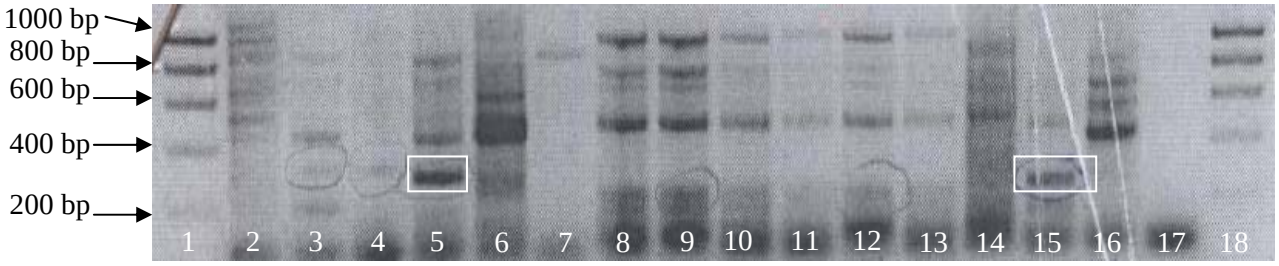

Fig. 1. PCR amplicons generated using primers NcHMG1 and NcHMG2 on DNA extracted from *Phaeoacremonium* isolates used in this study. Lane 1 contains the 1- Kb DNA marker (Hyperladder I, Bioline). The 300 bp fragment, is present noticeably in Lane 5 corresponding to DNA of *Pm. aleophilum*. Lane 15; PCR positive control: DNA of *Gibberella xylarioides* CBS 74979, (MAT 1-2 gene for mating type protein, accession number AM072520). Lane 17; PCR negative control (sterile distilled control).

The amplified products of interest were subsequently cloned and supplied to a second PCR amplification. The results of PCR amplification carried out on recombinant DNA are showed on Fig. 2.

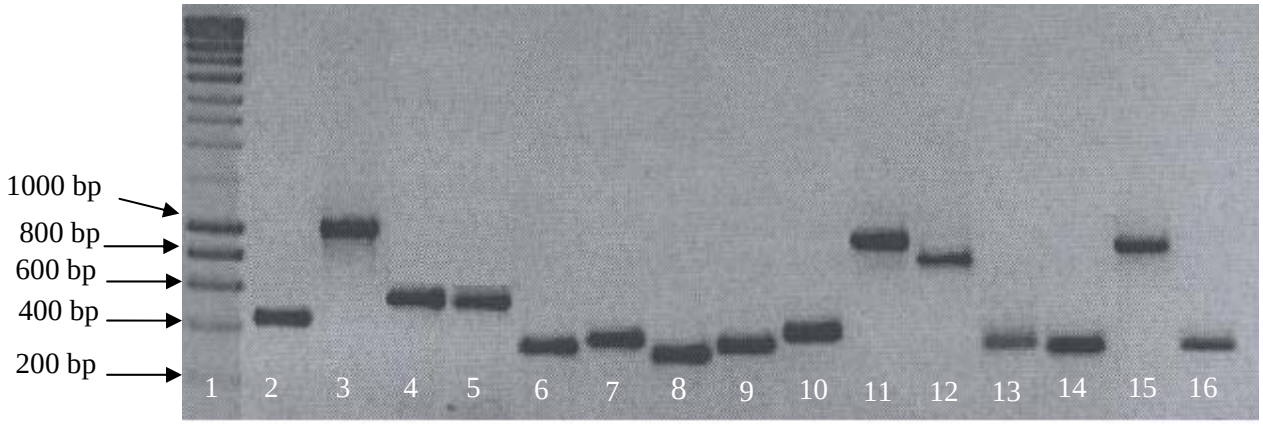

Fig. 2. PCR amplicons generated on the recombinant DNA using the universal primers M13F and M13R.

The expected product of interest, which have approximately a size of 450 bp (300 bp for the expected product of the mating type gene + 150 bp for the vector) were subsequently purified and sequenced (see Fig 2. Lane 2, 4 & 5).

Nucleotide Sequence of a portion of *MAT 1-2* in *Pm. aleophilum*

> 52Pal
TCCTCGTCCTCCCAATGCGTATATTCTTTATCGCAAAGATAATCATCCTATCGTCA
AGGCAGCTCATCCAGGCATTCGCAACAATGACATCTGTAAGTTTCATGAGGCACA
ACCCCTTCCTTTTCTTTCTTGTCTCTTCTTATCCTCCTGTGATGAAGTTCATGAAAC
TGATTTAATCTAGCTGTCATTCTGGGCAAGCGCTGGCACATGGAGACTCCTGAGG
TCCGCCTCCATTACAAGGACTTGGCTGATGAGTGCAAGCGCAAGCACTACGAGAA
GTATCCTCACTATCGCTACAATCCCAA

The nucleotide sequence (~300bp) of this idiomorph showed homology to the HMG-box of other related ascomycetes. In fact, sequence data from this fragment revealed substantial homology on nucleotide level with the *MAT 1-2* gene of *Fusarium sacchari* deposited in GenBank; accession number AB242272.1. Blast analyses showed also another best hits with MAT 1-2 gene of *Gaeumannomyces graminis* var. *tritici* strain GGT1 deposited in GenBank; accession number AY435485 as illustrated respectively in Fig 3 & 4.

```
52Pal  1     TCCTCGTCCTCCCAATGCGTATATTCTTTATCGCAAAGATAATCATCCTATCG-TCAAGG
59
             ||| || |||||||||||| ||||| ||||| || || ||   ||| ||  ||| ||||||
Fusarim3662  TCCACGACCTCCCAATGCTTATATCCTTTACCGTAAGGAGCGTCA-CCACTCGATCAAGG
3720

52Pal  60    CAGCTCATCCAGGCATTCGCAACAATGACATCTGTAAGT--TTCAT  103
             |    || || | |||   ||||||||| ||||||||||  |||||
Fusarim3721  CTCAACACCCTGACATCACCAACAATGAAATCTGTAAGTAGTTCAT  37
```

Fig 3. Alignment given by blast analysis of nucleotide sequences of isolate 52Pal and the sequence of MAT1-2 of *Fusarium sacchari* AB242272.

```
52Pal  179  TAGCTGTCATTCTGGGCAAGCGCTGG-CACATGGAGACTCCTGAGGTCCGCCTCCATTAC
237
            ||||||| |||||||| ||||  ||| || ||| ||||||| | | | |||  | | |||
Sbjct  109  TAGCTGTGATTCTGGGAAAGCAGTGGGCAAATG-AGACTCCCGTGATGCGCTCCAAGTAC
167

52Pal  238  AAGGACTTGGCTGATGAGTGCAAGCGCAAGCACTACGAGAAG  279
            ||| |  |||| || ||   ||||||||||   ||||||||
Sbjct  168  AAGAAGATGGCCGACGACATCAAGCGCAAGCTTCACGAGAAG  209
```

Fig 4. Aligmnet given by blast analysis of nucleotide sequences of isolate 52Pal and the sequence of MAT1-2 of *Gaeumannomyces graminis* var. *tritici* strain GGT1 deposited in GenBank; accession number AY435485

Subsequently, genome chromosome-walking steps were performed in both downstream and upstream directions to obtain the full sequence of the mating type gene as well as the whole idiomorph. Chromosome walking strategy is based on the known piece of *MAT1-2* sequenced and involved several steps of primer design, polymerase chain reaction, cloning, and sequencing.

Chromosome-walking strategy

Nested primer sets were designed based upon contigs corresponding to the *Mat 1-2* sequence, in order to obtain the full-length genes, then to determine the regions flanking sequences. These internal primer sets were used in combination with primers provided with the DNA Walking SpeedUp kit (Seegene Inc., Rockville, USA) to amplify fragments adjacent to the initially cloned fragments (Fig. 5).

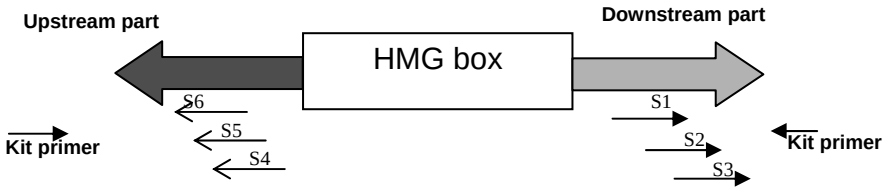

Fig. 5. Schematic representation showing the position of the primers used for chromosome - walking.

In total, 6 primers were designed and used for chromosome walking to obtain the full-length of the *MAT1-2* gene (Table 3).

Table 3. Primers designed in this study for chromosome walking

Name	**Direction**	**Sequence 5' → 3'**
S1	downstream	TCGCAACAATGACATCTGTAAG
S2	downstream	TGTCATTCTGGGCAAGCGCTG
S3	downstream	CCATTACAAGGACTTGGCTGATG
S4	upstream	TCATCAGCCAAGTCCTTGTAAT
S5	upstream	GTTTCATGAACTTCATCACAGGAGG
S6	upstream	ATGAGCTGCCTTGACGATAGGAT

Chromosome walking involves tree rounds of PCR using the DNA Walking SpeedUp kit (Seegene Inc., Rockville, USA), according to the manufacturer's instructions and as shown in Table 4.

Table 4. PCR protocol used for Chromosome-walking steps

First Run	Reagents	Volume (µl)
	DNA of *Pm. aleophilum* 52 Pal	1,5
	TSP primers **S1/S4** 10 µM	1
	Primer provided by the Kit diluted to 2.5 µM	0,5
	Master mix	5,1
	Dntp 1mM	0,5
	Taq 5u/µl	0,15
	MQ	4
	total	12,5
Second Run		
	PCR p. of first run diluted 1:10	1
	TSP primers **S2/S5** 10 µM	0,5
	Primer provided by the Kit diluted to 2.5 µM	0,5
	Master mix	5,85
	Dntp 1mM	0,5
	Taq 5u/µl	0,15
	MQ	4
	total	12,5
Third Run		
	PCR p. of second run diluted 1:10	1
	TSP **S3/S6** 10 µM	0,5
	Primer provided by the Kit diluted to 2.5 µM	0,5
	Master mix	5,6
	Dntp 1mM	0,5
	Taq 5u/µl	0,15
	MQ	4,25
	total	12,5

First run of PCR includes two different mixtures, from one hand, the mixture for the amplification of the upstream part using primers S4 combined with the primer provided by the DNA Walking SpeedUp kit (Seegene Inc., Rockville, USA), from the other hand, the amplification of the downstream part using primers S1 combined with the primer provided by the DNA Walking SpeedUp kit (Seegene Inc., Rockville, USA). It was performed in a GeneAmp PCR System 9600 (Applied Biosystems, Foster City, CA) using 1 cycle at 94 °C for 30 s, 42 °C for 60 s, 72 °C for 2 min, followed by 30 cycles at 94 °C for 40 s, 55 °C for 40 s, 72 °C for 3 min, with a final elongation step at 72 °C for 10 min.

Second run of PCR includes two different mixtures, from one hand, the mixture for the amplification of the upstream part using primers S5 combined with the primer provided by the DNA Walking SpeedUp kit (Seegene Inc., Rockville, USA), from the other hand, the amplification of the downstream part using primers S2 combined with the primer provided by the DNA Walking SpeedUp kit (Seegene Inc., Rockville, USA). It was performed in a GeneAmp PCR System 9600 (Applied Biosystems, Foster City, CA). DNA was initially denatured for 5 min at 94 °C, followed by 35 cycles at 94 °C for 40 s, 60 °C for 40 s, 72 °C for 3 min, and with a final elongation step at 72 °C for 7 min.

Third run of PCR includes two different mixtures, from one hand, the mixture for the amplification of the upstream part using primers S6 combined with the primer provided by the DNA Walking SpeedUp kit (Seegene Inc., Rockville, USA), from the other hand, the amplification of the downstream part using primers S3 combined with the primer provided by the DNA Walking SpeedUp kit (Seegene Inc., Rockville, USA) using the same conditions of the second run.

The PCR products were separated by electrophoresis at 100 V for 1h on 1% (w/v) agarose gel containing 0.1µg/ml ethidium bromide in 1 x TAE buffer (0.4M Tris, 0.05M NaAc, and 0.01 M EDTA, pH 7.85) and visualized under UV light. The results of the third PCR run are shown in Fig. 6.

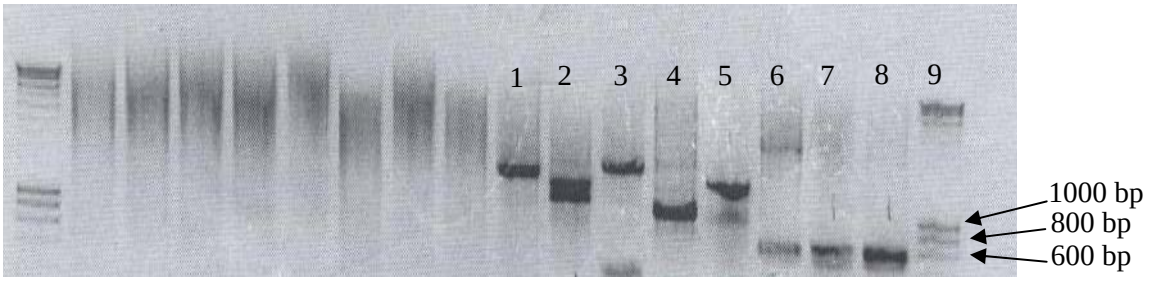

Fig. 6. PCR products generated in the the third PCR run. Lane 1,2,3 & 4; PCR products from the amplification of the downstream part. Lane 5,6,7 & 8; PCR products from the amplification of the upstream part.

PCR products were subsequently purified and sequenced as described previously. However, up to date, our attempts to obtain the sequence of these products were not successful. Further work is in progress to get these products sequenced and subsequently several chromosome-walking steps will be performed in both upstream and downstream directions to obtain the full sequence of the *MAT1-2* of *Pm. aleophilum* as well as the whole idiomorph.

DISCUSSION

In this study, we present our first attempt to describe the mating type gene of *Pm. aleophilum*, in order to understand the mating behaviour of this important pathogen associated with esca and related diseases of grapevine and its distribution.

While our attempts for the characterization of the MAT 1-1 using the primers Pair ChHMG1 and ChHMG2 were not successful, we could successfully characterize a portion of 300 bp of the MAT1-2 using the degenerate primer sets (NcHMG1 and NcHMG2). The nucleotide sequence (~300bp) of this idiomorph showed homology with the HMG-box of other related ascomycetes. In this chapter of the thesis, we described the method used and the future approach to be used for the characterisation of the full length of the MAT1-2. Based on this sequence, different primers were designed to be used in chromosome walking strategy to characterize the whole idimorph.

Identification of mating types using PCR is more reliable and faster than traditional methods, which are based on time consuming multiple pairings between a wide range of strains and selected tester strains of the two mating types. Therefore, the identification of mating type would be very useful and beneficial for population studies, first of all to gain knowledge about the mode of reproduction that is expected to be common, naturally, in each geographical region where the pathogen is a threat. Up to now, data on the mating type distribution of *Pm. aleophilum* is only available for California vineyards, where the two mating types occur in a nearly 1:1 ratio, in the country but also even in the same vineyard. This is consistent with the reports of naturally occurring sexual reproduction structures of *T. minima* (Rooney *et al*., 2004) in that country.

Togninia minima has been proved to be an heterothallic species, it is then important to understand the distribution of mating types and, in case they are not always both present, to restrict them to their present locations. This could be achieved trough refining quarantine regulations based on the knowledge that only one of the mating type of the pathogen is present in the country.

In conclusion, the mating type specific PCR could be implemented as a control method to test for the presence of the mating types of *Pm. aleophilum*, and, if applied on isolates obtained in different areas, will provide new insight into the distribution of mating types of *Pm. aleophilum* and on its epidemiological implications.

Chapter 7

General Discussion and Conclusions

General discussion and conclusions

The main aim of the research described in this thesis, was to improve the knowledge on esca complex diseases of grapevine, with particular attention to species of *Phaeoacremonium* and *Phaeomoniella chlamydospora*, which together with *Fomitiporia mediterranea* represent the main agents of esca complex diseases of grapevine. Research carried out in the last 20 years clarified many aspects of the aetiology and epidemiology of the disease, leading to consider species of *Phaeoacremonium* and *Phaeomoniella* as the main agents of at least three of the syndromes (Brown wood streaking of rooted cuttings, Petri disease, Young esca). Research studies focuses mainly, on the mechanisms by which these fungi produce symptoms, the metabolites they produce and the way the plant reacts to infection. However, in order to fully understand these processes, a deeper knowledge of the variability within the populations of the two pathogens is needed, as they are both found in all grape growing countries, associated with various sets of symptoms that have a different frequency in different areas – more frequently esca – ; " Tiger - like" foliar symptoms are often observed in Europe, while decline in young vines of Petri disease is more frequent in Australia, South Africa and California. Therefore, the present study investigates the identity of a global collection of strains of *Phaeoacremonium* and *Phaeomoniella* isolates from grapevine, collected mainly from very old vines, in isolated locations in Italy and other countries, in order to gain a better understanding of the epidemiology of these destructive diseases of grapevine. In addition to this population study, a molecular assay for the simultaneous detection of the major agents of the grapevine trunk diseases closely related to esca was partially developed.
This concluding chapter discusses the results obtained in the previous chapters and addresses areas of potential future research.

Newly identified species, new records on grapevine and new reports in different countries

During the course of this study, four new species of *Phaeoacremonium* namely *Pm. croatiense, Pm. hungaricum, Pm. sicilianum* and *Pm. tuscanum* have been distinguished from the existing *Phaeoacremonium* spp. based on a combined cultural, morphological and DNA sequence data sets of the actin and ß-Tubulin genes. It is of interest to notice that all the four new species described were isolated from very old vines growing in Italy, Hungary and Croatia. This finding is in agreement with the hypothesis that the disease was introduced the last century, from North America to other countries in Europe, when the rootstock varieties resistant to *Phylloxera* were exported all over the grape growing countries.

Growth temperature studies carried out for new species described in this study showed that *Pm. tuscanum* has a maximum growth temperature at 37–40 °C, reaching a radius of 7 mm after 8 d. This finding is quite interesting in relation to the ecology of *Phaeoacremonium* spp., as several thermotolerant *Phaeoacremonium* spp. are associated with phaeohyphomycosis in humans (Mostert *et al*. 2005). *Phaeoacremonium parasiticum, Pm. rubrigenum* and *Pm. inflatipes* (later identified as *Pm. alvesii*), were initially reported from human phaeohyphomycosis (Padhye et al. 1998, Guarro et al. 2003). Four *Phaeoacremonium* species have heretofore been isolated only from human infections: *Pm. tardicrescens, Pm. amstelodamense, Pm. griseorubrum,* and *Pm. rubrigenum* (Mostert et al. 2005). The present study revealed that *Pm. alvesii, Pm. griseorubrum* and *Pm. rubrigenum* are also associated with grapevine. This finding further supports the hypothesis that human pathogenic *Phaeoacremonium* spp. may have originated from woody host plants. The same hypothesis has been proposed for some other groups of human opportunistic pathogens such as the black yeasts (de Hoog et al. 2007). To confirm this hypothesis, studies are needed inoculating strains from woody hosts into an animal model, and strains from human cases into woody plants.

Pm. viticola, Pm. scolyti and *Pm. griseorubrum* represent new records for Italy, *Pm. mortoniae* and *Pm. rubrigenum* for Croatia, *Pm. alvesii* for Turkey and *Pm. mortoniae* for Hungary.

Given the occurrence of various *Phaeoacremonium* species on grapevine and the involvement of some of those species in human infections, accurate identification is critical. Since, as mentioned, morphological identification is not reliable, robust molecular-based detection tools are of great help in species detection. An existing multiplex PCR assay based on use of species-specific TUB and ACT primers can identify 22 species of *Phaeoacremonium* (Mostert *et al*., 2006b). It is necessary to test these primer sets on DNA from the new species described here to determine if target specificity remains intact. The ACT and TUB gene barcodes generated in the present study can be used to develop species-specific molecular detection tools that will facilitate ecological and epidemiological studies.

Up to date, 20 *Phaeocremonium* species have been isolated from grapevine. Given the fact that the current study has added other four new species, sampled from very old vineyards and isolated locations, where farmers grow local grape varieties, it is interesting to consider the possibility that there may be yet more unknown species of this genus in other areas waiting to be discovered. The following question arise; what their ecological niche would be?

Do they occur as endophytes or pathogens ? and what is the degree of their involvement in esca and related diseases of grapevine?

More extensive sampling from areas where farmers grow local grape varieties in other countries, would aid in understanding these questions. Knowledge pertaining to the involvement of *Phaeoacremonium* species in esca diseases complex, should shed light on the epidemiology of these destructive diseases of grapevine, with the final aim of helping in refining control strategies.

Genetic variation among isolates of *Phaeomoniella*

During the present study, the genetic variation among isolates of *Phaeomoniella chlamydospora* collected mainly from very old vines, in isolated locations in Italy and other countries was investigated. A multi-gene phylogeny was derived from these isolates using the ß-tubulin and elongation factor 1-α gene, in order to gain a better understanding of the genotype geographic distribution of this important pathogen. Contrary to our expectations, a high level of similarity was found among the isolates of *Pa. chlamydospora* used in this study, despite the fact that they were collected from areas not used in previous studies and despite the difference observed in cultural characteristics. The results of the present study are in general agreement with previous studies (Tegli *et al*., 2000; Borie *et al*., 2002; Pottinger *et al*.,2002, Mostert *et al*., 2006, Cobos and Martin, 2008) that also noted a low degree of genetic diversity among *Pa. chlamydospora* strains. However, is it common that a population of a pathogen like *Pa. chlamydospora* that is worldwide spread is so stable? Why a pathogen like *Pa. chlamydospora* that has been well adapted to so different climatic conditions, in such a wide variety of environments has practically no genetic variation?

The goal of this study was to answer these questions by carrying out an extensive sampling mainly from areas not included in previous research programmes. Actually, previous studies conducted on the genetic variability of *Pa. chlamydospora* focused mainly on variation within countries without paying specific attention to variation of the plant material. However, isolates coming from different countries may have the same initial propagation material. As a matter of fact, similar clonal lineages of *Pa. chlamydopora* in different countries could be due to single introduction events from the same inoculum source (grapevine cuttings).

Moreover, these studies had not been previously applied on isolates coming from isolated regions like islands for example. Sampling from areas where cultivars used in viticulture originated, namely Eurasia and USA, from isolated locations in Italy and other countries, from vineyards using minor cultivars (different from the international ones usually sampled in

previous papers as: cv. Savignon, cv. Chardonnay, cv. Sangiovese), from spontaneous grapevine growing areas (*Vitis vinifera* subsp. *sylvestris*) and from other countries selecting vines of local varieties (Chile, Croatia, France, Germany, Greece, Hungary, Iran, New Zealand, Palestine, Portugal, Serbia, South Africa, Turkey, and the USA) were therefore carried out. Furthermore old vines were, where available, included in this study as a source of esca tracheomycotic fungi with the specific objective of gathering a population of both genera, *Phaeomoniella* and *Phaeoacremonium*, showing as wide as possible variability within the population of the two fungi. In this chapter, we report the results so far obtained in *Phaeomoniella*.

Sequence data analysis of the β- tubulin and EF 1-α genes of the isolates sampled from these geographic areas did not show important genetic diversity and did not reveal any subdivision in this population. Phylogenetic analyses showed a high percentage of similarity and clustered all the isolates in a single clade, together with previously published species, available in GenBank, indicating the absence of genotype- geographic structure.

The occurrence of the same genotype in different countries and geographic areas supports the fact that *Pa. chlamydospora* can occur in apparently healthy rooted grapevine cuttings (Bertelli *et al*., 1998), and apparently disease-free grapevines and could be distributed via exported grapevine material.

Finally, a high frequency of isolation of *Pa. chlamydospora* was noticed from the plant material collected in this study, since it has been isolated much more frequently than species of *Phaeoacremonium* and it was present in all the Petri disease/esca diseased plant material sampled, and considerable differences were observed in term of cultural characteristics (different shape and colour of the grown colony of *Pa. chlamydospora*). Nevertheless, these differences were not supported by molecular analyses and showed that the population of *Pa. chlamydospora* sampled in the present work is not subdivided, and revealed a high level of similarity. This finding might be explained by the fact that *Pa. chlamydospora* most likely reproduces asexually in the field. Till now, no sexual state has yet been identified for this species, which suggests it is either rare, may be only present in the area of origin, or nonexistent, and possibly accounts for the genetic uniformity in this species.

The high homogeneity noticed in population of *Pa. chlamydospora* could also suggests that the fungus has spread all around the world quite recently, probably via grafting, as if spores were the main way of spreading of the pathogen, some mutations could have been found. The hypothesis of an origin of the disease in North America, where the rootstock varieties

resistant to *Phylloxera* were exported all over the grape growing countries, should be tested by a wider sampling in that area.

In our study of the genetic variability, multi gene phylogeny was used. However, further insights into the evolution and population genetics of *Pa. chlamydospora* may benefit from the development of highly informative molecular markers, and the use of other techniques developed for the study of genetic variation, and showed to give the greatest amount of variation and good insight into genotype distribution like, the AFLPs one (Amplified Fragment Length Polymorphisms), RFLPs (Restriction Fragment Length Polymorphisms), or using Multilocus sequence typing (MLST).

Development of a molecular approach for the simultaneous detection of fungi associated to esca and related diseases of grapevine

In recent years, molecular tools are becoming very important for the early and sensitive detection of the fungi involved in esca on propagation material. For this purpose, taxon specific priming multiplex PCR were developed in the current study, in order to achieve a detection method for the simultaneous detection of fungi associated with esca. In fact, the primers developed based on the internal transcribed spacer region (ITS1-5.8S-ITS2) of the nuclear ribosomal DNA of *Botryosphaeria* spp. was successfully used in the semi-nested PCR and Nested PCR and Semi nested multiplex PCR assays. The specific identity of the amplified DNA was determined by sequencing both the strands and pairwise comparisons with the homologous sequences available in the GenBank database. The trials indicated that by far the most common Botryosphaeriaceae species were *Botryosphaeria dothidea* and "*Botryosphaeria obtusa"*. These two species are not considered the most virulent of the Botryosphaeriaceae species that attack grapevine, but the most widespread. On the other hand, the presence of different products amplified by the specific primers designed for the detection of Botryosphaeriaceae spp., *Phaeomoniella chlamydospora* and *Phaeoacremonium aleophilum* and the forward universal primer ITS -5 confirms that the multiplex PCR was successful. However, the specific primer for the detection of *Fomitiporia mediterranea* could not be successfully combined in the multiplex reaction.

The multiplex approach developed in this study, still need to be optimized and tested on other diseased and plant propagation material to assess their application for detection and disease diagnosis of fungi associated with esca and related diseases of grapevine.

The results of this study showed also that the potential exists for *Pa. chlamydospora* infection to occur during grapevine propagation operations also in Italy. This is in accord with previous

findings in New Zealand (Whiteman *et al*., 2004) and South Africa (Retief *et al*., 2006) nurseries.

In this study, using traditional methods for isolation and culturing, none of the fungi detected with PCR could be isolated, while with PCR, DNA target had been amplified also in apparently healthy propagation material sampled from two Italian nurseries, suggesting that if *Pa. chlamydospora*, Botryosphaeriaceae spp., *Phaeoacremonium aleophilum* were present in the grapevine samples, they were either on the outside of the cuttings and removed during surface sterilisation, or they were at undetectable levels. The molecular techniques used in this study proved to be highly sensitive; the nested PCR assay was more sensitive for detection than the single PCR.

Management of esca diseases complex relies on the use of pathogen-free plants for new planting. Infection may take place in nurseries during the propagation process, and may also happen because of the use of infected mother plants. In order to assess the use of healthy mother plant and propagation material, such a method is of the utmost necessary for testing of plant material designated for nursery production of plant propagation material. In addition, this detection system could also be used for ecological studies of esca on grapevine.

WORK IN PROGRESS

In this thesis, we present also the work which was started, as a follow up of the outcome of the main research carried out.

In chapter 5, we describe the trial set up to investigate the pathogenicity of the newly described *Phaeoacremonium* species reported from grapevine (this work). Pathogenicity studies were conducted, to determine the potential of the species previously described mainly as human pathogenic one, namely *Phaeoacremonium* (*Pm) alvesii*, *Pm. griseorubrum* and *Pm. rubrigenum*, which were newly reported on grapevine from Turkey, Italy and Croatia, respectively, and of the four new species of *Phaeoacremonium,* namely *Pm. croatiense, Pm. hungaricum, Pm. sicilianum* and *Pm. tuscanum* newly described and isolated from vascular tissues of grapevines, in order to determine their potential as vascular pathogens and to test their effective capability to colonise pruning wounds and to cause vascular discoloration, similar to that seen in esca diseased grapevines, with the final aim to understand their effective involvement in esca diseases complex. Pathogenicity tests, which are still underway, were conducted with grapevine nursery plants of 1yr.old (cv. R24 *Sangiovese*, rootstock 161/49) in a greenhouse using wound inoculations. This trial includes also the inoculation of

Phaeomoniella chlamydoposra and *Pm. aleophilum* to verify the efficacy of this pathogenicity test.

Some of the inoculated plants including positive controls, were collected from the greenhouse after two months and examined in the laboratory. Plants were split lengthwise through the inoculation hole to reveal the xylem and pith regions for observation and measurement of the lesions. At the time being, the information obtained was difficult to interpret due to the small lesions observed both in control as in inoculated vines, because of the short length of time between the starting of the inoculation experiment and the evaluation. Future surveys will be carried out up to the observation of significant lesions in the positive controls, and subsequently statistical analysis and re – isolation will be undertaken.

In Chapter 6**,** we present our first attempt to describe the mating type gene of *Pm. aleophilum*, in order to understand the distribution of mating types and the mating behaviour of this important pathogen associated with esca and related diseases of grapevine. Up to now, our attempts for the characterization of the MAT 1-1 using the primers Pair ChHMG1 and ChHMG2 were not successful, whereas for the MAT1-2 we could successfully characterize a portion of 300 bp for using the degenerate primer sets (NcHMG1 and NcHMG2). The nucleotide sequence (~300bp) of this idiomorph showed homology to the HMG-box of other related ascomycetes. In this chapter of the thesis, we described the method used and the future approach to be used for the characterisation of the full length of the MAT1-2. Based on this sequence, different primers were designed to be used in chromosome walking strategy to characterize the whole dimorph.

Further work is in progress to obtain the whole idiomorph by means of chromosome walking and genomic analyses. Identification of mating types using PCR is faster than traditional methods and would be very useful and beneficial for population studies, especially in geographic regions where only the anamorph of *Togninia minima* has been identified.

Togninia minima has been proved to be an heterothallic species, it is then important to restrict the one type of mating isolates to their present locations. This can be achieved trough refining quarantine regulations based on the knowledge that only one of the mating type of the pathogen is present in the country. The mating type specific PCR could be implemented as a control method to test for the presence of the mating types for *Pm. aleophilum* species, and will provide new insight into the distribution of mating types of *Pm. aleophilum*, that should enhance the quality of quarantine regulations in the future.

SUMMARY

In recent years, vineyards have experienced a dramatic increase in grapevine trunk diseases, that cause a drastic decline in the health of grapevines, affect the viability of newly planted vineyards, limit the long-term sustainability of wine grape production and inhibit the productivity of, and in many cases kill, mature vines and consequently leads to important losses in the majority of the grapevine producing countries of the world.

In this thesis, special interest was given to esca complex diseases, the most destructive trunk diseases of grapevine worldwide, which present a great concern to most producers, viticulturists and researchers. In addition, specific attention has been focused on *Phaeoacremonium* and *Phaeomoniella* genera, the principal hyphomycetes associated with Brown wood streaking, Petri disease and esca (young esca) of grapevine. Accordingly, the main objective of this study was to better understand the level of genetic diversity that exist within and among *Phaeoacremonium* and *Phaeomoniella* populations of a global collection, with the purpose of improving our knowledge on esca complex diseases of grapevine. Furthermore, a molecular assay for the simultaneous detection of the major agents of the esca complex disease was developed.

Chapter 1 gives an introduction to the esca complex disease, and the problems associated with its detection and management are summarised. Moreover, a general overview of the state of the art of *Pa. chlamydospora* and *Phaeoacremonium* is provided.

Chapter 2 describes a multiplex PCR approach developed in this study, which can permit to detect *Phaeomoniella chlamydospora***,** *Phaeoacremonium aleophilum*, and five species of Botryosphaeriaceae using one reaction, in order to offer nursery owners and growers a very sensitive method for detecting the presence of pathogens associated with esca and related diseases of grapevine, especially, for testing plant material designated for nursery production of plant propagation material. This assay has been tested on DNA extracted from samples showing esca symptoms and asymptomatic ones (natural infected plant material and apparently healthy propagation material), in order to assess the suitability and applicability of such procedure in the grapevine sanitary assessment.

Chapter 3 investigated the genetic variation among isolates of *Phaeomoniella chlamydospora* on grapevines, which is still insufficiently understood. A global collection of *Pa. chlamydospora* strains collected mainly from very old vines, in isolated locations in Italy and other countries, was established as part of this study. A multi-gene phylogeny was derived from these isolates using the β-tubulin and elongation factor 1-α gene. The aim of this part of study is to elucidate the probable presence of novel species of *Phaeomoniella* from grapevine and to gain a better understanding of the genotype geographic distribution of this important pathogen. Findings obtained by this population genetics study are relevant to shed light on the sources of inoculum and pathogen dispersal, and on host-pathogen co-evolution, and improve our understanding of the biology of this important pathogen, its disease cycle and epidemiology. Isolates of *Phaeomoniella chlamydospora* from different vineyards and production areas used in this study, had a high percentage of similarity indicating that long range dispersal trough aerial inoculum or infected plant material play an important role in genotype distribution and confirms that asexual reproduction most likely is dominant in the field.

Chapter 4 analyses the phylogeny of a global collection of 118 *Phaeoacremonium* isolates from grapevines, in order to gain a better understanding of their involvement in esca complex diseases of grapevine. Phylogenetic analyses of combined DNA sequence datasets of actin and β-tubulin genes revealed the presence of 13 species of *Phaeoacremonium* among the isolated obtained from esca diseased grapevines. Species previously described mainly as human pathogenic species, namely *Pm. alvesii*, *Pm. griseorubrum* and *Pm. rubrigenum* are newly reported on grapevine from Turkey, Italy and Croatia, respectively. *Phaeoacremonium viticola* and *Pm. scotyli* represent new records for Italy, as well as *Pm. mortoniae* for Hungary and Croatia. In addition, four new species of *Phaeoacremonium,* namely *Pm. croatiense*, *Pm. hungaricum, Pm. sicilianum* and *Pm. tuscanum* are newly described from grapevine based on morphology, cultural characteristics, as well as molecular phylogeny.

In **Chapter 5** the trial set up to investigate the pathogenicity of the newly *Phaeoacremonium* species reported on grapevine (this study) is illustrated. In fact, the relative importance of the different *Phaeoacremonium* species in esca complex diseases is still insufficiently well understood, since several new species have only recently been described and their status as pathogens still unknown. Hence, pathogenicity tests were carried out to determine their potential as decline pathogens and to understand their effective involvement in esca complex diseases of grapevine.

Chapter 6 describes the characterisation of a portion of the mating type gene (MAT1-2) of *Pm. aleophilum.* Further work is in progress to obtain the whole idiomorph by means of chromosome walking and genomic analyses. Identification of mating types using PCR is faster and cleaner than traditional methods and would be very useful and beneficial for population studies, especially in geographic regions where only the anamorph of *Togninia minima* has been identified.

Chapter 7 summarises the research presented in this thesis, discusses the important findings of this dissertation and lead to several propositions for future research initiatives.

RIASSUNTO

Negli ultimi anni nei vigneti si è verificato un drammatico incremento dell'incidenza di malattie del tronco della vite. Oltre a provocare una generale drastica diminuzione della sanità delle piante, queste malattie hanno effetto sulla durata della vita dei nuovi impianti e sulla sostenibilità a lungo termine della produzione di uva da vino, limitano la produttività delle piante adulte che in molti casi muoiono e di conseguenza comportano perdite economicamente importanti nella maggior parte dei paesi viti-vinicoli del mondo.
In questa tesi sono state trattate in particolare la malattia di Petri ed il mal dell'esca, due delle più importanti malattie della vite che preoccupano la maggior parte dei produttori, dei viticoltori e dei ricercatori di tutto il mondo. Un'attenzione particolare è stata rivolta ai generi *Phaeoacremonium* e *Phaeomoniella*, i principali ifomiceti associati alle due malattie. In particolare, l'obiettivo principale di questo studio è stato quello di indagare il livello di diversità genetica esistente all'interno e tra popolazioni di *Phaeomoniella* e *Phaeoacremonium* facenti parte di un'ampia collezione, con lo scopo di ottenere dati che aiutino a comprendere il comportamento patogenetico delle due specie con un areale di distribuzione così ampio e così strettamente legate alla vite come ospite preferenziale o unico (*P. chlamydospora*). Inoltre, è stato messo a punto, anche se ancora deve essere ottimizzato, un protocollo molecolare per la diagnosi simultanea della presenza dei più importanti agenti causali del complesso dell'Esca e di alcuni importanti patogeni del legno che sono spesso presenti su vite, frequentemente in associazione con gli agenti dell'esca.

Il **Capitolo 1** introduce il complesso del mal dell'esca e riassume le problematiche associate alla sua diagnosi e gestione. Inoltre, viene divulgato lo stato dell'arte sui generi *Phaeomoniella* e *Phaeoacremonium.*

Il **Capitolo 2** descrive un approccio di multiplex PCR per la diagnosi della presenza di *Phaeomoniella chlamydospora***,** *Phaeoacremonium* sp.*, Fomitiporia mediterranea* e specie di Botryopshaeriaceae. Il protocollo ha lo scopo di offrire ai vivaisti ed ai produttori uno strumento diagnostico estremamente sensibile per diagnosticare la presenza dei patogeni associati al Mal dell'esca e ad altre malattie correlate, in modo particolare nel materiale di propagazione sospetto all'interno di programmi di certificazione. Questo protocollo è stato saggiato sul DNA estratto da campioni vegetali con e senza sintomi di Esca (materiale

vegetale naturalmente infetto e materiale di propagazione apparentemente sano) in modo da validarne l'idoneità e l'applicabilità nelle procedure di controllo sanitario della vite.

Il **Capitolo 3** indaga sulla variabilità genetica esistente tra isolati di *Phaeomoniella chlamydospora* da vite, argomento che non è ancora sufficientemente chiaro. Parte di questo studio è stato dedicato alla costituzione di un'ampia collezione di ceppi di *Pa. chlamydospora* per la maggior parte isolati da viti molto vecchie provenienti da diverse località dell'Italia e di altri Paesi. Una filogenesi multigenica è stata derivata da questi isolati sulla base delle informazioni ottenute dall'analisi dei geni per la β-tubulina e l' Elongation factor. Scopo di questa fase dello studio è stato quello di verificare il livello di variabilità genetica all'interno della specie in relazione ad esempio alla provenienza geografica, alla specie ospite, alle caratteristiche dei campioni analizzati e della loro origine. I risultati ottenuti da questo studio filogenetico sono importanti per delucidare le fonti dell'inoculo, la dispersione del patogeno, la co-evoluzione di ospite e patogeno, e per incrementare le conoscenze sulla biologia e l'epidemiologia di questo importante patogeno. Gli isolati di *Pa. chlamydospora* utilizzati in questo studio provenienti da diversi vigneti e da diverse aree di produzione hanno mostrato un elevato livello di similitudine suggerendo che la dispersione sulla lunga distanza attraverso l'inoculo aereo, ma più probabilmente il materiale vegetale infetto, giocano un ruolo importante nella distribuzione del genotipo e confermando che nel vigneto la riproduzione asessuale è dominante.

Il **Capitolo 4** analizza la filogenesi di una collezione di 118 isolati di *Phaeoacremonium* da vite per meglio comprendere il loro ruolo nella malattia di Petri e nell'esca. L'analisi filogenetica basata sulle sequenze nucleotidiche combinate dei geni per l'actina e la β-tubulina rivelano un livello di variabilità genetica all'interno della specie *Pm. aleophilum* molto superiore a quella evidenziata in *P. chlamydospora*, come da attendersi in una specie che presenta riproduzione sessuata. Inoltre l'analisi filogenetica ha evidenziato una grande variabilità anche fra gli isolati *Phaeocremonium* ottenuti da piante malate di esca, individuando la presenza di ben 13 diverse specie di *Phaeoacremonium*. Specie che erano state in precedenza descritte come patogene per l'uomo quali *Pm. alvesii*, *Pm. griseorubrum* e *Pm. rubrigenum* vengono per la prima volta segnalate su viti provenienti dalla Turchia, l'Italia e la Croazia. *Phaeoacremonium viticola* e *Pm. scotyli* sono nuove segnalazioni per l'Italia così come *Pm. mortoniae* per l'Ungheria e la Croazia. Inoltre, vengono descritte quattro nuove specie di *Phaeoacremonium*, denominate *Pm. croatiense*, *Pm. hungaricum*, *Pm.*

sicilianum e *Pm. tuscanum* sulla base della morfologia, di caratteristiche colturali e della filogenesi molecolare.

Il **Capitolo 5** illustra la patogenicità delle nuove specie di *Phaeoacremonium* e di quelle precedentemente descritte come patogene per l'uomo per la prima volta segnalata su vite (questo studio). Infatti, l'eventuale ruolo delle diverse specie di *Phaeoacremonium* nella malattia di Petri e nell'esca non è sufficientemente chiaro dal momento che varie fra le nuove specie sono state descritte solo recentemente. Questi saggi sono stati condotti in serra mediante inoculazioni sul tronco.

Il **Capitolo 6** descrive la caratterizzazione di una porzione del mating type gene (MAT1-2) di *Pm. aleophilum.* Lo studio ancora in corso mira ad ottenere l'intero idiomorfo mediante il "chromosome walking" e l'analisi genomica. L'identificazione dei "mating types" attraverso la PCR è più veloce dei metodi tradizionali e potrebbe essere molto utile per studi di popolazione specialmente in quelle regioni geografiche in cui è stato identificato solo l'anamorfo di *Togninia minima.*

Il **Capitolo 7** riassume la ricerca presentata in questa tesi, discute i nuovi dati acquisiti, nel corso di questo studio e propone varie ipotesi per future iniziative di ricerca.

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