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RVRALIS ET SILVESTRIS SAPIENTIA

Indirizzo Monitoraggio e Controllo degli Ecosistemi Forestali
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Botryosphaeriaceae species associated with cankers and dieback of grapevine and other woody hosts in agricultural and forestry ecosystems

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Chapter 1 Introduction

The *Botryosphaeriaceae*

Historical overview

The history of the family *Botryosphaeriaceae* Theiss. & Syd. has been rather complicated as documented by the several taxonomic revisions carried out over the years. This fungal family was introduced by Theissen and Sydow in 1918 as a sub-family of the *Pseudosphaeriaceae*. The *Pseudosphaeriaceae* were considered by Theissen (1916) in the order *Myriangiales* while a year later Theissen and Sydow (1917) thought it more appropriate to unite the *Pseudosphaeriaceae* with the *Dothideaceae* (Luttrell 1951). Theissen and Sydow (1918) erected the sub-class of the *Dothideinae* and included the order *Pseudosphaeriales*, the family *Botryosphaeriaceae* and the genus *Botryosphaeria* within this sub-class. This classification was not accepted by Petrak (1923) who placed *Botryosphaeria* in the sub-family *Pseudosphaeriae* within the *Pleosporaceae*, order *Sphaeriales*. Miller (1928) observed substantial differences among species in the *Sphaeriales* characterised by having true perithecial ascomata and paraphyses, and those allocated in the *Dothideales* characterised by ascostromatic ascomata lacking paraphyses, and proposed that *Botryosphaeria* species should be well accommodated in the *Dothideales* because their lack of true perithecial walls. A few years later Miller (1938), studying in more detail morphological characteristics resulting from the ontogeny of the perithecial wall and the development of tissues in the centrum, observed that the *Sphaeriales* formed perithecia and paraphyses, while ascostromatic forms with interthecial threads that appeared in the ascomatal cavity before the development of the asci were distinctive characters of the *Dothideales*. For this reason he kept *Botryosphaeria* in the family *Pseudosphaeriaceae* and placed the genus in the order *Pseudosphaeriales*. Subsequently, on the basis of the type of centrum development, the name *Pseudosphaeriales* was replaced with *Pleosporales* by Luttrell (1955) and *Botryosphaeria* was accommodated in the *Pleosporales*. Barr (1972, 1976) initially ignored specimens of *B. dothidea* characterised by interthecial tissues, and classified *Botryosphaeria* in the *Dothideales*. A few years later, Barr (1979) recognised that *Botryosphaeria* species had a typical centrum of the *Pleosporales* and so were well-accommodated in this order. von Arx and Müller (1975) and von Arx (1981, 1987) observed the presence of unrelated genera and the overlap of taxonomic characters used

to separate the orders in the classification proposed by Luttrell (1955, 1973) and Barr (1972, 1987). In addition, given the close resemblance of *Guignardia* and *Botryosphaeria* in morphological terms, von Arx and Müller (1975) did not accept their placement in the distinct orders *Dothideales* and *Pleosporales* respectively, and came to the conclusion that *Guignardia* and *Botryosphaeria* should be combined in the *Dothideales*. Hawksworth et al. (1995) accepted *Guignardia* under the *Dothideales* but included this genus in the family *Mycosphaerellaceae,* retaining *Botryosphaeria* under the *Botryosphaeriaceae*.

The introduction of molecular tools and phylogenetic analyses of nucleotide sequence data has in part contributed to clarifying the confused taxonomic history of the *Botryosphaeriaceae*. Several phylogenetic studies have been carried out using different nuclear and mitochondrial rDNA genes in order to establish the ordinal placement of the *Botryosphaeriaceae*. However, these studies yielded conflicting results and did not allow unequivocal clarification of the taxonomic position of the family within the *Dothideales* or the *Pleosporales* (Berbee 1996; Silva-Hanlin and Hanlin 1999; Lumbsch et al. 2000; Lutzoni et al. 2004). Scoch et al. (2006) using a multi-gene approach revealed that *Botryosphaeria* and *Guignardia* species clustered in a separate clade that could not be well accommodated in any other order. For this reason they introduced the order *Botryosphaeriales* Schoch, Crous & Shoemaker to accommodate the family *Botryosphaeriaceae*. It remained the only family under the *Botryosphaeriales* until Minnis et al. (2012) introduced the *Planistromellaceae* into this order. Species in the genus *Botryosphaeria* are easily distinguishable from those of *Guignardia* because of their larger ascomata and ascospores, a multilocular stroma, as well as different growth characteristics in culture (Van der Aa 1973; Punithalingam 1974). As a consequence of the massive use of DNA sequencing methods to link asexual and sexual morphs and the end of dual nomenclature the name *Guignardia* was replaced with the older name *Phyllosticta* (Glienke et al. 2011; Sultan et al. 2011; Wikee et al. 2011; Wong et al. 2012). Wikee et al. (2013) using a multi-gene phylogeny based on ITS, 28S rRNA gene (LSU), translation elongation factor 1-alpha (EF1- α), actin (ACT), and glyceraldehyde-3-phosphate dehydrogenase (GPDH) sequence data combined with morphological characteristics, proposed to resurrect the *Phyllostictaceae* to accommodate *Phyllosticta* within

the order *Botryosphaeriales*. Slippers et al. (2013) introduced three additional new families, namely *Aplosporellaceae*, *Melanopsaceae* and *Saccharataceae*. Therefore, the order *Botryosphaeriales* currently includes six distinct families.

Genera in the *Botryosphaeriaceae*

When it was introduced, the family *Botryosphaeriaceae* included three distinct genera: *Botryosphaeria*, *Phaeobotryon* and *Dibotryon* (Theissen and Sydow 1918). The first botryosphaeriaceous fungus was described in the 1820s as a species of *Sphaeria* (Fries) (Slippers and Wingfield 2007). The genus *Botryosphaeria* Ces. & De Not. was introduced in 1863 by Cesati and De Notaris to accommodate species transferred from the genera *Sphaeria* and *Gibbera* and is based on the type species *B*. *dothidea* (Moug. : Fr.) Ces. & De Not. (Barr 1972; Slippers et al. 2004). It included ascomycetous fungi producing typically uni- to multi-locular ascomata and bi-tunicate asci with hyaline, aseptate ascospores which may become brown with age. A number of anamorphs genera linked to *Botryosphaeria* have been described over the years. The taxonomy of genera and species in the *Botryosphaeriaceae* has relied for a long time on the morphology of sexual (Barr 1987, 1989; Hsieh and Chen 1994; Phillips et al. 2008) and asexual morphs (Denman et al. 2000; Crous et al. 2006; Phillips et al. 2008). However, the identification of genera and species based exclusively on morphological features of teleomorphs and anamorphs has led in the past to a proliferation of names. It is now clear that morphological characters alone are by no means sufficient to circumscribe genera within this family.

The advent of DNA sequencing methods has contributed significantly to unravelling the mixed up taxonomy of this fungal family. Over time, many genera have been reduced to synonymy, some other have been either removed to other families or newly introduced and some of the older genera have been resurrected. Denman et al. (2000) attempted to revise species in the genus *Botryosphaeria* and proposed to reduce anamorphs of *Botryosphaeria* into two groups, *Fusicoccum* and *Diplodia*. These two genera can essentially be differentiated on the basis of conidial morphology. Species in the genus *Fusicoccum* produce hyaline, fusiform, small (mainly <10 μm), thin-walled conidia, while *Diplodia* species are characterised by

having larger (mainly >10 μm), thick-walled conidia, becoming pigmented when mature. This taxonomic subdivision was supported by ITS rDNA phylogeny and several later studies accepted this view (Zhou and Stanosz 2001; Alves et al. 2004; Slippers et al. 2004). Crous et al. (2006) using DNA sequence data of the 28S rRNA gene combined with the analysis of morphological characters resolved 10 distinct lineages, each corresponding to a distinct genus within the *Botryosphaeriaceae*. In this study the authors restricted the name *Botryosphaeria* to only *B*. *dothidea* and *B*. *corticis* introducing the single-name nomenclatural system based on the use of a single genus name, typically referring to the asexual morph, regardless of whether a sexual morph is known or not. More recently, in an in-depth taxonomic study on phylogenetic lineages in the *Botryosphaeriales*, Slippers et al. (2013) evaluated the phylogenetic relationships of all the genera known from culture on the basis of DNA sequence data from six loci (SSU, LSU, ITS, EF1-α, β-tubulin and mtSSU). In this study the authors concluded that 17 genera, including *Barriopsis*, *Botryobambusa*, *Botryosphaeria*, *Cophinforma*, *Diplodia*, *Dothiorella*, *Endomelanconiopsis*, *Lasiodiplodia*, *Macrophomina*, *Neodeightonia*, *Neofusicoccum*, *Neoscytalidium*, *Phaeobotryon*, *Phaeobotryosphaeria*, *Pseudofusicoccum*, *Spencermartinsia* and *Tiarosporella*, can now be accepted within the *Botryosphaeriaceae*.

Ecology

Species in the *Botryosphaeriaceae* have a cosmopolitan distribution and are frequently reported associated with many woody hosts (Barr 1972; Punithalingam 1980; von Arx 1987; Slippers and Wingfield 2007). They were initially described as saprophytes on dead tissue of woody plants. Later studies on *Botryosphaeria* and its related anamorphs have demonstrated that these microorganisms are important pathogens associated with different disease symptoms such as leaf spots, twig blight, cankers, fruit rots, dieback and the death of whole plants on woody perennial crops, forest trees and ornamental plants (Phillips et al. 2013). The ability of *Botryosphaeriaceae* species to infect host plants through natural openings it has long been known. In addition, since the 1980s it has been recognised that *Botryosphaeriaceae* species are able to live endophytically on their hosts. Petrini and Fisher (1988) isolated *Diplodia*

sapinea (syn. *Sphaeropsis sapinea*), an important pathogen of several forest trees, from stem and xylematic tissue of asymptomatic *Pinus* trees. A few years later Johnson et al. (1992) isolated several fungal species belonging to the genera *Fusicoccum*, *Neofusicoccum*, *Pseudofusicoccum* and *Lasiodiplodia* from apparently healthy mango trees in Australia. Since then, several other species have been isolated and described as pathogens with an endophytic phase. As a consequence of the growing attention which has been addressed at studying these fungi in asymptomatic plants it is now clear that the *Botryosphaeriaceae* represent the dominant component of endophytic communities that live on different woody plants and, in particular, on *Pinus* and *Eucalyptus* species (Fisher et al. 1993; Smith et al. 1996a, b; Burgess et al. 2006). The exact ecological role of the *Botryosphaeriaceae* is still unclear for most of the species. Although several species are reported as pathogens, they do not always exhibit high levels of aggressiveness. *B*. *dothidea*, for example, has been reported as an aggressive pathogen on fruit trees and Pistachio in the USA (Michailides 1991; Ma et al. 2001, 2004), but it proved to be a weak pathogen on *Vitis*, *Eucalyptus* and *Syzygium* in South Africa (van Niekerk et al. 2004; Pavlic et al. 2007; Slippers et al. 2007). This may be due to the effect of environmental conditions and/or other abiotic factors. Several species belonging principally to the genus *Dothiorella* show low or insignificant levels of aggressiveness when artificially inoculated (van Niekerk et al. 2004; Pavlic et al. 2007). These species are sporadically associated with severe diseases. In fact it is still unclear what is the effective role that weakly or not pathogenic species play within natural communities. The latency period is often related to the occurrence of abiotic stress which can alter plant defence and favour the parasitic activity of some *Botryosphaeriaceae* (Schoeneweiss 1981; Swart and Wingfield 1991; Blodgett and Stanosz 1995; Desprez-Loustau et al. 2006). In particular, environmental stress that may promote infections by *Botryosphaeriaceae* species include: drought, hail, frost and wind injuries, lesions caused by other pathogens or insect pests, competition with other plants for water and mineral elements, plantations established in unsuitable environments (Zwolinski et al. 1995; Paoletti et al. 2001). In addition, several *Botryosphaeriaceae* species are known as aggressive pathogens, highly competitive and able to colonise plant host tissues very quickly. Symptoms may develop rapidly on infected trees resulting in serious damage on extensive

areas (Gezahgne et al. 2004; Linaldeddu et al. 2014). Because of their endophytic nature the exchange of asymptomatic plants represents an important means for the dissemination of these fungi which hampers the application of suitable control strategies against these pathogens. Therefore, it may be concluded that species belonging to the family *Botryosphaeriaceae* represent a serious threat to several agricultural and forest ecosystems.

Emerging *Botryosphaeriaceae* in agro-forestry ecosystems

Species in the *Botryosphaeriaceae* are well known as agents of cankers and dieback on many woody hosts of agricultural, forestry and ornamental importance. In the past it was thought that *Botryosphaeriaceae* were host-specific or, otherwise, able to infect a limited number of hosts. As a consequence, many species have been described on the basis of host-specificity. These include *Botryosphaeria quercuum* from *Quercus* sp., *Botryosphaeria ribis* from *Ribes* sp., *Botryosphaeria mali* from *Malus* spp., *Botryosphaeria vitis* from *Vitis* spp. (Slippers and Wingfield 2007). In contrast, recent studies have demonstrated that many species in the *Botryosphaeriaceae* have a low level of host-specificity and some species, such as *Diplodia seriata* and *Neofusicoccum parvum* are highly polyphagous (Phillips et al. 2013). However, some species are commonly associated with specific hosts or with specific plant families, such as *Diplodia sapinea* and *D. scrobiculata* on *Pinus* spp. and occasionally on other conifers, *N. eucalyptorum* and *N. eucalypticola* on *Eucalyptus* spp. and *N. protearum* on *Proteaceae* (De Wet et al. 2008). Furthermore, *Botryosphaeriaceae* are characterised by a high ecological adaptability, as their survival in a specific environment is not influenced by the presence of a specific host. Future climatic models concord for a gradual increase in global temperature together with extreme weather events (Coakley et al. 1999). In this context many pathogenic and invasive species of *Botryosphaeriaceae* may find optimal conditions to grow and cause serious damage (Desprez-Loustau et al. 2006; Linaldeddu et al. 2014).

Several species of *Botryosphaeriaceae* are currently recognised as the most important pathogens on grapevine throughout the world (Taylor et al. 2005). To date, more than 20 species in this family have been reported as occurring on grapevine (Úrbez-Torres 2011). Since the 1990s, *D. mutila* has been reported as the causal agent associated with the decline of

Fraxinus spp., especially *F. excelsior*, in Central, Eastern and Northern Europe. In addition, this pathogen has also been reported in association with cankers and branch dieback of *F. ornus* in Italy (Przybyl 2002; Sidoti and Granata 2004; Lygis et al. 2005; Pukacki and Przybyl 2005; Bakys et al. 2009). Likewise, several *Botryosphaeriaceae* species are involved in the aetiology of cankers and dieback of eucalypt trees in native and introduced areas (Webb 1983; Barnard et al. 1987; Shearer et al. 1987; Crous et al. 1989; Old et al. 1990; Fisher et al. 1993; Smith et al. 1994).

Nowadays *Botryosphaeriaceae* represent an emerging threat to agricultural and forestry ecosystems in Sardinia. In recent years epidemic attacks of *Diplodia* and *Neofusicoccum* species have gradually increased. These outbreaks have involved grapevine (Linaldeddu et al. 2010) as well as different forest trees such as holm oak and cork oak (Linaldeddu et al. 2007, 2009, 2014) and Juniper (Linaldeddu et al. 2011).

Aims of the thesis

On the basis of the aforementioned on *Botryosphaeriaceae*, and in consideration of the role played by these pathogens in the aetiology of many emerging diseases of woody plants in Sardinia, all characterised by a strong economic and ecological impact, it was considered appropriate in this PhD thesis to expand knowledge on five patho-systems by studying taxonomy, morphology, phylogeny and pathogenicity of the main species of *Botryosphaeriaceae* involved.

Reported below are the main results obtained for each of the five patho-systems studied which include the following hosts: grapevine, hazelnut, ash, eucalypt and tree heath.

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Chapter 2

Fungal Diversity
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Diversity of *Botryosphaeriaceae* species associated with grapevine and other woody hosts in Italy, Algeria and Tunisia, with descriptions of *Lasiodiplodia exigua* and *Lasiodiplodia mediterranea* sp. nov.

Abstract The diversity of *Botryosphaeriaceae* species associated with "Botryosphaeria dieback" of grapevine was investigated in 18 vineyards in Sardinia, Italy. *Lasiodiplodia* isolates obtained from different woody hosts including holm oak, sweet orange and broom bush in Italy, Algeria and Tunisia were also characterized. Morphological and cultural characteristics as well as ITS and EF1-α sequence data were used to identify the fungal isolates. Forty-eight botryosphaeriaceous isolates were obtained from 113 symptomatic grapevine samples, from which ten species were identified. *Diplodia seriata* was the dominant species (25 % of isolates), followed by *Neofusicoccum parvum* (21.7 %). Two species, *Diplodia olivarum* and *D. africana* are reported for the first time on grapevine. In addition, two new species namely *Lasiodiplodia mediterranea* sp. nov. from grapevine, holm oak and sweet orange and *Lasiodiplodia exigua* sp. nov. from broom bush are described. In artificial inoculation experiments conducted on excised green grapevine shoots and lignified canes as well as holm oak seedlings, *L. mediterranea* was shown to be an aggressive pathogen.

Keywords *Citrus* × *sinensis* - *Diplodia* - *Lasiodiplodia* - *Neofusicoccum* - *Quercus ilex* - *Retama raetam*

Introduction

During the last decades an increase in grapevine trunk diseases, due to attack by several fungal pathogens belonging mainly to the family *Botryosphaeriaceae*, has been reported in both traditional and emerging grape-producing countries worldwide (Larignon et al. 2001; Phillips 2002; van Niekerk et al. 2004; Úrbez-Torres et al. 2006; Luque et al. 2009; Pitt et al. 2010; Mohammadi et al. 2013; Mondello et al. 2013; Yan et al. 2013). Common external symptoms caused by infection of *Botryosphaeriaceae* on grapevine include leaf spots, leaf wilting, fruit rots, bud necrosis and perennial cankers which are often associated with a poor

vine growth, cordon dieback and sudden death of whole plant. Internal wood symptoms consist mainly of wedge-shaped necrotic sectors and brown stripes below the bark. The name "Botryosphaeria dieback" has recently been proposed to include all these decline-associated symptoms caused by species of *Botryosphaeriaceae* (Úrbez-Torres 2011). Similar to other grape-growing regions also in Sardinia (Italy), Botryosphaeria dieback represents a worrying problem for grape and wine production (Linaldeddu et al. 2010). In Sardinia, grapevine trunk diseases were originally linked to infections by *Eutypa lata* (Pers.) Tul. & C. Tul. and other fungi commonly associated with "esca" disease (Serra et al. 2010), but recent investigations have shown that in fact many species of *Botryosphaeriaceae* are directly involved in the aetiology of wood symptoms on trunks and cordons (Deidda et al. 2012). However, the information currently available about the occurrence, distribution and identity of the species of *Botryosphaeriaceae* associated with the different grapevine wood symptoms in Sardinia is still limited.

To date, at least 23 different taxa of *Botryosphaeriaceae* have been reported as weak or aggressive pathogens on grapevine worldwide, many of which have been described as new species during the last decade. Four species namely *Botryosphaeria dothidea* (Moug.) Ces. & De Not., *Diplodia seriata* De Not., *Neofusicoccum parvum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips and *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl. are usually recognised as the predominant species associated with grapevine cankers and dieback worldwide (Úrbez-Torres 2011). Recent studies, based on sequence data of the Internal Transcribed Spacers (ITS) of the ribosomal RNA cluster and part of the translation Elongation Factor 1-alpha (EF1-α) gene, have led to the identification of cryptic species within the *L. theobromae* species complex (Alves et al. 2008; Abdollahzadeh et al. 2010; Begoude et al. 2010; Liu et al. 2012; Úrbez-Torres et al. 2012). Currently, six species of *Lasiodiplodia*, including *L. crassispora* T.I. Burgess & Barber, *L. missouriana* Úrbez-Torres, Peduto & Gubler, *L. parva* A.J.L. Phillips, A. Alves & Crous, *L. pseudotheobromae* A.J.L. Phillips, A. Alves & Crous, *L. theobromae* and *L. viticola* Úrbez-Torres, Peduto & Gubler have been isolated from grapevine (Úrbez-Torres 2011; Úrbez-Torres et al. 2012; Correia et al. 2013; Yan et al. 2013). It is likely that over the years the name *L. theobromae* has been applied to

more than one species. In order to stabilize the name and allow its unambiguous application, a neotype specimen and an ex-neotype culture have recently been designated for *L. theobromae* (Phillips et al. 2013). *Lasiodiplodia theobromae* is a plurivorous pathogen with a worldwide distribution especially in tropical and subtropical regions where it occurs mainly on woody plants including fruit and forest trees (Mohali et al. 2005; Alves et al. 2008; Liu et al. 2012). Despite this, in Mediterranean countries few studies have focused on geographic distribution, host range or genetic variability of this pathogen and other species of *Lasiodiplodia*. *Lasiodiplodia theobromae* was previously reported associated with dieback of grapevine in Sicily (Italy) and Spain (Aroca et al. 2008; Burruano et al. 2008). In recent years, during surveys carried out in Algeria, Tunisia and Sardinia aimed at clarifying the causes of decline affecting different woody plants such as sweet orange (*Citrus×sinensis*), broom bush (*Retama raetam* (Forssk.) Webb & Berthel.) and holm oak (*Quercus ilex* L.), a large collection of *L. theobromae*-like strains were isolated from trees showing cankers and a progressive dieback of branches.

The main aims of the work described here were: 1) to study the species diversity and distribution of *Botryosphaeriaceae* associated with grapevine Botryosphaeria dieback in Sardinia; 2) to characterize a collection of *Lasiodiplodia* isolates obtained from different hosts and geographic origins in terms of morphological and phylogenetic relationships to all *Lasiodiplodia* species known from culture.

Materials and Methods

Sampling, fungal isolation and identification

From February 2010 to August 2013, 33 declining grapevine plants were collected from 18 vineyards of different ages representing nine of the most widely planted grapevine cultivars in Sardinia (Table 1). In addition, three samples from cankered branches of sweet orange collected in May 2013 in Algeria and fifteen samples from cankered branches of broom bush collected in Tunisia in June 2012 were processed and the results are included in this study. Furthermore, an unidentified *Lasiodiplodia* isolate obtained from a cankered branch of holm oak collected in May 2004 in Sardinia was studied.

Grapevine plants were brought to the laboratory to be inspected and symptomatic samples were initially cleaned of loose bark and then the outer bark surface tissue was cut away by a scalpel. Longitudinal and transversal cuts from symptomatic canes, cordons, and trunks were made to observe any internal symptom (Fig. 1). Isolations were made from chips of xylem tissues, approx. 5 mm^2, cut by a sterile scalpel from the margin of necrotic lesions. All chips were cultured on potato dextrose agar (PDA, Oxoid Ltd.) in Petri dishes. After incubation at 25 °C for 1 week, fungal colonies were sub-cultured onto half-strength PDA or on water agar supplemented with autoclaved poplar or holm oak twigs to enhance sporulation. All colonies were kept on the laboratory bench at about 20–25 °C where they received diffused daylight. Putative botryosphaeriaceous isolates were identified by reference to the keys and descriptions data provided in Phillips et al. (2013).

Monoconidial cultures were obtained by spreading conidia on the surface of PDA and incubating overnight at 25 °C. Individual germinating conidia were transferred to fresh plates of PDA. Representative isolates of each species were stored on PDA slants under oil in the culture collection of the Sez. di Patologia vegetale ed Entomologia, Dipartimento di Agraria, at the University of Sassari. In addition, three strains of the two new *Lasiodiplodia* species were also deposited at the Centraalbureau voor Schimmelcultures (CBS), Utrecht, the Netherlands and nomenclatural data in MycoBank (Crous et al. 2004). Specimens were lodged with the herbarium of Estação Agronómica Nacional, Oeiras, Portugal (LISE).

Table 1. Geographic origin and number of symptomatic grapevine samples from different cultivars processed in this study.

Locality	Site	Cultivar	Number of plants	Number of samples
Santa Maria La Palma	a	Cannonau	1	7
		Carignano	1	5
		Monica	1	10
	b	Vermentino	1	5
Dolianova	a	Vermentino	5	8
	b	Vermentino	4	10
Badesi	a	Cannonau	1	6
		Regina	1	8
	b	Cannonau	1	5
Oliena	a	Cannonau	2	6
Orgosolo	a	Cannonau	2	4
Sorgono	a	Cabernet Sauvignon	1	1
	b	Cannonau	1	5
	c	Monica	1	3
	d	Monica	2	1
	e	Muristellu	1	3
	f	Monica	1	4
Ula Tirso	a	Barbera	1	5
	b	Cannonau	1	2
Neoneli	a	Barbera	1	2
		Muristellu	1	5
		Pascale	1	3
	b	Muristellu	1	5

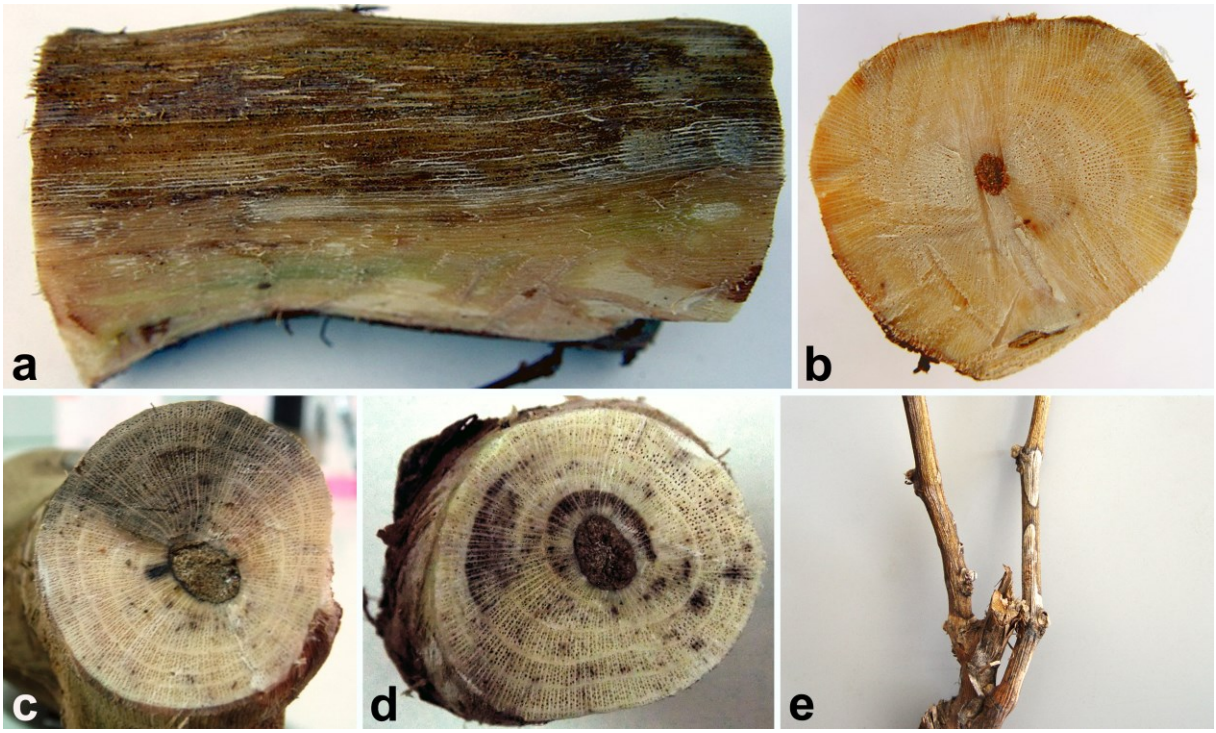

Figure 1. Symptoms observed on investigated grapevine plants: trunk sample showing brown stripes, visible after bark removal (a) and in cross section (b); cross section of a cordon showing a characteristic wedge shaped necrotic sector (c); black spots visible in a cross-sectioned arm (d); necrotic lesions around bleached areas on mature canes (e).

Morphology and cultural characteristics

For the new species described here, colony growth characteristics, including surface and reverse colony appearance were recorded after 7 days of incubation at 25 °C in the dark on PDA. Cardinal temperatures for growth were determined on PDA plates incubated at 5, 10, 15, 20, 25, 30, 35 and 40 °C (±0.5 °C) in the dark. Five replicate plates for each isolate were made and colony diameters were measured after 4 days.

For microscopy, the contents of conidiomata were dissected out and mounted in 100 % lactic acid. Measurements of conidiogenous cells, conidia and paraphyses were made with the Leica IM 500 measurement module from images recorded on a Leica DFC 320 digital camera. From measurements of 50 conidia the mean, standard deviation and 95 % confidence intervals were calculated. Spore dimensions are presented as mean values with extreme values in

parentheses. Dimensions of other structures are given as mean of at least 20 measurements.

DNA extraction, PCR amplification and sequencing

Following morphological identification, a subset of isolates of each species of *Botryosphaeriaceae* obtained in this study was selected for DNA sequence analysis. Instagene Matrix (BioRad Laboratories, Hercules, CA) was used to extract genomic DNA from 5-day-old cultures grown on PDA and incubated at 25 °C. The ITS region was amplified and sequenced with primers ITS1 and ITS4 (White et al. 1990), while the primers EF446f and EF1035r (Inderbitzin et al. 2005, 2010) were used to amplify and sequence part of the EF1-α gene. Polymerase chain reaction (PCR) mixtures and amplification conditions were conducted as described by Linaldeddu et al. (2013). The PCR products were purified using the EUROGOLD gel extraction kit (EuroClone S.p.A.) following manufacturer's instructions. ITS and EF1-α regions were sequenced in both directions by the BMR Genomics DNA sequencing service (www.bmrgenomics.it). The nucleotide sequences were read and edited with FinchTV 1.4.0 (Geospiza, Inc.; http://www.geospiza.com/finchtv) and then compared with reference sequences retrieved from GenBank in BLAST searches (Altschul et al. 1990). Nucleotide sequences of additional isolates included in this study were retrieved from GenBank (Table 2).

Table 2. *Lasiodiplodia* isolates included in this study.

Isolate number[a]	Species	Host	Locality	Collector	GenBank	
					ITS[b]	EF
CBS 124707[c]	*Lasiodiplodia citricola*	*Citrus* sp.	Iran	Abdollahzadeh & Javadi	*GU945354*	*GU945340*
CBS 124706	*L. citricola*	*Citrus* sp.	Iran	A.Shekari	*GU945353*	*GU94533*
CBS 118741	*L. crassispora*	*Santalum album*	Australia	Burgess & Dell	*DQ103550*	*EU673303*
CBS 110492	*L. crassispora*	Unknown	Unknown	Unknown	*EF622086*	*EF622066*
CBS 130992	*L. egyptiacae*	*Mangifera indica*	Egypt	A.M. Ismail	*JN814397*	*JN814424*
BOT-29	*L. egyptiacae*	*Mangifera indica*	Egypt	A.M. Ismail	*JN814401*	*JN814428*
CMM 3609	*L. euphorbiicola*	*Jatropha curcas*	Brazil	Machado & Pereira	*KF234543*	*KF226689*
CMM 3651	*L. euphorbiicola*	*Jatropha curcas*	Brazil	Machado & Pereira	*KF234553*	*KF226711*
CMM 3652	*L. euphorbiicola*	*Jatropha curcas*	Brazil	Machado & Pereira	*KF234554*	*KF226715*
CBS 137785	*L. exigua*	*Retama raetam*	Tunisia	B.T. Linaldeddu	KJ638317	KJ638336
BL 184	*L. exigua*	*Retama raetam*	Tunisia	B.T. Linaldeddu	KJ638318	KJ638337
BL 185	*L. exigua*	*Retama raetam*	Tunisia	B.T. Linaldeddu	KJ638319	KJ638338
BL 186	*L. exigua*	*Retama raetam*	Tunisia	B.T. Linaldeddu	KJ638320	KJ638339
BL 187	*L. exigua*	*Retama raetam*	Tunisia	B.T. Linaldeddu	KJ638321	KJ638340
PD161	*L. exigua*	*Pistacia vera*	USA	Cook & PI	*GU251122*	*GU251254*
CBS 124704	*L. gilanensis*	Unknown	Iran	Abdollahzadeh & Javadi	*GU945351*	*GU945342*
CBS 124705	*L. gilanensis*	Unknown	Iran	Abdollahzadeh & Javadi	*GU945352*	*GU945341*
CBS 115812	*L. gonubiensis*	*Syzygium cordatum*	South Africa	D. Pavlic	*AY639595*	*DQ103566*
CBS 116355	*L. gonubiensis*	*Syzygium cordatum*	South Africa	D. Pavlic	*AY639594*	*DQ103567*
CBS 124709	*L. hormozganensis*	*Olea* sp.	Iran	Abdollahzadeh & Javadi	*GU945355*	*GU945343*
CBS 124708	*L. hormozganensis*	*Mangifera indica*	Iran	Abdollahzadeh & Javadi	*GU945356*	*GU945344*
CBS 124710	*L. iraniensis*	*Mangifera indica*	Iran	N. Khezrinejad	*GU945346*	*GU945334*
CBS 124711	*L. iraniensis*	*Juglans* sp.	Iran	A. Javadi	*GU945347*	*GU945335*
CMM 3610	*L. jatrophicola*	*Jatropha curcas*	Brazil	Machado & Pereira	*KF234544*	*KF226690*
CMM 3833	*L. macrospora*	*Jatropha curcas*	Brazil	Machado & Pereira	*KF234557*	*KF226718*
CBS 124925	*L. mahajangana*	*Terminalia catappa*	Madagascar	J. Roux	*FJ900597*	*FJ900643*
CBS 124927	*L. mahajangana*	*Terminalia catappa*	Madagascar	J. Roux	*FJ900595*	*FJ900641*
CBS122519	*L. margaritacea*	*Adansonia gibbosa*	Western Australia	T.I. Burgess	*EU144050*	*EU144065*
CBS 122065	*L. margaritacea*	*Adansonia gibbosa*	Western Australia	T.I. Burgess	*EU144051*	*EU144066*
CBS 137783	*L. mediterranea*	*Quercus ilex*	Italy	B.T. Linaldeddu	KJ638312	KJ638331
CBS 137784	*L. mediterranea*	*Vitis vinifera*	Italy	S. Serra	KJ638311	KJ638330

CAD 012	*L. mediterranea*	*Vitis vinifera*	Italy	A. Deidda	KJ638313	KJ638332
ALG 36	*L. mediterranea*	*Citrus sinensis*	Algeria	A. Berraf-Tebbal	KJ638314	KJ638333
ALG 40	*L. mediterranea*	*Citrus sinensis*	Algeria	A. Berraf-Tebbal	KJ638316	KJ638335
ALG 41	*L. mediterranea*	*Citrus sinensis*	Algeria	A. Berraf-Tebbal	KJ638315	KJ638334
CBS128311	*L. missouriana*	*Vitis* sp.	USA	Striegler & Leavitt	*HQ288225*	*HQ288267*
CBS 128312	*L. missouriana*	*Vitis* sp.	USA	Striegler & Leavitt	*HQ288226*	*HQ288268*
CBS 456.78	*L. parva*	Cassava field-soil	Colombia	O. Rangel	*EF622083*	*EF622063*
CBS 494.78	*L. parva*	Cassava field-soil	Colombia	O. Rangel	*EF622084*	*EF622064*
CBS 120832	*L. plurivora*	*Prunus salicina*	South Africa	U. Damm	*EF445362*	*EF445395*
CBS 121103	*L. plurivora*	*Vitis vinifera*	South Africa	F. Halleen	*AY343482*	*EF445396*
CBS 116459	*L. pseudotheobromae*	*Gmelina arborea*	Costa Rica	J. Carranza-Velásquez	*EF622077*	*EF622057*
CBS 447.62	*L. pseudotheobromae*	*Citrus aurantium*	Suriname	C. Smulders	*EF622081*	*EF622060*
CBS118740	*L. rubropurpurea*	*Eucalyptus grandis*	Australia	Burgess & Pegg	*DQ103553*	*EU673304*
WAC 12536	*L. rubropurpurea*	*Eucalyptus grandis*	Australia	Burgess & Pegg	*DQ103554*	*DQ103572*
CMM 3872	*L. subglobosa*	*Jatropha curcas*	Brazil	Machado & Pereira	*KF234558*	*KF226721*
CMM 4046	*L. subglobosa*	*Jatropha curcas*	Brazil	Machado & Pereira	*KF234560*	*KF226723*
CBS 164.96	*L. theobromae*	Fruit along coral reef coast	Papua New Guinea	A. Aptroot	*AY640255*	*AY640258*
CBS 111530	*L. theobromae*	Unknown	Unknown	Unknown	*EF622074*	*EF622054*
CBS118739	*L. venezuelensis*	*Acacia mangium*	Venezuela	S. Mohali	*DQ103547*	*DQ103568*
WAC 12540	*L. venezuelensis*	*Acacia mangium*	Venezuela	S. Mohali	*DQ103548*	*DQ103569*
CBS128313	*L. viticola*	*Vitis* sp.	USA	Cartwright & Gubler	*HQ288227*	*HQ288269*
CBS 128315	*L. viticola*	*Vitis* sp.	USA	Striegler & Gubler	*HQ288228*	*HQ288270*
CBS 136015	*D. mutila*	*Populus alba*	Portugal	A. Alves	*KJ361838*	*KJ361830*
CBS 112555	*D. seriata*	*Vitis vinifera*	Portugal	A.J.L. Phillips	*AY259094*	*AY573220*

[a]Acronyms of culture collections: ALG, Personal culture collection A. Berraf-Tebbal; BL, B.T. Linaldeddu, Università degli Studi di Sassari, Italy; CAD, A. Deidda, Università degli Studi di Sassari, Italy; CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CMM, Culture Collection of Phytopathogenic Fungi "Prof. Maria Menezes", Universidade Federal Rural de Pernambuco, Recife, Brazil; MFLUCC, Mae Fah Luang University Culture Collection, Chiang Rai, Thailand; WAC, Department of Agriculture, Western Australia Plant Pathogen Collection, South Perth, Western Australia

[b]Sequence numbers in italics were retrieved from GenBank. All others were obtained in the present study

[c]Ex-type strains in bold face

Phylogenetic analysis

The ITS and EF1-α sequences of *Lasiodiplodia* isolates obtained in this study were combined and the dataset, including sequences of 21 other species of *Lasiodiplodia* downloaded from GenBank, was compiled with the outgroups *Diplodia mutila* (Fr.) Fr. and *D. seriata* (Table 2). Sequences were aligned with ClustalX v. 1.83 (Thompson et al. 1997), using the default parameters. Alignments were checked and manual adjustments were made where necessary. Incomplete portions at either end of the alignment and the ambiguously aligned portion spanning the first 60 bases of the EF1-α locus were excluded from the analyses. A comparison of highly supported clades (bootstrap support values≥70 %) among trees generated from Maximum likelihood (ML) analyses of individual data sets was performed in order to detect conflict between individual phylogenies (Alves et al. 2008).

Maximum likelihood analyses were done using RAxML (Stamatakis 2006) on the webserver (Stamatakis et al. 2008) at http://phylobench.vital-it.ch/raxml-bb.php with the gamma model of rate heterogeneity in effect and maximum likelihood search. Bayesian analyses were done with Mr Bayes v.3.0b4 (Ronquist and Huelsenbeck 2003) employing a Markov Chain Monte Carlo (MCMC) method. The general time-reversible model of evolution (Rodriguez et al. 1990), including estimation of invariable sites and assuming a discrete gamma distribution with six rate categories was used. Four MCMC chains were run simultaneously, starting from random trees for 106 generations. Trees were sampled every 100th generation for a total of 104 trees. The first 103 trees were discarded as the burn-in phase of each analysis. Posterior probabilities (Rannala and Yang 1996) were determined from a 50 % majority-rule consensus tree generated with the remaining 9,000 trees. This analysis was repeated three times starting from different random trees to ensure trees from the same tree space were sampled during each analysis. Trees were visualized with TreeView (Page 1996).

Pathogenicity tests

Pathogenicity of two *Lasiodiplodia mediterranea* strains (BL1 and BL101) was verified by inoculating ten 2-year-old holm oak seedlings (strain BL1) and five excised grapevine shoots

and five lignified canes from cv. Cannonau (strain BL101). A mycelial plug (3–4 mm^2) taken from the margin of an actively growing colony on PDA was placed in a shallow wound (~3 mm) made with a scalpel at the middle of each shoot and cane and at the stem base of each seedling. The inoculation point was covered with cotton wool soaked in sterile water and wrapped with Parafilm®. The inoculated shoots were placed in a beaker containing 200 mL of sterile distilled water and then enclosed in a transparent plastic bag for 12 days, whereas the bottom and top end of each cane was sealed with a synthetic grafting resin to prevent drying and contamination and then enclosed in a transparent plastic bag for 50 days. Inoculated grapevine samples were kept in the laboratory in daylight and at 18–26 °C. Inoculated seedlings were watered every 3 days and kept in the laboratory for 2 months. Five grapevine shoots and canes and ten holm oak seedlings inoculated with a PDA plug were used as control. At the end of each experiment, re-isolation was attempted by transferring to PDA 10 surface-sterilized pieces of inner bark and xylem tissue taken around the margin of each lesion on grapevine samples and stem of holm oak seedlings.

Results

Botryosphaeriaceous species associated with symptomatic grapevines

Wedge-shaped necrotic sectors on cordon and trunk represented the most frequent symptom detected (58.5 % of the samples processed) on investigated grapevine plants. Black stripes in the wood was the second most frequent symptom (23 %) followed by black spots in the wood (11.5 %) and necrotic cane lesions (7 %). From 113 grapevine samples processed, 48 botryosphaeriaceous isolates representing 10 distinct species namely *Botryosphaeria dothidea*, *Diplodia africana* Damm & Crous, *D. mutila*, *D. olivarum* A.J.L. Phillips, Frisullo & Lazzizera, *D. seriata*, *Lasiodiplodia mediterranea* sp. nov., *Neofusicoccum australe* (Slippers, Crous & M.J. Wingf.) Crous, Slippers & A.J.L. Phillips, *N. cryptoaustrale* Pavlic, Maleme, Slippers & M.J. Wingf., *N. luteum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips and *N. parvum* were isolated and identified from the 6 grapevine cultivars and 18 sites sampled (Table 3).

Table 3. GenBank accession numbers, number of samples of the different cultivars and sites from which each pathogen was isolated.

Species	GenBank accession no.		Symptoms														No. sites
			V-shaped necrosis					Brown stripe					Black spot	Cane lesions			
	ITS	EF1-α	Cn	Cr	Mo	Mu	Ve	Ba	Cn	Mo	Mu	Ve	Ba	Cn	Cr	Ve	
B. dothidea CAD018	KJ638322	KJ790254	4	-	-	1	-	-	1	-	1	-	-	-	-	-	**3**
D. africana CAD014	KJ638326	KJ638344	-	-	-	-	-	-	-	-	-	-	-	-	-	1	**1**
D. mutila BL98	KF307718	KF318765	-	-	-	-	-	-	-	-	-	-	-	1	-	-	**1**
D olivarum CAD019	KJ638323	KJ638341	-	-	1	-	-	-	-	-	-	-	-	-	-	-	**1**
D. seriata CAD020	KJ638324	KJ638342	3	-	-	2	1	1	2	2	-	1	-	-	-	-	**9**
L. mediterranea BL101	KJ638311	KJ638330	5	-	-	-	-	-	2	-	-	-	-	-	-	-	**1**
N. australe CAD021	KJ638325	KJ638343	-	-	3	-	-	-	-	-	-	-	-	-	-	1	**2**
N. cryptoaustrale CAD023	KJ638328	KJ638346	1	-	-	-	-	-	-	-	-	-	-	-	-	-	**1**
N. luteum CAD022	KJ638327	KJ638345	-	1	-	-	-	-	-	-	-	-	-	-	2	-	**1**
N. parvum CAD024	KJ638329	KJ790253	3	-	4	-	-	1	1	1	-	-	1	-	-	-	**4**

[a] Ba = Barbera; Cn = Cannonau; Cr = Carignano; Mo = Monica; Mu = Muristellu; Ve = Vermentino

The identity of isolates of each species was confirmed by analysis of ITS and EF1-α sequences. For all species BLAST searches in GenBank showed 99–100 % similarity with reference sequences of representative strains including those of ex-type isolates. New sequences were deposited in GenBank (Table 3).

Neofusicoccum was the genus most frequently isolated: 16.8%of samples from 8 sites, whereas *D. seriata* was the most frequently isolated species (10.6 % of samples from 9 sites), followed by *N. parvum* (9.7 % of samples from 4 sites). These two species together with *B. dothidea* and *L. mediterranea* (here described as a new species) were the only species associated with both V-shaped necrosis and brown vascular stripes on cordons. Isolations from V-shaped necrotic sectors overall yielded 8 botryosphaeriaceous species (Table 3). However, apart from *D. seriata* and *N. parvum*, the other 6 species were isolated only in one site. In particular, *L. mediterranea* was obtained from all samples collected in one vineyard located in the north of Sardinia. From sectioned cordons showing black spots the only species isolated was *N. parvum*, no other fungal pathogens such as *Phaeoacremonium* spp. and *Phaeomoniella chlamydospora* (W. Gams, Crous, M.J. Wingf. & Mugnai) Crous & W. Gams, typically associated with this grapevine symptom, were obtained. Four species, namely *D. africana*, *D. mutila*, *N. australe* and *N. luteum* were isolated from cane samples showing inner bark necrotic lesions.

DNA phylogeny of *Lasiodiplodia* isolates

Fragments of approximately 500 and 300 bases were determined for ITS and EF1-α regions, respectively. New sequences were deposited in GenBank (Table 2) and the alignment in TreeBase (15565). Individual gene phylogenies revealed no major conflicts thus indicating that the two loci could be combined. The combined ITS and EF1-α dataset consisted of 850 characters (including alignment gaps) for 53 ingroup and 2 outgroup taxa. ML and Bayesian analyses generated trees with essentially the same topology (TreeBase 15565). The ML tree is shown in Fig. 2 with ML bootstrap support values and Bayesian posterior probability scores at the nodes. In the phylogenetic analysis 22 clades corresponding to species were recognized. Of these, 20 included all *Lasiodiplodia* spp. previously known from culture and for which

molecular data are available (Fig. 2). The other two well supported clades included the *Lasiodiplodia* isolates obtained in this study.

The first clade (ML bootstrap=98 %, posterior probability= 1.00) containing the *Lasiodiplodia* isolates from broom bush in Tunisia and pistachio (*Pistacia vera* L.) in the USA represents a previously unrecognized species, which is described here as *Lasiodiplodia exigua* sp. nov. The second clade (ML bootstrap=90 %, posterior probability=0.86) including the *Lasiodiplodia* isolates obtained from grapevine and holm oak in Italy and sweet orange in Algeria was considered to represent a further distinct species, which is described here as *Lasiodiplodia mediterranea* sp. nov. The isolate of *Lasiodiplodia jatrophicola* A.R. Machado & O.L. Pereira (CMM 3610), a species recently described by Machado et al. (2014), clustered in the *Lasiodiplodia iraniensis* Abdollahz., Zare & A.J.L. Phillips clade.

Figure 2. Maximum likelihood tree resulting from the combined analysis of ITS and EF1-α sequence data. ML Bootstrap support values and Bayesian posterior probability scores are given at the nodes. The tree was rooted to *Diplodia mutila* and *Diplodia seriata*. Ex-type isolates are in bold. The scale bar represents the number of substitutions per site.

Taxonomy

Lasiodiplodia euphorbiicola A.R. Machado & O.L. Pereira, Fungal Diversity 67:231–247

MycoBank: MB 804872 *Lasiodiplodia euphorbicola* A.R. Machado & O.L. Pereira, Fungal Diversity 67:231–247

Notes: Orthography of the epithet is herein corrected.

Lasiodiplodia exigua Linaldeddu, Deidda & A.J.L. Phillips sp. nov.

MycoBank: MB 808355 (Fig. 3)

Etymology: in reference to the small conidia.

Sexual state: Not seen. Asexual state: Conidiomata pycnidial formed on poplar twigs in culture within 3–4 weeks, solitary and covered by mycelium, dark brown to black. Paraphyses hyaline, cylindrical, mostly septate, ends rounded, 80.1±19×2.9±0.5 µm (mean±S.D., n=20). Conidiogenous cells 15.6±3.2×4.2±1 µm (mean±S.D., n=20), hyaline, smooth, cylindrical, sometimes slightly swollen at the base, holoblastic forming conidia at their tips. Conidia ellipsoid to ovoid, apex and base rounded, thick-walled, initially hyaline and aseptate, becoming one septate and then dark brown with age, with longitudinal striations (19.6–)21.8(−24.3)×(10.8–)12.3(−13.3) µm, 95 % confidence limits=21.5–22.1×12.1–12.4 µm (mean±S.D. = 21.8±1.1×12.3±0.5 µm, l/w ratio=1.8±0.1).

Cultural characteristics: Colonies initially white to light-brown with fluffy, aerial mycelium, becoming olivaceous-grey on the surface after 3–4 days; reverse side of the colonies dark-brown.

Cardinal temperatures for growth: Minimum<10 °C, maximum <40 °C and optimum 25–30 °C, covering the medium surface (90 mm) before 7 days in the dark.

Habitat: Twigs and branches of *Retama raetam* and *Pistacia vera*.

Known geographic distribution: Tunisia and Arizona (USA).

Specimens examined: TUNISIA, Nabeul, isolated from a branch canker of *Retama raetam*, 27 June 2012, Benedetto T. Linaldeddu, HOLOTYPE LISE 96302, a dried culture sporulating on *Quercus ilex* twigs, culture ex-holotype CBS 137785=BL104. Other isolates examined are

listed in Table 2.

Notes: Phylogenetically *L. exigua* is closely related to *Lasiodiplodia mahajangana* Begoude, Jol. Roux & Slippers, but can easily be distinguished on average conidial dimensions and l/w ratio. Moreover, average size of the septate paraphyses of *L. exigua* are 80.1×2.9 µm, whereas aseptate paraphyses of *L. mahajangana* are 43×3 µm.

Lasiodiplodia mediterranea Linaldeddu, Deidda & Berraf-Tebbal sp. nov.

MycoBank: MB 808356 (Fig. 4).

Etymology: Named for the Mediterranean region where this fungus was isolated for the first time.

Sexual state: Not seen. Asexual state: Conidiomata pycnidial formed on poplar twigs in culture within 2–3 weeks, uniloculate, dark brown to black, immersed in the host becoming erumpent when mature. Paraphyses hyaline, cylindrical, septate, sometimes branched, ends rounded, measuring 87±19.9×2.7±0.6 µm (mean±S.D., n=20). Conidiogenous cells 13.6±2.2×3.7±1 µm (mean±S.D., n=20), hyaline, smooth, cylindrical, sometimes slightly swollen at the base, holoblastic forming conidia at their tips, proliferating internally giving rise to periclinal thickenings. Conidia subcylindrical to elliptical, apex and base rounded, typically widest at the middle, thick-walled, initially hyaline and aseptate and remaining so for a long time, becoming one or two-septate and dark brown with age, with longitudinal striations (26.3–)30.6(−37)×(13.5–)16.1(−18) µm, 95 % confidence limits=30–31.1×15.9–16.3 µm (mean±S.D. = 30.6± 2.8×16.1±0.9 µm, l/w ratio=1.9±0.2).

Cultural characteristics: Colonies on PDA grew rapidly, reaching 90 mm in diameter before 7 days at 25 °C, the mycelium was moderately aerial, surface white at first and later turned pale to dark grey from the centre to the margin and greyish to dark in reverse. Isolates growing at 35 °C produced a diffusible pink pigment within 3 days (Fig. 4).

Cardinal temperatures for growth: Minimum <10 °C, maximum <40 °C and optimum 25–30 °C.

Habitat: On trunk and branches of *Vitis vinifera*, *Quercus ilex* and *Citrus×sinensis*.

Known distribution: Italy and Algeria

Specimens examined: ITALY, Bortigiadas, isolated from a branch canker of *Quercus ilex*, June 2004, Benedetto T. Linaldeddu, HOLOTYPE LISE 96303, a dried culture sporulating on *Quercus ilex*, culture ex-holotype CBS 137783=BL1. ITALY, Badesi, isolated from a brown stripe under the bark on *Vitis vinifera*, 11 February 2010, Salvatorica Serra (culture BL101=CBS 137784). Other isolates examined are listed in Table 2.

Notes: *Lasiodiplodia mediterranea* is phylogenetically closely related to *L. pseudotheobromae*, but can be distinguished based on the shape and dimensions of conidia and paraphyses.

Figure 3. *Lasiodiplodia exigua*: colony morphology of *L. exigua* after 7 days growth at 25 °C on PDA (a); septate paraphyses (b); conidia developing on conidiogenous cells (c); hyaline thick-walled conidia (d); hyaline aseptate conidia and one septate conidium (e); hyaline conidium and one pale brown aseptate conidium (f); aged and one septate conidium in two different focal planes to show the longitudinal striations (g-h). Bars=10 μm.

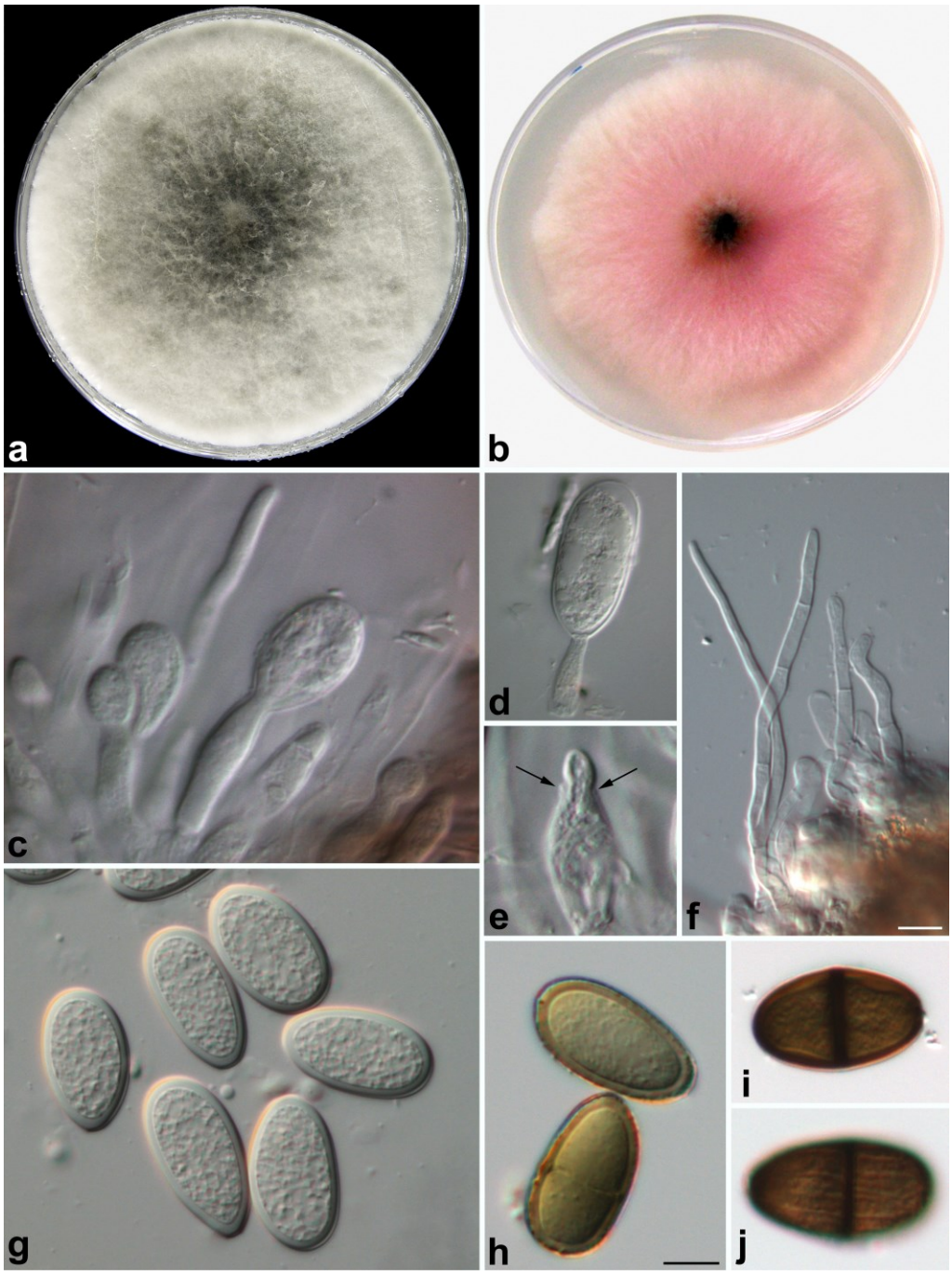

Figure 4. *Lasiodiplodia mediterranea*: colony morphology of *L. mediterranea* after 7 days growth at 25 °C on PDA (a); colony showing typical pink pigmentation at 35 °C on PDA (b); conidia developing on conidiogenous cells (c-d); conidiogenous cell with periclinal thickenings (arrowed) (e); septate paraphyses (f); hyaline thick-walled conidia (g); aseptate and septate light brown conidia (h); aged one septate conidium in two different focal planes to show the longitudinal striations (i-j). Bars=10 µm.

Pathogenicity tests

Pathogenicity of *L. mediterranea* was verified by wound inoculation of excised grapevine shoots and lignified canes as well as holm oak seedlings under controlled laboratory conditions.

Twelve days after inoculation, all grapevine shoots inoculated with the pathogen displayed dark-brown to black discoloration on bark and vascular tissues, measuring 10.3±3 cm (mean±S.D.). Fifty days after inoculation, the lignified canes displayed dark-brown to black discoloration on bark and vascular tissues, measuring 8.1±1.8 cm (mean±S.D.). In cross section all canes showed a wedge-shaped necrotic sector. On holm oak seedlings *L. mediterranea* caused extensive necrotic lesions, which often girdled the stem, causing leaf chlorosis and in most cases wilting of the distal portion of the canopy. Wood necrosis on stems measured 8.3±2.7 cm (mean±S.D.). The wilted seedlings reacted by producing new shoots below the point of inoculation. Artificially obtained symptoms were congruent with field observations. The pathogen was successfully re-isolated from the margin of all symptomatic tissues, thus fulfilling Koch's postulates. Control grapevine shoots and canes and holm oak seedlings inoculated with sterile PDA plugs remained symptomless.

Discussion

There has been much recent phylogenetic and morphological study on genera of the *Botryosphaeriaceae* and it is now relatively easy to identify taxa to genera and species (Phillips et al. 2008; 2013; Liu et al. 2012). Thus the studies of *Botryosphaeriaceae* on various hosts has multiplied. The present study represents the first survey aimed at studying the occurrence and diversity of species of *Botryosphaeriaceae* associated with grapevine in Sardinia. The results obtained have given new insights into the complex aetiology associated with Botryosphaeria dieback. Morphological studies and DNA sequence analyses allowed us to identify 10 different botryosphaeriaceous species from V-shaped necrotic sectors, brown vascular stripes, black spots and cane necrotic lesions, including *Botryosphaeria dothidea*, *Diplodia africana*, *D. mutila*, *D. olivarum*, *D. seriata*, *Lasiodiplodia mediterranea* sp. nov., *Neofusicoccum australe*, *N. cryptoaustrale*, *N. luteum* and *N. parvum*. All species found in this

study, except *D. africana*, *D. olivarum* and *L. mediterranea*, which are reported for the first time on grapevine, have been detected in other grape-growing areas worldwide, and are associated with a broad range of grapevine disease symptoms including leaf spots, fruit rot, shoot dieback, bud necrosis, vascular discoloration of the wood and perennial cankers (Úrbez-Torres 2011).

Diplodia seriata was the dominant species, sampled from nine sites and five grapevine cultivars, followed by *Neofusicoccum parvum* and *Botryosphaeria dothidea*. The high frequency of isolation of *Diplodia seriata* obtained in this study is in accordance with results of previous studies conducted in France (Larignon et al. 2001), Australia (Taylor et al. 2005; Pitt et al. 2010), Spain (Luque et al. 2009) and Chile (Morales et al. 2012) where this pathogen was found as the dominant species isolated from symptomatic grapevine samples. Although *D. seriata* has been reported from a wide range of grapevine cultivars worldwide, there are conflicting reports regarding its pathogenicity on this host: in particular, it has been considered to be a primary pathogen by Larignon et al. (2001) in France, Auger et al. (2004) in Chile and van Niekerk et al. (2004) in South Africa, as a secondary pathogen by Phillips (1998) in Portugal and Úrbez-Torres and Gubler (2009) in California and as not pathogenic in Australia by Taylor et al. (2005). These conflicting data may be a result of differences in inoculation methods and experimental conditions, susceptibility among the various grapevine cultivars, age and type of host tissue but may also be due to differences in strain virulence. In this regard, Larignon et al. (2001) found significant differences in the mean lesion lengths caused on 1-year-old canes by ten strains of *D. seriata* used in a pathogenicity assay.

Apart from *Diplodia seriata*, *Neofusicoccum parvum* and *Botryosphaeria dothidea* the other seven species of *Botryosphaeriaceae* obtained in this study were, in most cases, isolated from a single site each thus suggesting that various site-specific conditions (microclimate conditions, source of propagation material and the occurrence of alternative hosts surrounding the vineyard) may influence the presence of these species within vineyards. This aspect is supported by the fact that all of the species isolated in this study are polyphagous and some of them are known to be able to infect several forest trees such as cork oak, holm oak and juniper in Sardinia (Linaldeddu et al. 2006, 2007, 2011, 2014). In addition, given the low frequency of

isolation of these seven species, at the moment it is not possible to establish the exact role that they play in the aetiology of Botryosphaeria dieback in Sardinia, or their possible synergistic interaction.

In this study 22 clades were resolved within *Lasiodiplodia*. Species names are available for 20 of these clades, for the other two clades, which represent two new species obtained from different woody hosts in Italy, Algeria and Tunisia the names *L. mediterranea* and *L. exigua* are introduced here.

Lasiodiplodia mediterranea was found associated with grapevine V-shaped necrotic sectors. To date, six species of *Lasiodiplodia* have been associated with grapevine wood diseases (Úrbez-Torres et al. 2012; Correia et al. 2013; Yan et al. 2013). All six species were chiefly linked to cankers and wood symptoms according to results obtained in this study for *L. mediterranea*. Phylogenetically, *L. mediterranea* is closely related to *L. pseudotheobromae* but the two species differed in three bp in ITS and nine bp in EF1-α. Morphologically *L. mediterranea* resembles *L. macrospora* A.R. Machado & O.L. Pereira, a species recently described in Brazil on *Jatropha curcas* L. (Machado et al. 2014). However, *L. mediterranea* can be distinguished from other species on the basis of its larger conidia (Table 4), and the size of its septate and branched paraphyses. Besides grapevine, *L. mediterranea* has also been isolated from a cankered branch of holm oak in Sardinia and cankered branches of sweet orange in Algeria indicating the polyphagous nature of this new *Lasiodiplodia* species.

The second clade, which includes isolates from broom bush in Tunisia and one isolate from pistachio in the USA previously identified as *L. theobromae* (strain PD161), represents a previously unrecognized *Lasiodiplodia* species, which we described here as *L. exigua* sp. nov. Although this species is phylogenetically closely related to *L. mahajangana*, it is easily separated by its larger conidia (av. = 21.8×12.3 µm) as compared with *L. mahajangana* (av. = 17.5×11.5 µm). Because it was impossible in this study to obtain broom bush seedlings the pathogenicity of *L. exigua* was not assessed and thus Koch's postulates have not been satisfied. The data presented here support the plurivorous nature of *L. exigua* and at the same time add further evidence to the fact that the name *L. theobromae* has been applied to a

Table 4. Conidial sizes and L/W ratios of *Lasiodiplodia* species.

Species	Conidia Size (µm)	L/W ratio	References
L. citricola	(20–)22–27(–31) × (11–)12–17(–19)	1.6	Abdollahzadeh et al. (2010)
L. crassispora	27–30(–33) × 14–17	1.8	Burgess et al. (2006)
L. egyptiacae	(17–)20–24(−27) × 11–12(−13)	2.0	Ismail et al. (2012)
L. euphorbiicola	15−23 × 9−12	-	Machado et al. (2013)
L. gilanensis	(25–)28–35(–39) × (14.5–)15–18(–19)	1.9	Abdollahzadeh et al. (2010)
L. gonubiensis	(28–)32–36(–39) × (14–)16–18.5(–21)	1.9	Pavlic et al. (2004)
L. hormozganensis	(15.5–)18–24(–25) × 11–14	1.7	Abdollahzadeh et al. (2010)
L. iraniensis	(15.5–) 17–23(–29.5) × 11–14	1.6	Abdollahzadeh et al. (2010)
L. jatrophicola	22−26 × 14−17	-	Machado et al. (2013)
L. lignicola	(15–)16–17.5 × (8–)8.5–10.5(–11)	1.7	Phillips et al. (2013)
L. macrospora	28−35 × 15−17	-	Machado et al. (2013)
L. mahajangana	(13.5–)15.5–19(–21.5) × (10–)11.5–13(–14)	1.4	Begoude et al. (2009)
L. margaritacea	(12–)14–17(–19) × (10–)11–12(–12.5)	1.3	Pavlic et al. (2008)
L. missouriana	(16–)17.5–19.5(–21) × (8–)9–10.5(–11.5)	1.9	Úrbez-Torres et al. (2011)
L. parva	(15.5–)16–23.5(–24.5) × (10–)10.5–13(–14.5)	1.8	Alves et al. (2008)
L. plurivora	(22–)26.5–32.5(–35) × (13–)14.5–17(–18.5)	1.9	Damm et al. (2007)
L. pseudotheobromae	(22.5–)23.5–32(–33) × (13.5–)14–18(–20)	1.7	Alves et al. (2008)
L. rubropurpurea	24–33 × 13–17	1.9	Burgess et al. (2006)
L. subglobosa	16−23×11−17	-	Machado et al. (2013)
L. theobromae	(19–)21.5–31.5(–32.5) × (12–)13–17 (–18.5)	1.9	Phillips et al. (2013)
L. venezuelensis	26–33 × 12–15	2.1	Burgess et al. (2006)
L. viticola	(16.5–)18–20.5(–23) × (8–)9–10.1(–10.5)	2.05	Úrbez-Torres et al. (2011)
L. exigua	(19.6–)20–22(–24) × (10.9–)11–12(–13)	1.8	This study
L. mediterranea	(26.3–)28.5–30(–35) × (13.5–)15.5–16.5(–19)	1.8	This study

number of cryptic species. Given that a neotype specimen and ex-culture with related molecular data were established for *L. theobromae* (Phillips et al. 2013), a more detailed analysis of the current 990 sequences accessible in GenBank under the name *L. theobromae* will be possible in the future.

In conclusion, this study shows that *D. seriata* and *N. parvum* are the predominant botryosphaeriaceous taxa associated with V-shaped necrotic sectors and other wood symptoms of diseased grapevine in Sardinia. However, given the high number of *Botryosphaeriaceae* taxa found and their different assemblage among sites, the exact relationship between fungal species and grapevine wood disease symptoms has been difficult to determine without accurate diagnostic laboratory investigations. In addition, on the basis of combined phylogenetic and morphological analyses, two new *Lasiodiplodia* species were recognized inside the *L. theobromae* complex. The detection of *Lasiodiplodia* spp. from different hosts and countries suggests a wide distribution of members of this genus in the Mediterranean basin.

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Chapter 3

Phylogeny, morphology and pathogenicity of *Botryosphaeriaceae*, *Diatrypaceae* and *Gnomoniaceae* associated with branch canker and dieback of hazelnut in Sardinia (Italy)

Abstract Severe trunk and branch diseases of hazelnut trees have recently been observed in several groves in Sardinia (Italy). Because the aetiology had not yet been sufficiently investigated, from autumn 2012 to spring 2014, sixty samples of twigs and branches of hazelnut trees showing exudates and different types of canker (sunken with wedge-shaped necrotic sector, open canker and Cytospora canker) were collected in the main hazelnut growing area in the centre of the island. Based on morphology, colony appearance and DNA sequence data, seven species belonging to four genera and three families were isolated and identified. These included *Diplodia sapinea*, *D. seriata*, *Dothiorella iberica*, *D. parva* and *D. symphoricarposicola* (*Botryosphaeriaceae*), *Gnomoniopsis castanea* (*Gnomoniaceae*) and *Anthostoma decipiens* (*Diatrypaceae*). In addition, two undescribed species, here reported as *Diaporthella* sp. and *Dothiorella* sp., were isolated. Results obtained have allowed us to clarify, almost a century after its first description, the aetiology of the disease known as Cytospora canker and to reveal the existence of three evolutionarily distinct lineages for its causal agent *A. decipiens*. Pathogenicity trials carried out on wounded hazelnut logs showed that three species, *Anthostoma decipiens*, *Diaporthella* sp. and *Diplodia seriata* are aggressive pathogens on hazelnut. Finally, results obtained show that the diversity of fungal pathogens associated with twig and branch cankers of hazelnut is greater than previously recognised and that further studies are necessary to determine the exact role played by each species and their potential synergistic interaction.

Keywords *Anthostoma* - *Diaporthella* - *Dothiorella* - taxonomy - pathogenicity

Introduction

Hazelnut (*Corylus avellana* L.) is an important stone fruit tree worldwide. It is cultivated in many Mediterranean countries as well as in Azerbaijan, Georgia, China, Iran and North

America. Italy is the second largest hazelnut-producing country after Turkey (Me and Valentini 2006).

In both traditional and emerging hazelnut-producing countries worldwide, hazelnut trees are affected by several diseases which can reduce yields, increase production costs and threaten plant survival. Among the most serious diseases that affect this crop, branch cankers play a primary role. Some canker-causing agents such as *Cytospora corylicola* Sacc. ex Fuckel and *Anisogramma anomala* (Peck) E. Müller and more recently a *Dothiorella* sp. are reported as important limiting factors (Gottwald and Cameron 1980; Guerrero and Pérez 2013; Lamichhane et al. 2014).

In Italy, Cytospora canker caused by the ascomycete *C. corylicola* is reported across the hazelnut growing areas including Sardinia. Despite several studies reporting detailed information about the symptoms caused by *C. corylicola* on hazelnut (Salerno 1961; Granata 1985; Scortichini and Di Prospero 2002), until now no information is available about the DNA phylogeny of this fungus. For *C. corylicola* there are no nucleotide sequences available in public databases, and there are no cultures linked to a type of the species. In addition, there are conflicting reports about its pathogenicity. Salerno (1961) reported *C. corylicola* as a pathogen capable of causing cankers on hazelnut both in field and laboratory conditions, whereas, Servazzi (1950) and Graniti (1957) define *C. corylicola* as a saprophyte capable of colonising old plants that have been strongly weakened by abiotic factors. More recently, Tavella and Giannetti (2006) reported that Cytospora canker has significantly increased over the years in northern Italy, even in young, properly managed groves. This aspect is further confirmed by Lamichhane et al. (2014) who found that plant age is not correlated to the severity of Cytospora canker and that some pedoclimatic factors could have an impact on establishment, epidemiology and pathogenicity of *C. corylicola*. However, in both studies, there is little detail about the pathogen identification.

Eastern filbert blight caused by *Anisogramma anomala* is one of the most destructive diseases of hazelnut in North America (Gottwald and Cameron 1979; Pinkerton et al. 1992). The disease became commercially important in the 1970s, and currently is a major threat to the hazelnut industry (Chen et al. 2007). Symptoms include lethal cankers on branches which

have been to cause canopy dieback, and death in mature trees. This disease has a major impact on crop productions and require expensive chemical control (Mehlenbacher 1994; Johnson et al. 1996; Julian et al. 2008).

Recently the occurrence of hazelnut shoot blight and canker was reported in Chile (Guerrero and Perez 2013). From symptomatic trees a species identified as *Diplodia coryli* Fuckel was consistently isolated. The pathogenicity tests carried out on 1-year-old plants showed that *D. coryli* caused necrosis and discolouration of vascular tissues on inoculated shoots and twigs. According to Phillips et al. (2008) the species *D. coryli* as well as *Diplodia juglandis* (Fr.) Fr. belong to the genus *Dothiorella*, but to date the lack of authentic isolates has hampered their correct identification.

Unlike other countries, in Sardinia (Italy) few studies have been conducted to assess the sanitary problems that affect hazelnut trees. Given that knowledge of infectious diseases of commercial groves is crucial for their proper management, in 2008 a preliminary field survey was carried out in one grove located in the main producing area in the centre of the island. The complexity of the symptoms observed during the investigation, some of which of unknown aetiology, suggested the need to expand the investigation: 1) to isolate and identify the pathogens associated with the different hazelnut diseases observed; 2) to evaluate the pathogenicity of the species most directly associated with each symptom.

Materials and Methods

Sampling and fungal isolation

From autumn 2012 to summer 2014, the healthy status of hazelnut trees, from 2 groves located in the centre of Sardinia was monitored and samples of branches and twigs showing shoot blight, black exudates, canker and dieback were collected from 60 symptomatic trees (Table 1). All samples were brought to the laboratory to be inspected and the outer bark surface was cut away with a scalpel. Longitudinal and transversal cuts were made to observe any internal symptoms.

Isolations were made from chips of inner bark and xylem tissues, approx. 5 mm^2, aseptically cut from the margin of infected tissues. All samples were cultured on potato

dextrose agar (PDA, Oxoid Ltd.) in Petri dishes. After incubation at 25 °C for 1 wk, fungal colonies were sub-cultured onto half-strength PDA or on water agar supplemented with autoclaved poplar and holm oak twigs to enhance sporulation. All colonies were kept on the laboratory bench at about 20–25 °C where they received diffused daylight. Furthermore, four *Dothiorella* isolates obtained from four cankered branches of hazelnut trees collected in autumn 2008 in the grove 1 were studied.

Representative isolates of each species were stored on PDA slants under oil in the culture collection of the Sez. di Patologia vegetale ed Entomologia, Dipartimento di Agraria at the University of Sassari.

Table 1. Site characteristics of hazelnut groves and number of symptomatic samples collected.

Sampling sites	Geographical coordinates		Elevation (m a.s.l.)	Surface (hectares)	Cultivar	Number of samples
	Lon. N (°)	Lat. E (°)				
Site 1	39°56'59.1"	9°11'41.8"	906	2	TGR – TGL*	41**
Site 2	39°58'01.9"	9°11'18.7"	625	1	Unknown	23

*TGR = Tonda Gentile Romana; TGL = Tonda Gentile delle Langhe
**Including four branch cankers collected in 2008

Morphological identification and characterisation

For each fungal species, colony growth characteristics including surface and reverse colony appearance were observed and recorded after 7 days at 25 °C in the dark on PDA. For undescribed species, cardinal temperatures for growth of isolates were determined on PDA and malt extract agar (MEA, 20 g/L malt extract, 20 g/L agar, Oxoid Ltd.), incubating plates at 5, 10, 15, 20, 25, 30, 35 and 40 °C (±0.5 °C) in the dark. Five replicate plates for each isolate were made and colony diameters were measured after 4 days.

Identification of diatrypaceous and gnomoniaceous isolates to species level were based principally on the analysis of DNA sequence data, whereas species of *Botryosphaeriaceae*

were initially identified based on colony and conidial morphology as described by Phillips et al. (2013).

For microscopy, the contents of conidiomata were dissected out and mounted in 100 % lactic acid. Measurements of conidiogenous cells and conidia were made with the Leica IM 500 measurement module from images recorded on a Leica DFC 320 digital camera. From measurements of 50 conidia the mean, standard deviation and 95 % confidence intervals were calculated. Spore dimensions are presented as mean values with extreme values in parentheses. Dimensions of other structures are given as mean of at least 20 measurements.

DNA extraction, PCR amplification and sequencing

Molecular analysis was used to confirm identification of all isolates to species level. Genomic DNA was extracted from 5-day-old cultures grown on PDA and incubated at 25°C using Instagene Matrix (BioRad Laboratories, Hercules, CA). The ITS region was amplified and sequenced with primers ITS1 and ITS4 (White et al. 1990), while the primers EF446f and EF1035r (Inderbitzin et al. 2010) were used to amplify and sequence part of the translation elongation factor 1- alpha gene (EF1-α). Polymerase chain reaction (PCR) mixtures and amplification conditions were conducted as described by Linaldeddu et al. (2013). The PCR products were purified using the EUROGOLD gel extraction kit (EuroClone S.p.A.) following the manufacturer's instructions. ITS and EF1-α regions were sequenced in both directions by the BMR Genomics DNA sequencing service (www.bmr-genomics.it). The nucleotide sequences were read and edited with FinchTV 1.4.0 (Geospiza, Inc. http://www.geospiza.com/finchtv) and then compared with reference sequences available at the GenBank database using BLAST analysis (Altschul et al. 1990). Nucleotide sequences of additional isolates included in this study were retrieved from GenBank (Table 2).

Phylogenetic analysis

Phylogenetic analysis was performed only for *Dothiorella* species. The ITS and EF1-α sequences of 13 *Dothiorella* isolates obtained in this study were combined and the dataset, including 49 sequences of other *Dothiorella* isolates downloaded from GenBank, was

compiled with the outgroup *Spencermartinsia viticola* (A.J.L. Phillips & J. Luque) A.J.L. Phillips, A. Alves & Crous (Table 2). Sequences were aligned with ClustalX v. 1.83 (Thompson et al. 1997), using the following parameters: pairwise alignment parameters (gap opening = 10, gap extension = 0.1) and multiple alignment parameters (gap opening = 10, gap extension = 0.2, transition weight = 0.5, delay divergent sequences = 25 %). Alignments were checked and manual adjustments were made where necessary.

Maximum parsimony (MP) analysis was performed with PAUP* v.4.0b10 (Swofford 2003) using the heuristic search option with 1000 random taxon additions and tree bisection and reconnection as the branch-swapping algorithm. All characters were unordered and of equal weight, and gaps were treated as missing data. Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. The robustness of the most parsimonious trees was evaluated by 1000 bootstrap replications (Hillis and Bull 1993). Other measures used were consistency index (CI), retention index (RI) and homoplasy index (HI).

Bayesian analyses were done with Mr Bayes v.3.0b4 (Ronquist and Huelsenbeck 2003) employing a Markov Chain Monte Carlo (MCMC) method. The general time-reversible model of evolution (Rodriguez et al. 1990), including estimation of invariable sites and assuming a discrete gamma distribution with six rate categories was used. Four MCMC chains were run simultaneously, starting from random trees for 10^6 generations. Trees were sampled every 100^{th} generation for a total of 10^4 trees. The first 10^3 trees were discarded as the burn-in phase of each analysis. Posterior probabilities (Rannala and Yang 1996) were determined from a 50 % majority-rule consensus tree generated with the remaining 9,000 trees. This analysis was repeated three times starting from different random trees to ensure trees from the same tree space were sampled during each analysis. Trees were visualized with TreeView (Page 1996).

Table 2. List of isolates used in this study.

Species	Isolate number[1]	Host	Locality	Collector	GenBank	
					ITS[2]	EF
D. americana	**CBS 128309**	*V. vinifera*	USA	Striegler & Leavitt	*HQ288218*	*HQ288262*
D. americana	CBS 128310	*V. vinifera*	USA	Striegler & Leavitt	*HQ288219*	*HQ288263*
D. brevicollis	**CBS 130411**	*A. karroo*	South Africa	Jami & Gryzenhout	*JQ239403*	*JQ239390*
D. brevicollis	CBS 130412	*A. karroo*	South Africa	Jami & Gryzenhout	*JQ239404*	*JQ239391*
D. capri-amissi	**CBS 121763**	*A. erioloba*	South Africa	van der Walt & Marais	*EU101323*	*EU101368*
D. capri-amissi	CBS 121878	*A. erioloba*	South Africa	van der Walt & Marais	*EU101324*	*EU101369*
D. casuarini	**CBS 120688**	*Casuarina* sp.	Australia	M.J. Wingfield	*DQ846773*	*DQ875331*
D. casuarini	CBS 120690	*Casuarina* sp.	Australia	M.J. Wingfield	*DQ846774*	*DQ875333*
D. dulcispinae	**CBS 130413**	*A. karroo*	South Africa	Jami & Gryzenhout	*JQ239400*	*JQ239387*
D. dulcispinae	CBS 130415	*A. karroo*	South Africa	Jami & Gryzenhout	*JQ239402*	*JQ239389*
D. dulcispinae	CBS 121764	*A. mellifera*	Namibia	van der Walt & Roux	*EU101299*	*EU101344*
D. dulcispinae	CBS 121765	*A. mellifera*	South Africa	van der Walt & Heath	*EU101300*	*EU101345*
D. dulcispinae	CBS 121766	*A. mellifera*	South Africa	van der Walt & Heath	*EU101301*	*EU101346*
D. iberica	**CBS 115041**	*Q. ilex*	Spain	J. Luque	*AY573202*	*AY573222*
D. iberica	CAA005	*P. vera*	USA	Unknown	*EU673312*	*EU673279*
D. iberica	BL159	*C. avellana*	Italy	B.T. Linaldeddu	KP205486	KP205459
D. iberica	BL162	*C. avellana*	Italy	B.T. Linaldeddu	KP205487	KP205460
D. iberica	BL163	*C. avellana*	Italy	B.T. Linaldeddu	KP205488	KP205461
D. iberica	BL170	*C. avellana*	Italy	B.T. Linaldeddu	KP205489	KP205462
D. iranica	**CBS 124722**	*O. europaea*	Iran	A. Javadi	*KC898231*	*KC898214*
D. longicollis	**CBS122068**	*L. cunninghamii*	Australia	T.I. Burgess	*EU144054*	*EU144069*
D. longicollis	CBS 122067	*L. cunninghamii*	Australia	T.I. Burgess	*EU144053*	*EU144068*
D. moneti	**MUCC505**	*A. rostellifera*	Australia	K.M. Taylor	*EF591920*	*EF591971*
D. moneti	MUCC506	*A. rostellifera*	Australia	K.M. Taylor	*EF591921*	*EF591972*
D. parva	**CBS 124720**	*C. avellana*	Iran	Abdollahzadeh & Javadi	*KC898234*	*KC898217*
D. parva	CBS 124721	*C. avellana*	Iran	Abdollahzadeh & Javadi	*KC898235*	*KC898218*
D. parva	BL172	*C. avellana*	Italy	B.T. Linaldeddu	KP205490	KP205463
D. parva	JL599	*C. avellana*	Spain	J. Luque	*EU673314*	*EU673281*
D. pretoriensis	**CBS 130404**	*A. karroo*	South Africa	Jami & Gryzenhout	*JQ239405*	*JQ239392*
D. pretoriensis	CBS 130403	*A. karroo*	South Africa	Jami & Gryzenhout	*JQ239406*	*JQ239393*
D. prunicola	**CBS 124723**	*P. dulcis*	Portugal	E. Diogo	*EU673313*	*EU673280*
D. santali	**MUCC509**	*S. acuminatum*	Australia	K.M. Taylor	*EF591924*	*EF591975*
D. santali	MUCC508	*S. acuminatum*	Australia	K.M. Taylor	*EF591923*	*EF591974*
D. sarmentorum	**IMI63581b**	*Ulmus* sp.	England	E.A. Ellis	*AY573212*	*AY573235*
D. sarmentorum	CBS 115038	*M. pumila*	Netherlands	A.J.L. Phillips	*AY573206*	*AY573223*

D. sempervirentis	**CBS 124718**	*C. sempervirens*	Iran	M.A. Aghajani	*KC898236*	*KC898219*
D. sempervirentis	CBS 124719	*C. sempervirens*	Iran	M.A. Aghajani	*KC898237*	*KC898220*
D. striata	**CBS 124731**	*C. sinensis*	New Zealand	Pennycook & Johnston	*EU673320*	*EU673287*
D. striata	CBS 124730	*C. sinensis*	New Zealand	Pennycook & Johnston	*EU673321*	*EU673288*
D. symphoricarposicola	BL12	*C. avellana*	Italy	B.T. Linaldeddu	KP205491	KP205464
D. symphoricarposicola	BL53	*C. avellana*	Italy	B.T. Linaldeddu	KP205492	KP205465
D. symphoricarposicola	BL158	*C. avellana*	Italy	B.T. Linaldeddu	KP205493	KP205466
D. symphoricarposicola	BL167	*C. avellana*	Italy	B.T. Linaldeddu	KP205496	KP205469
D. symphoricarposicola	BL174	*C. avellana*	Italy	B.T. Linaldeddu	KP205495	KP205468
D. symphoricarposicola	BL175	*C. avellana*	Italy	B.T. Linaldeddu	KP205494	KP205467
D. symphoricarposicola	CMW39372	*C. atlantica*	Serbia	Unknown	*KF575015*	KF575050
D. symphoricarposicola	CMW39371	*C. atlantica*	Serbia	Unknown	*KF575016*	*KF575051*
D. symphoricarposicola	**MFLUCC13-0497**	*Symphoricarpos* sp.	Italy	Erio Camporesi	*KJ742378*	*KJ742381*
D. symphoricarposicola	MFLUCC13-0498	*Symphoricarpos* sp.	Italy	Erio Camporesi	*KJ742379*	*KJ742382*
D. thailandica	**CBS 133991**	*Bambusa* sp.	Thailand	Dongqin Dai	*JX646796*	*JX646861*
D. uruguayensis	**CBS 124908**	*H. edulis*	Uruguay	C. Perez	*EU080923*	*EU863180*
D. vidmadera	**DAR78992**	*V. vinifera*	Australia	Pitt & Loschiavo	*EU768874*	*EU768881*
D. vidmadera	DAR78994	*V. vinifera*	Australia	Pitt & Loschiavo	*EU768877*	*EU768883*
D. vidmadera	IMI501235a	*C. avellana*	Chile	Unknown	*JX163116*	
D. vidmadera	IRAN1571C	Unknown	Unknown	Unknown	*KF890200*	*KF890182*
Dothiorella sp.	CBS 188.87	*J. regia*	France	Meylan	*EU673316*	*EU673283*
Dothiorella sp.	CBS 242.51	Unknown	Italy	R. Ciferri	*EU673317*	*EU673284*
Dothiorella sp.	BL13	*C. avellana*	Italy	B.T. Linaldeddu	KP205498	KP205471
Dothiorella sp.	BL52	*C. avellana*	Italy	B.T. Linaldeddu	KP205497	KP205470
Dothiorella sp.	CMW39360	*F. excelsior*	Bosnia	Unknown	*KF575012*	*KF575052*
Dothiorella sp.	CMW39362	*T. occidentalis*	Serbia	Unknown	*KF575013*	*KF575053*
Dothiorella sp.	CMW39363	*C. lawsoniana*	Serbia	Unknown	*KF575014*	*KF575054*
S. viticola	**CBS 117009**	*V. vinifera*	Spain	J. Luque & S. Martos	*AY905554*	*AY905559*
S. viticola	CBS 302.75	*P. gilliesii*	France	Unknown	*EU673319*	*EU673286*

[1]Acronyms of culture collections: BL – B.T. Linaldeddu, Università degli Studi di Sassari, Italy; CAA – A. Alves, Universidade de Aveiro, Portugal; CBS – Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CMW – M.J. Wingfield, FABI, University of Pretoria, South Africa; DAR – Plant Pathology Herbarium, Orange Agricultural Institute, DPI, Orange, NSW, Australia; IMI – International Mycological Institute, CBI-Bioscience, Egham, Bakeham Lane, UK; IRAN – Iranian Fungal Culture Collection, Iranian Research Institute of Plant Protection, Iran; JL – J. Luque, IRTA, Spain; MFLUCC – Mae Fah Luang University Culture Collection, Chiang Rai, Thailand; MUCC – Murdoch University Culture Collection, Perth, Australia. Isolates in bold are cultures ex-type.

[2]Sequence numbers in italics were retrieved from GenBank. All others were obtained in the present study.

Pathogenicity test

Pathogenicity of the species most frequently isolated was verified by a log inoculation trial. Logs (1 m long and 3-4 cm in diameter) were taken from asymptomatic hazelnut trees and transferred immediately to the laboratory for inoculation. The logs were marked and the bottom and top ends of each sealed with a synthetic grafting resin to prevent drying and contamination. Ten logs were inoculated with a representative isolate of each fungal species and ten were used as controls. For each log, a piece of outer bark (about 0.3 × 0.3 cm) was removed with a scalpel on the middle of each log and replaced with an agar-mycelium plug of the same size taken from the margin of a colony growing actively on PDA with the aerial mycelium facing the inner bark. The inoculation site was covered with cotton-wool soaked in sterile water and wrapped with a piece of aluminium foil secured with Parafilm. A sterile PDA plug was placed on the wound in control logs. The logs were enclosed in a transparent plastic bag for thirty days, and kept in the laboratory in daylight at 25 °C. At the end of the experiment, the length of necrotic lesions caused by each isolate tested was measured after removal of the outer bark. Re-isolation was attempted by transferring to PDA 10 pieces of inner bark and woody tissues taken around the margin of each lesion. Cultures were grown at room in daylight temperature until full colony development.

Statistical analyses

Data from pathogenicity assays were first checked for normality and then subjected to analysis of variance (ANOVA). Significant differences among mean values were determined using Least Significant Difference (LSD) multiple range test ($P = 0.05$) following one-way ANOVA. Statistical analyses were performed using XLSTAT software (Addinsoft, Paris, France).

Results

Field surveys

Field inspections carried out in both groves over a two-year period showed the presence of disease symptoms in 64 hazelnut trees (Table 1). Symptomatic trees showed a variety of symptoms including twig and shoot blight (Fig. 1), large sunken cankers with blackish exudates (Fig. 2), small reddish-brown bark exudates with necrosis of the underlying bark and cambium (Fig. 3), slightly sunken cankers with discoloured areas in the bark with a red/orange mass of conidia (Cytospora canker) (Fig. 4) and elongated open cankers (Fig. 5).

Twig and shoot blight symptoms usually appeared in late spring with the sudden wilting of new shoots (Fig. 1a). The infection spreads from shoots to twigs causing bark and xylem necrotic lesions. Leaves on affected shoots turned yellow, then dull red and finally red-brown (Fig. 1b). They often remain attached for some time after the death of the twigs. In autumn affected plants showed extensive twig dieback (Fig. 1c). The appearance of this symptom during the investigation was observed in ten plants. On the same plants, the presence of old twig dieback symptoms was also recorded, suggesting that this disease had also occurred in previous years.

When branch and trunk samples with sunken cankers were cross-sectioned, internal wood symptoms included characteristic V-shaped necrotic sectors (Fig. 2c). Canopy symptoms associated with this type of canker included dead or dying shoots and branches, often with dead leaves still attached, scattered among healthy foliage. Sunken cankers with V-shaped necrotic sectors were the most common disease symptoms observed on symptomatic hazelnut trees (29 plants).

Nine plants located in grove 1 showed discoloured spots on the bark of the trunk or branches which exuded a reddish-brown fluid that spread out over the bark during the summer. The necrotic lesion was usually confined to the inner bark, but occasionally also sapwood was infected (Fig. 3). Plants with these symptoms showed no additional symptoms in the twigs and leaves.

Eleven plants showed typical symptoms of Cytospora canker (known as *Mal dello stacco* in Italian). Cankers on stem and branches were often elongated, slightly sunken, with irregular

reddish-brown areas in the bark which appeared with longitudinal cracks (Fig. 4a). In the presence of high humidity, conidia oozing from the conidiomata in a gelatinous dull orange matrix were observed around the margin of the canker on symptomatic branches (Fig. 4b). In cross section, branches with Cytospora canker showed light-brown to brown alterations of inner-bark and sapwood, which became soft as the disease progressed (Fig. 4c, d). After the canker girdled the branch, the distal part withered and leaves remained attached.

Finally on five plants an unusual type of branch canker, here reported as open canker, was observed (Fig. 5). The early stage of the infections was characterised by longitudinal streaks along the inner bark and sapwood. Callus tissue around the cankered area forming ridges under the bark were usually observed (Fig. 5b). Cankers often exceeded a metre in length and developed from the bottom upwards girdling the branches.

Figure 1. Shoots and twigs disease symptoms detected on hazelnut trees: early dieback on shoots (a); branch dieback with brown leaves still attached during the summer (b); extensive twig and branch dieback (c); colony morphology of *Diaporthella* sp. after 7 days growth at 25 °C on PDA (d).

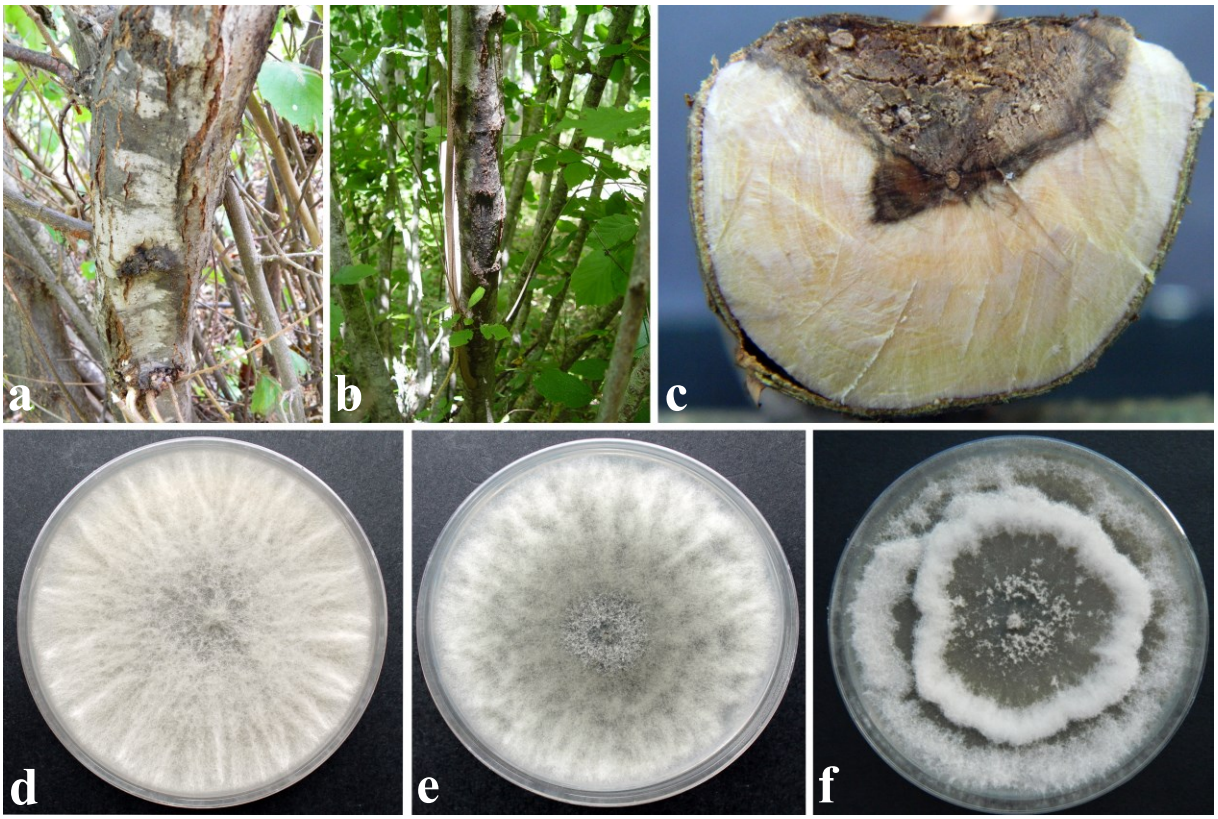

Figure 2. Hazelnut trees with active sunken cankers, wet due to the emission of blackish exudates (a) and (b); cross section of a branch canker with in evidence the wedge-shaped necrotic sector (c); colony morphology after 7 days growth at 25 °C on PDA of the main fungal species associated: *Diplodia seriata* (d), *Dothiorella iberica* (e) and *Dothiorella symphoricarposicola* (f).

Figure 3. Hazelnut branches with reddish-brown exudates and extensive necrotic lesion of inner bark and xylematic tissues (a), (b) and (c); colony morphology of *Gnomoniopsis castanea* after 7 days growth at 25 °C on PDA (d).

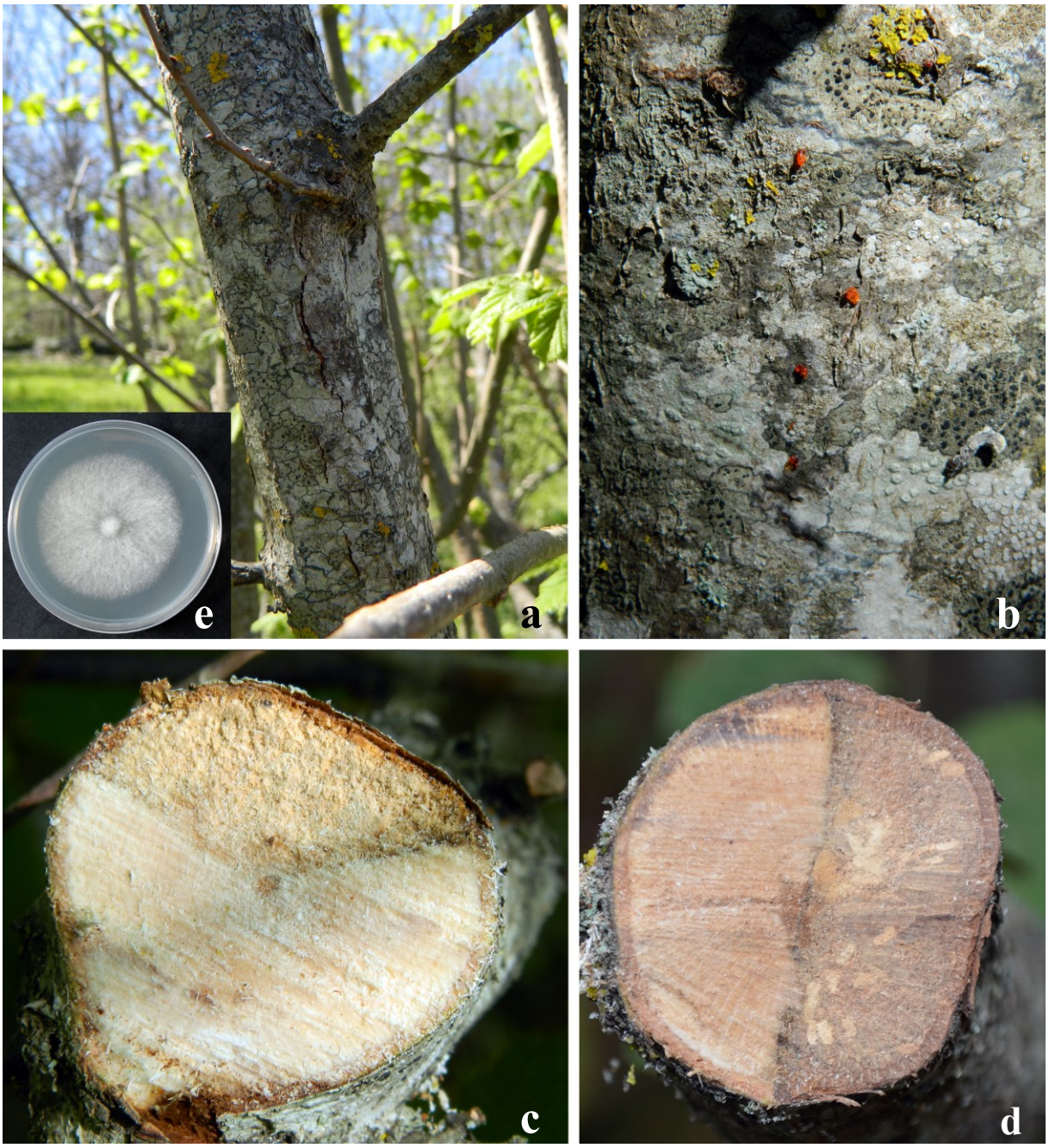

Figure 4. Hazelnut trees with characteristic symptoms of Cytospora canker: slightly sunken canker, with irregular reddish-brown areas in the bark (a); red mass of conidia oozing from the margin of the canker (b); cross section of the main trunk showing the progression of the disease (c) and (d); colony morphology of *Anthostoma decipiens* after 7 days growth at 25 °C on PDA (e).

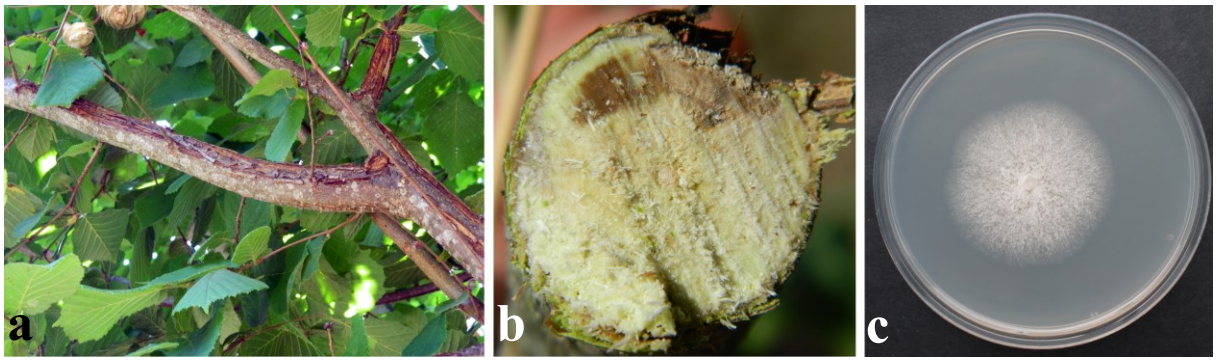

Figure 5. Hazelnut branch with an "open canker" characterised by longitudinal streaks along the inner bark and sapwood (a); cross section of a branch canker with in evidence the callus tissue around the cankered area forming ridges and pale brown wood necrosis (b); colony morphology of *Diaporthella* sp. after 7 days growth at 25 °C on PDA.

Fungal isolation and identification

Fungal colonies with similar cultural characteristics were consistently isolated from inner bark and xylem tissues of all symptomatic hazelnut shoots and twigs processed. After 7 days at 25 °C the fungal colonies were cottony white with a pale-pink pigmentation on the reverse side of the agar plates (Fig. 1d). All colonies remained sterile in culture and after two months of incubation at 25 °C showy hyphal clusters were often observed. The ITS and EF1-α sequences of three representative isolates (ITS accession no. KP205483, KP205484, KP205484; EF1-α: KP205456, KP205457, KP205458) showed respectively 94% and 87% of similarity with sequence of the strain CBS 121124 of *Diaporthella corylina* Lar. N. Vassiljeva (accession no. KC343004 and KC343730), a pathogenic species recently described on *Corylus* spp. in China and Russia by Vasilyeva et al. (2007). No other sequences of fungal species generated significant alignments in GenBank in the returned list of a BLAST search. Based on these results it is possible to affirm that the isolates obtained from hazelnut represent a new distinct species within the small genus *Diaporthella* Petr. which consists of four species and only for *D. corylina* are DNA sequences currently available. We refrain from giving a species name in this study because further morphological investigations are required to describe it unequivocally. Therefore, in this thesis it will continue to be referred to as *Diaporthella* sp.

Isolations from branch samples showing sunken cankers with V-shaped necrotic sector yielded a total of 35 fungal isolates belonging to two distinct families, namely *Botryosphaeriaceae* Theiss. & P. Syd. and *Gnomoniaceae* G. Winter. Botryosphaeriaceous fungi were the most commonly isolated species from this type of canker (29 isolates). On the basis of morphological features and DNA sequence data (ITS and EF1-α), five distinct species namely *Diplodia sapinea* (1 isolate), *D. seriata* (11 isolates), *Dothiorella parva* (2 isolates), *D. iberica* (5 isolates) and *D. symphoricarposicola* (6 isolates) were identified (Table 3). For all species BLAST searches in GenBank showed 99-100 % similarity with ITS reference sequences of representative strains including those of ex-type cultures. In addition, four *Dothiorella* isolates on the basis of morphological features and ITS sequence data could not be assigned to any known species of *Dothiorella* and were here reported as *Dothiorella* sp. This species is being further studied in relation to other *Dothiorella* species and its formal

description is in preparation. Among the gnomoniaceous fungi two isolates with white to pale grey aerial mycelium developing in concentric circles on PDA were identified as *Gnomoniopsis castanea* G. Tamietti. Sequences obtained (ITS accession no. KP205499, KP205500, KP205501) had 100% similarity with the ITS sequence HM142946 of ex-type culture Rb7. Four additional isolates belonged to *Diaporthella* sp. Isolates of both these last two species were always isolated together with colonies of *Dothiorella* spp.

Based on their appearance in culture and morphology, the nine isolates obtained from the inner bark of branches with reddish-brown exudates, could be assigned to four fungal species. Six isolates were identified as *Gnomoniopsis castanea*, one isolate as *Dothiorella iberica*, one isolate as *D. symphoricarposicola* and one as *Diaporthella* sp.

White to pale-grey *Cytospora*-like cultures were consistently isolated from active Cytospora cankers on PDA. Fungal colonies had white cottony aerial hyphae that became pale grey after 4 to 6 days on PDA at 25 °C. The reverse of colonies turned yellowish to faintly rosy with irregular blackened areas within 2 weeks of incubation on PDA. All colonies had ITS nucleotide sequences with 99-100% similarity to those of representative sequences of *Anthostoma decipiens* (accession No. AM399021, JN975370, KC774565) available in GenBank, and were therefore identified as *A. decipiens* (Rocchi et al. 2010; Luque et al. 2012; Jaklitsch et al. 2014). Sequence alignments of ITS regions of *A. decipiens* strains downloaded from GenBank, in combination with sequence data from isolates collected in this study, showed the presence of intraspecific variability in ITS1 sequences, resulting in three linked lineages (L1-L3) (Table 4).

Finally, isolations made on PDA from the margin of wood necrosis of open cankers, yielded fungal colonies identical to those obtained from symptomatic shoot samples. ITS sequencing confirmed that these isolates were *Diaporthella* sp.

Table 3. Number of samples examined for each symptomatology and related pathogens isolated.

Fungi	Symptoms					Sites
	Shoot blight	Sunken cankers	Bark necrosis	Cytospora canker	Open cankers	
Anthostoma decipiens	-	-	-	11/11	-	1 and 2
Diaporthella sp.	10/10	4/29	1/9	-	5/5	1 and 2
Diplodia sapinea	-	1/29	-	-	-	1
Diplodia seriata	-	11/29	-	-	-	1 and 2
Dothiorella iberica	-	5/29	1/9	-	-	1 and 2
Dothiorella parva	-	2/29	-	-	-	2
Dothiorella symphoricarposicola	-	6/29	1/9	-	-	1 and 2
Dothiorella sp.	-	4/29	-	-	-	1 and 2
Gnomoniopsis castanea	-	2/29	6/9	-	-	1 and 2

Table 4. Polymorphic nucleotides from aligned sequence data of ITS1 region showing the variation among *Anthostoma decipiens* isolates. Green shading denotes polymorphisms found in *A*. *decipiens* lineage 1. Light blue shading is for those found in lineage 2. Grey is for those found in lineage 3.

Identity	Culture no.	Accession no.	References	ITS		Lineage
				150	169	
Anthostoma decipiens	IPV-FW349	AM399021	Rocchi et al. (2010)	T	C	L1
	BL164	KP205472	This study	T	C	
	CAD056	KP205475	This study	T	C	
	CAD057	KP205476	This study	T	C	
	CAD058	KP205477	This study	T	C	
	CAD059	KP205478	This study	T	C	
	CAD061	KP205480	This study	T	C	
	CAD062	KP205481	This study	T	C	
	CAD063	KP205482	This study	T	C	
	CD	KC774565	Jaklitsch et al. (2014)	T	T	L2
	BL177	KP205473	This study	T	T	
	BL178	KP205474	This study	T	T	
	JL567	JN975370	Luque et al. (2012)	G	T	L3
	CAD060	KP205479	This study	G	T	

DNA phylogeny of *Dothiorella* isolates

Fragments of approximately 500 and 300 bases were determined for ITS and EF1-α regions, respectively. New sequences were deposited in GenBank (Table 2). Individual gene phylogenies revealed no major conflicts thus indicating that the two loci could be combined. The combined ITS and EF1-α sequences for 62 ingroup and 2 outgroup taxa contained 772 characters including alignment gaps, of which 581 were constant, 23 were variable and parsimony-uninformative and 168 were parsimony-informative. Other parameters: CI = 0.656, HI = 0.344, RI = 0.899. The Bayesian and MP analyses produced phylogenetic trees essentially with the same topology. A MP tree is shown in Fig. 6 with bootstrap support and Bayesian posterior probability scores at the nodes. In the phylogenetic analysis 21 well-supported clades, representing 21 species of *Dothiorella* were resolved. The 13 *Dothiorella* isolates studied were distributed into four clades. In particular, one isolate clustered with *D. parva* strains; four isolates clustered in the *D. iberica* clade and six isolates clustered in a larger group identified as *D. symphoricarposicola*. Of note, the *D. symphoricarposicola* isolates were divided in two sub-clades separated by 4 bp differences in EF1-α sequences. The two sub-clades were considered as two distinct lineages of *D. symphoricarposicola* and therefore reported as lineage A and lineage B (Fig. 6). ITS sequences for all *D. symphoricarposicola* isolates were identical. The remaining two isolates clustered in a well-supported clade recognised here as representative of a new *Dothiorella* species reported in this thesis as *Dothiorella* sp. Phylogenetically, *Dothiorella* sp. is closely related to *D. vidmadera* and *D. sempervirentis*, but can be distinguished on account of the number of differences in ITS and EF1-α sequences.

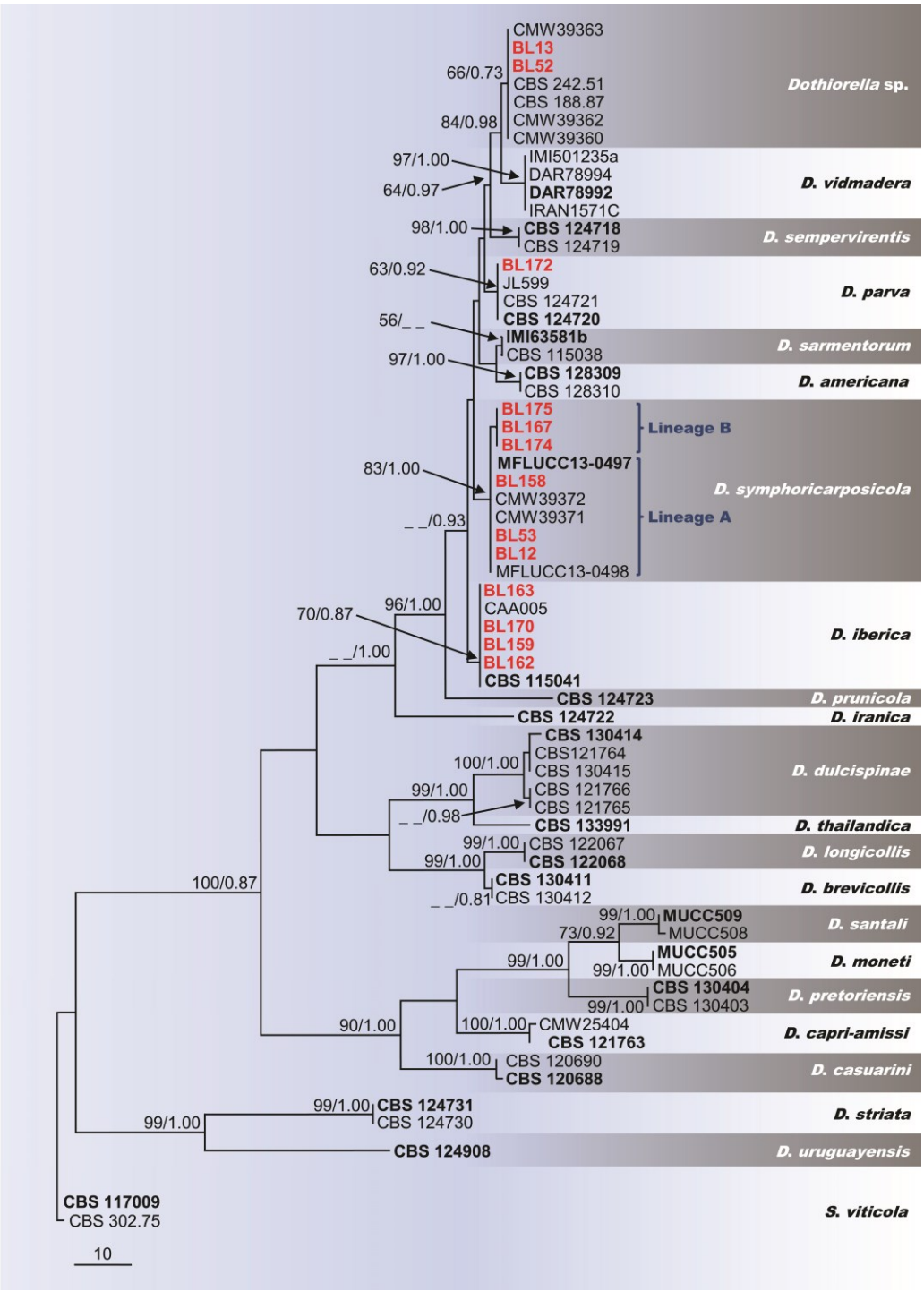

Figure 6. One of the most parsimonious trees resulting from the combined analysis of ITS and EF1-α sequence data. MP Bootstrap support values and Bayesian posterior probability scores are given at the nodes. The tree was rooted to *Spencermartinsia viticola*. Ex-type isolates are in bold. Isolates obtained in this study are in red. The bar shows ten changes.

Taxonomy

Based on DNA sequence phylogeny conidial morphology and cultural characteristics we conclude that the isolates of *Dothiorella* sp. represent an undescribed *taxon*. We thus provide the following morphological descriptions of it here.

***Dothiorella* sp.** Fig. 7

Teleomorph not seen. Conidiomata pycnidial, produced on poplar twigs on ½ PDA within 2–4 wk, solitary or aggregated, dark-brown to black, globose, uniloculate, immersed in the host and becoming erumpent when mature. Conidiophores absent. Conidiogenous cells cylindrical, discrete or integrated, holoblastic, hyaline, thin-walled, smooth, 11.53 ± 2.40 × 3.13 ± 0.61 µm (mean ± S.D., n=20). Conidia ellipsoid to ovoid, brown, 1-septate, occasionally two-septate, moderately thick-walled, externally smooth, ends rounded, sometimes with a truncate base, (19.3–)23.3(–25.5) × (7.5–)8.7(–10.6) µm, 95 % confidence limits = 22.9–23.7 × 8.5–8.9 µm (av. ± S.D. = 23.3 ± 1.4 × 8.7 ± 0.6 µm, l/w ratio = 2.7 ± 0.2).

Culture characteristics: Colonies on PDA grew quickly, reaching 90 mm diameter in 4 days in the dark at 20 °C, the mycelium was sparsely to moderately aerial, surface white at first and later turned pale grey to dark olivaceous and dark in reverse.

Cardinal temperatures for growth: min. ≤ 5 °C, max. ≤ 35 °C, opt. 20 °C.

Substrate: cankered branch of *Corylus avellana*; *Chamaecyparis lawsoniana*; *Fraxinus excelsior*; *Juglans regia*; *Thuja occidentalis*.

Distribution: Bosnia and Herzegovina, France, Italy, Serbia.

Specimens examined. Italy, Aritzo, on cankered branch of *Corylus avellana*, 09 July 2008, collected by B.T. Linaldeddu, strain BL13. Italy, Aritzo, on cankered branch of *Corylus avellana*, 09 July 2008, collected by B.T. Linaldeddu, strain BL52. Additional isolates are given in Table 2.

Notes: Morphologically, *Dothiorella* sp. is similar to *D. vidmadera*, but the two species are readily distinguishable by conidia size and shape as expressed as the L/W ratio of about 2.2 for *D. vidmadera* and about 2.7 for *Dothiorella* sp.

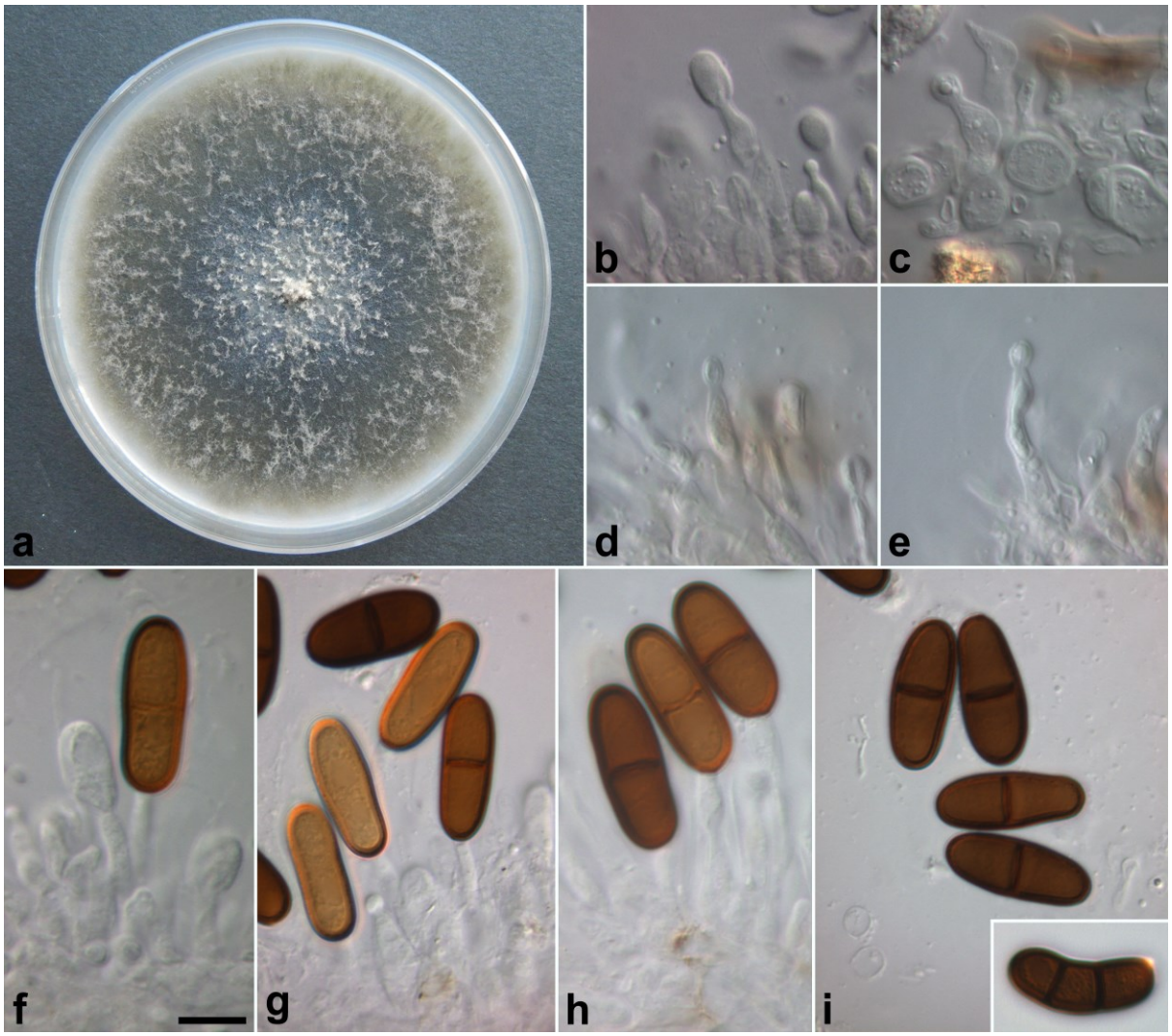

Figure 7. Colony morphology of *Dothiorella* sp. after 7 d growth at 25 °C on PDA (a); hyaline immature conidia developing on conidiogenous cells (b-e); pale brown aseptate and septate conidia on conidiogenous cell (g-h); old one and two septate conidia (i). Bars = 10 µm.

Pathogenicity test

A pathogenicity trial comprising 7 strains representing seven species in three genera was conducted to determine whether the collected fungi were pathogenic to hazelnut. When inoculated into wounded hazelnut logs, three species, including *Anthostoma decipiens*, *Diaporthella* sp. and *Diplodia seriata* produced necrotic lesions significantly longer than on controls (Fig. 8). *Diaporthella* sp. proved to be the most virulent species. It caused more extensive necrotic lesions (Fig. 9), which often girdled the logs. When logs were cross-sectioned internal wood symptoms included pale-brown discolorations congruent with field observations conducted on open cankers. In cross section logs inoculated with *D. seriata* showed a wedge-shaped necrotic sector (Fig. 9). *Dothiorella iberica*, *D. symphoricarposicola*, *Dothiorella* sp. and *Gnomoniopsis castanea* caused small necrotic lesions around the inoculation point that did not differ statistically from the control (Fig. 9). Control hazelnut logs inoculated with sterile PDA plugs remained symptomless.

All seven species were successfully re-isolated from the margins of developing lesions, thus fulfilling Koch's postulates. Furthermore, *A. decipiens* and *D. seriata* were re-isolated at varying distances on wood below the point of inoculation following a cross section of the logs.

Discussion

Research in this study represents the most comprehensive investigation of hazelnut canker-causing agents to date in Italy. Results obtained have allowed us to clarify the aetiology related to five serious diseases occurring on hazelnut trees in Sardinia. Using comparisons of ITS and EF1-α sequence data combined with the conidia morphology and cultural features, 9 species belonging to five genera and three families were identified from a relatively large collection of symptomatic hazelnut twig and branch samples. These included *Diplodia sapinea*, *D. seriata*, *Dothiorella iberica*, *D. parva*, *D. symphoricarposicola* and *Dothiorella* sp. (*Botryosphaeriaceae*), *Gnomoniopsis castanea* and *Diaporthella* sp. (*Gnomoniaceae*) and *Anthostoma decipiens* (*Diatrypaceae*).

These findings show that the diversity of fungal species associated with twig and branch

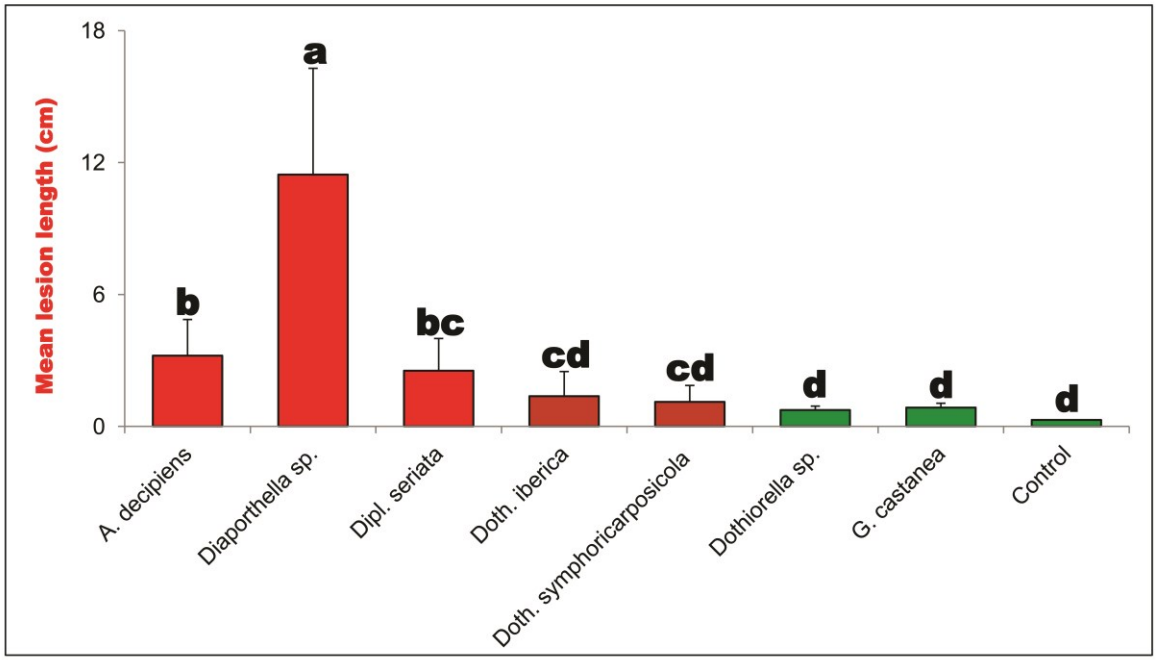

Figure 8. Mean lesion length (cm) caused by isolates of *Anthostoma decipiens*, *Diaporthella* sp., *Diplodia seriata*, *Dothiorella iberica*, *Dothiorella symphoricarposicola*, *Dothiorella* sp. and *Gnomoniopsis castanea* on hazelnut logs. Error bars represent the standard deviations from the mean. Values with the same letter above the bar do not differ significantly at $P = 0.05$ according to LSD multiple range test.

cankers of hazelnut is greater than previously recognised. To our knowledge, all species found in this study, except *D. parva,* are here reported for the first time on hazelnut. Species of *Botryosphaeriaceae* have a worldwide distribution and are known to cause diseases of significant economic importance in agriculture and forestry (Gezahgne et al. 2004; Úrbez-Torres 2011; Phillips et al. 2013; Linaldeddu et al. 2014). Species of this family are associated with a broad range of plant disease symptoms including leaf spots, fruit rot, bud necrosis, shoot dieback, vascular discoloration of the wood and perennial V-shaped cankers (Phillips et al. 2013). However, so far only one of the species recognised in this study was officially known on hazelnut. It was originally isolated from *Corylus avellana* in Spain and reported as *Dothiorella* sp. (strain JL599) by Phillips et al. (2008) and then formally described as *Dothiorella parva* by Abdollahzadeh et al. (2014).

While a separate mention should be made for the strain IMI-501235a, which was originally

found associated with shoot blight and twig and branch cankers of hazelnut cv. Barcelona in Chile and identified as *Diplodia coryli* Fuckel (Guerrero and Perez 2013), but phylogenetic analyses conducted in this study unequivocally identify this strain as *Dothiorella vidmadera* W.M. Pitt, J.R. Úrbez-Torres & Trouillas, a species recently described on grapevine in Australia (Pitt et al. 2013).

Figure 9. Symptoms observed on excised hazelnut branches 30 days after inoculation with *Dothiorella* sp. (a); *Gnomoniopsis castanea* (b); *Dothiorella symphoricarposicola* (c); *Dothiorella iberica* (d) and (e); *Diplodia seriata* (f) and (g); *Anthostoma decipiens* (h) and (i); *Diaporthella* sp. (l) and (m). Control branch (n) and (o).

The other known species of *Dothiorella* isolated in this study include *D. iberica* and *D. symphoricarposicola*. *Dothiorella iberica* is a common and cosmopolitan weak pathogen on a wide variety of hosts including *Cupressus* (Azouaoui-Idjer et al. 2012), *Juniperus communis* (Alves et al. 2013), *Malus* (Phillips et al. 2005), *Persea* (Mcdonald and Eskalen 2011), *Pistacia* (Phillips et al. 2008), *Quercus* (Phillips et al. 2005, 2008, Lynch et al. 2013), *Vitis* (Úrbez-Torres et al. 2007, Qiu et al. 2011, Baskaratevan et al. 2012). In contrast, *D. symphoricarposicola* has only recently been described as a new species associated with dead bark of *Symphoricarpos* sp. in Italy (Li et al., 2014). For this species phylogenetic analyses showed the existence of two evolutionarily distinct lineages within isolates, here reported as lineage A and lineage B. The other four *Dothiorella* isolates obtained in this study were phylogenetically related to *Diplodia coryli* CBS 242.51 and *Diplodia juglandis* CBS 188.87 with which they formed a distinct clade closely related to *Dothiorella vidmadera*. Phillips et al. (2008) considered neither of these two strains authentic, therefore it is not possible to recombine them as *Dothiorella coryli*. Morphologically, conidia of *Dothiorella* sp. differ from those of their two closest relatives, being significantly longer and narrower than *D. vidmadera* and *Dothiorella sempervirentis* (Pitt et al. 2013; Abdollahzadeh et al. 2014). On the basis of results obtained in this thesis a formal description for this new *Dothiorella* species is in preparation. In the pathogenicity trial conducted on excised hazelnut logs, all *Dothiorella* species tested showed to be weak pathogens, causing small necrotic lesions around the inoculation point. Nonetheless, these fungi still need to be monitored carefully, because some of them may spread and act as opportunistic pathogens in a suitable environment. At the moment it is not possible to establish the exact role played by these species in the aetiology of sunken cankers on hazelnut nor their possible synergistic interaction with *Diplodia seriata*, the species most directly associated with this symptom.

Diplodia seriata is a cosmopolitan and plurivorous fungus which occurs on many plant genera and families (Phillips et al. 2007, 2013). Although *D. seriata* has been reported from a wide range of woody hosts worldwide, there are conflicting reports regarding its pathogenicity. In a recent study Elena et al. (2014) demonstrated the existence of different virulence levels in *D. seriata*. Pathogenicity tests performed in this study showed that *D.*

seriata is a hazelnut pathogen and the symptoms artificially obtained were congruent with field observations. One botryosphaeriaceous isolate was identified as *Diplodia sapinea*. This species is a well-known pathogen of conifer worldwide (Phillips et al. 2013). It has also been reported on *Prunus* spp. in South Africa (Damm et al. 2007). The present study is the first report of *D. sapinea* on hazelnut. The hazelnut groves sampled were surrounded by a *Pinus radiata* plantation and it is likely that high inoculum pressure resulted in few infections of the hazelnut trees by *D. sapinea*.

Twenty-eight gnomoniaceous fungi belonging to two distinct genera, namely *Gnomoniopsis* and *Diaporthella*, were isolated in this study from symptomatic hazelnut samples. The genus *Gnomoniopsis* (*Gnomoniaceae*, *Diaporthales*) is currently composed of 17 species (Index Fungorum) which are endophytic and/or parasitic to plants in the families: *Fagaceae*, *Onagraceae* and *Rosaceae* (Walker et al. 2010). In this study the species *Gnomoniopsis castanea,* recently described in Italy associated with nut rot of *Castanea sativa* Miller, was constantly isolated from the inner bark of branch with reddish-brown exudates. Pathogenicity tests showed that *G. castanea* is a weak pathogen on hazelnut. Further studies are needed to elucidate the ecology of this fungus, taking into consideration both that brown rot on nuts of *C. sativa* represents an emerging disease in Italy and that hazelnut and chestnut share the same areas in Sardinia. Interestingly, attacks of brown rot have also occurred in chestnut cultivations in Australia, where the agent was recently described as *Gnomoniopsis smithogilvyi* (Shuttleworth et al. 2012). Phylogenetic analysis (data not shown) revealed that *G. castanea* and *G. smithogilvyi* are in fact the same species.

Twenty gnomoniaceous isolates were phylogenetically (ITS and EF1-α sequences data) distinct from all other known fungal species for which nucleotide sequences are available in public databases. A BLAST search generated significant alignments only with the species *Diaporthella corylina*, suggesting that the isolates studied belong to an undescribed species of *Diaporthella*. The genus *Diaporthella* Petr. is based on the type species *D. aristata* (Fr.) Petr. (Petrak 1924) and currently only four species are listed in Index Fungorum. Currently, a detailed morphological description and DNA sequence data are available only for *D. corylina*. *Diaporthella corylina* has been reported as a virulent pathogen on *Corylus* spp. by Vasilyeva

et al. (2007). These data agree with those obtained in this study regarding the virulence of *Diaporthella* sp. This fungus is directly involved in the aetiology of shoot blight and branch canker symptoms.

About a century after it was first reported in Italy, there were still doubts about the aetiology of the disease named Cytospora canker (*Mal dello Stacco*). Trotter (1933) initially attributed the disease to *Cytospora corylicola*, but subsequently other authors expressed doubts about the true identity of the Cytospora canker-agent reporting it as *Cytospora* sp. (Servazzi 1950; Graniti 1957). In addition, recently published papers on this disease do not report any information about the morphology and phylogeny of the pathogen. Investigations carried out in this study allowed us to clarify unequivocally that the pathogen responsible for this disease is *Anthostoma decipiens*. This fungus produces in culture Cytospora-like colonies and the appearance of these probably triggered the confusion over the identity of the fungus. Recently, *A. decipiens* has been associated with a decline of the European hornbeam (*Carpinus betulus* L.) in Italy, and its pathogenicity on this host has been verified (Saracchi et al. 2008; Rocchi et al. 2010). The intraspecific diversity of *A. decipiens* recognised in this study was not related to the geographical origin of isolates or plant hosts. The isolates obtained in this study clustered with all three lineages identified. Since Cytospora canker is an emerging disease in various hazelnut groves in Italy, further studies are needed to investigate the intraspecific variability of *A. decipiens* and the possible presence of pathotypes.

In conclusion, this study shows that *D. seriata* is the predominant botryosphaeriaceous fungi associated with V-shaped cankers on hazelnut. However, given the high number of *Botryosphaeriaceae* found and their different assemblage, the exact relationship between fungal species and wood disease symptoms has been difficult to determine and requires further investigations. In addition, the undescribed species of *Diaporthella* has been recognised as a very aggressive hazelnut pathogen suggesting the need to expand the knowledge about the ecology, epidemiology, biogeography and infection biology of this pathogen which represents a serious threat to the hazelnut industry.

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Chapter 4

Fungal Diversity
2014
67: 143-156

The complex of *Diplodia* species associated with *Fraxinus* and some other woody hosts in Italy and Portugal

Abstract Studies on the taxonomy and phylogeny of *Diplodia* have been hampered by the lack of an ex-type culture linked to the holotype of *D. mutila*, which is the type of the genus. In this study a large collection of *Diplodia* strains, obtained from ash and other woody hosts showing V-shaped cankers and branch dieback, were identified based on morphological characters and DNA sequence data from ITS and EF1-α loci. Results of combined morphological and phylogenetic analyses showed that the *Fraxinus* isolates from Italy, the Netherlands, Portugal and Spain belong to three distinct species namely *Diplodia fraxini*, *Diplodia mutila* and *Diplodia subglobosa* sp. nov. An epitype was designated for *Diplodia mutila*, with associated ex-epitype cultures. The name *D. fraxini* is re-instated and a neotype designated. Two species, *Diplodia seriata* and *Diplodia pseudoseriata* were reported for the first time on *Fraxinus* spp.

Keywords *Botryosphaeriaceae* - Epitype - Neotype - Phylogeny - Systematics - Taxonomy

Introduction

Fraxinus L. (ash) is a tree genus native to the temperate and subtropical regions of the Northern Hemisphere. It belongs to the family *Oleaceae* and includes 43 species, most of which are large or medium-sized trees, with some shrub species widespread in dry areas (Wallander 2008). Three species, *F. angustifolia* Vahl (narrow leaved ash), *F. excelsior* L. (European ash) and *F. ornus* L. (manna ash) are widely planted as ornamentals.

Since the early 1990s severe branch dieback of *F. excelsior* was observed in different countries of Central, Eastern and Northern Europe (Przybyl 2002; Lygis et al. 2005; Pukacki and Przybyl 2005; Bakys et al. 2009). All of these studies demonstrated the occurrence of several pathogenic fungi from necrotic shoots of ash. Among these, *Diplodia mutila* (Fr.) Fr. was one of the most consistently detected species. This pathogen was also reported associated with cankers and branch dieback of *F. ornus* in Sicily (Italy) (Sidoti and Granata 2004).

In recent years, during surveys carried out in Portugal and Sardinia (Italy) aimed at clarifying the causes of a decline affecting *Fraxinus* spp. in urban and natural areas, a large collection of *Diplodia mutila*-like strains were isolated from trees showing V-shaped cankers and a progressive dieback of shoots and branches. Although morphologically similar to *D. mutila*, some of these strains differed in their colony morphology, larger conidia and DNA sequence data (ITS and EF1-α) from other known strains of *D. mutila*. This species has been reported from a wide range of hosts of agricultural and forestry importance, where it has been associated with canker, dieback and fruit rot symptoms (Farr and Rossman 2013). However, there are conflicting reports regarding its pathogenicity, and in particular, on *Fraxinus* spp. (Przybyl 2002; Sidoti and Granata 2004; Bakys et al. 2009), which may be a result of differences in strain virulence but may also be due to the existence of cryptic species. Cryptic speciation is common in the family *Botryosphaeriaceae* and in the genus *Diplodia*, which renders species identification difficult when based solely on morphological characters (Phillips et al. 2012, 2013). Fries (1823) described the species *Sphaeria fraxini* Fr. on *Fraxinus* sp., and later (Fries 1849) he transferred it to *Diplodia* as *Diplodia fraxini* (Fr. : Fr.) Fr. Subsequently, this fungus was placed by Saccardo (1884) in the genus *Botryodiplodia* (Sacc.) Sacc. as *B. fraxini* (Fr. : Fr.) Sacc. Unfortunately, no ex-type cultures are available for this species.

Currently, 17 *Diplodia* species are known from culture (Phillips et al. 2013). These species have been recognised mainly on the basis of DNA sequence data (single or multilocus) and minor differences in conidial morphology (de Wet et al. 2003; Alves et al. 2004, 2006; Gure et al. 2005; Damm et al. 2007; Lazzizera et al. 2008; Pérez et al. 2010; Jami et al. 2012; Phillips et al. 2012, 2013; Linaldeddu et al. 2013; Lynch et al. 2013). For the majority of species there are ex-type or ex-epitype cultures deposited in publicly available culture collections that can serve as standards for the morphological and molecular characterisation of the species. The only exception is *D. mutila* for which several cultures are available but none has been linked to the type of the species. The lack of an ex-type culture, or other cultures linked to the holotype of *D. mutila* has hampered taxonomic studies on the genus *Diplodia* (which is typified by *D. mutila*), especially those based on DNA sequence data.

Therefore, the main aims of this work were: 1) to characterise collections of *D.* mutila-like

and other *Diplodia* spp. isolates in terms of morphological and phylogenetic relationships to known *Diplodia* species; 2) to select a suitable epitype specimen for *D. mutila* and a neotype specimen for *D. fraxini* with related cultures that can be made available for future studies.

Materials and methods

Isolates and morphology

Diplodia isolates used in this study were obtained from branches of *F. angustifolia* and *F. ornus* showing sunken cankers and dieback. *Diplodia* species isolated from other symptomatic trees including *Populus alba* L. (white poplar), *Cupressus sempervirens* L. (Italian cypress) and *Quercus coccifera* L. (kermes oak) were also included in this study. Isolations were made from chips of inner bark and wood tissues approx. 5 mm^2 cut aseptically from the margin of necrotic lesions or directly from pycnidia. All samples were cultured on potato dextrose agar (PDA, Oxoid Ltd.) in Petri dishes. After incubation at 25 °C for 1 week, colonies were sub-cultured onto half-strength PDA (1/2 PDA) or on water agar supplemented with autoclaved poplar twigs to enhance sporulation. All colonies were kept on the laboratory bench at about 20–25 °C where they received diffused daylight.

Monoconidial cultures were obtained by spreading conidia on the surface of PDA and incubating overnight at 25 °C. Single germinating spores were transferred to fresh plates of PDA. Cardinal temperatures for growth were determined on PDA plates (90 mm) incubated at 5, 10, 15, 20, 25, 30, 35 and 40 °C (±0.5 °C) in the dark. Five replicate plates for each isolate were made and colony diameters were measured after 4 days.

For microscopy, the contents of conidiomata were dissected out and mounted in 100 % lactic acid. For observations of conidiogenesis, the conidiogenous layer was dissected out and mounted in 100 % lactic acid. Measurements of conidia were made with the Leica IM 500 measurement module from images recorded on a Leica DFC 320 digital camera. From measurements of 50 conidia the mean, standard deviation and 95 % confidence intervals were calculated. Conidial dimensions are given as the range of dimensions with extremes in parentheses. Dimensions of other structures are given as the range of at least 20 measurements.

Representative isolates were deposited at the Centraalbureau voor Schimmelcultures (CBS), Utrecht, the Netherlands and nomenclatural data in MycoBank (Crous et al. 2004). Specimens were lodged with the herbarium of Estação Agronómica Nacional, Oeiras, Portugal (LISE). Isolates used for phylogenetic analyses in this study are provided (Table 1).

DNA extraction, PCR amplification and sequencing

DNA was isolated from fungal mycelium by the method of Santos and Phillips (2009). Procedures and protocols for DNA sequencing were as described by Alves et al. (2004). PCR reactions were carried out with Taq polymerase, nucleotides and buffers supplied by MBI Fermentas (Vilnius, Lithuania) and PCR reaction mixtures were prepared according to Alves et al. (2004), with the addition of 5 % DMSO to improve the amplification of some difficult DNA templates. All primers were synthesised by MWG Biotech AG (Elbersberg, Germany). The ITS region was amplified using the primers ITS1 and ITS4 (White et al. 1990) as described by Alves et al. (2004). The primers EF1-728F and EF1-986R (Carbone and Kohn 1999) were used to amplify part of the translation elongation factor 1-α gene (EF1-α) as described by Alves et al. (2006). The amplified PCR products were purified with the JETQUICK PCR Purification Spin Kit (GENOMED, Löhne, Germany). The PCR products were sequenced by STAB Vida Lda (Portugal).

Phylogenetic analysis

The ITS and EF1-α sequences were combined and the dataset, including sequences of other *Diplodia* species downloaded from GenBank, was compiled with the outgroup *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl. (Table 1). Sequences were aligned with ClustalX v. 1.83 (Thompson et al. 1997), using the following parameters: pairwise alignment parameters (gap opening=10, gap extension=0.1) and multiple alignment parameters (gap opening=10, gap extension=0.2, transition weight=0.5, delay divergent sequences=25 %). Alignments were checked and manual adjustments were made where necessary.

Table 1 Isolates used in this study

Isolate number[1]	Species	Host	Locality	Collector	GenBank	
					ITS[2]	EF
CBS 120835	*D. africana*	*Prunus persica*	South Africa	U. Damm	*EF445343*	*EF445382*
CBS 121104	*D. africana*	*Prunus persica*	South Africa	U. Damm	*EF445344*	*EF445383*
DA1	*D. africana*	*Juniperus phoenicea*	Italy	B.T. Linaldeddu	*JF302648*	*JN157807*
CBS 132777	*D. agrifolia*	*Quercus agrifolia*	USA	S.C. Lynch & A. Eskalen	*JN693507*	*JQ517317*
UCROK1429	*D. agrifolia*	*Quercus agrifolia*	USA	S.C. Lynch & A. Eskalen	*JQ411412*	*JQ512121*
CBS 124931	*D. alatafructa*	*Pterocarpus angolensis*	South Africa	J. Mehl & J. Roux	*FJ888460*	*FJ888444*
CBS 124933	*D. alatafructa*	*Pterocarpus angolensis*	South Africa	J. Mehl & J. Roux	*FJ888478*	*FJ888446*
CBS 130408	*D. allocellula*	*Acacia karroo*	South Africa	F. Jami & M. Gryzenhout	*JQ239397*	*JQ239384*
CBS 130410	*D. allocellula*	*Acacia karroo*	South Africa	F. Jami & M. Gryzenhout	*JQ239399*	*JQ239386*
CBS 124254	*D. bulgarica*	*Malus sylvestris*	Bulgaria	S. Bobev	*GQ923853*	*GQ923821*
CBS 124135	*D. bulgarica*	*Malus sylvestris*	Bulgaria	S. Bobev	*GQ923852*	*GQ923820*
CBS 112549	*D. corticola*	*Quercus suber*	Portugal	A. Alves	*AY259100*	*AY573227*
CBS 112547	*D. corticola*	*Quercus ilex*	Spain	Sanchez &. Trapero	*AY259110*	*DQ458872*
BL7	*D. corticola*	*Quercus afares*	Tunisia	B.T. Linaldeddu	*JX894190*	*JX894209*
BL11	*D. corticola*	*Quercus ilex*	Italy	B.T. Linaldeddu	*JX894192*	*JX894211*
CBS 168.87	*D. cupressi*	*Cupressus sempervirens*	Israel	Z. Solel	*DQ458893*	*DQ458878*
CBS 261.85	*D. cupressi*	*Cupressus sempervirens*	Israel	Z. Solel	*DQ458894*	*DQ458879*
BL102	*D. cupressi*	*Cupressus sempervirens*	Tunisia	B.T. Linaldeddu	KF307722	KF318769
CBS 431.82	*D. fraxini*	*Fraxinus excelsior*	Netherlands	H.A. van der Aa	*AY236955*	*AY236904*
BL16	*D. fraxini*	*Fraxinus angustifolia*	Italy	B.T. Linaldeddu	KF307710	KF318757
BL70	*D. fraxini*	*Fraxinus angustifolia*	Italy	B.T. Linaldeddu	KF307711	KF318758
BL135	*D. fraxini*	*Fraxinus angustifolia*	Italy	B.T. Linaldeddu	KF307712	KF318759
BL136	*D. fraxini*	*Fraxinus angustifolia*	Italy	B.T. Linaldeddu	KF307713	KF318760
BL137	*D. fraxini*	*Fraxinus angustifolia*	Italy	B.T. Linaldeddu	KF307714	KF318761
BL138	*D. fraxini*	*Fraxinus angustifolia*	Italy	B.T. Linaldeddu	KF307715	KF318762
CAD001	*D. fraxini*	*Fraxinus angustifolia*	Portugal	A. Deidda	KF307700	KF318747
CAD002	*D. fraxini*	*Fraxinus angustifolia*	Portugal	A. Deidda	KF307701	KF318748
CAD003	*D. fraxini*	*Fraxinus angustifolia*	Portugal	A. Deidda	KF307702	KF318749
CAD004	*D. fraxini*	*Fraxinus angustifolia*	Portugal	A. Deidda	KF307703	KF318750

CAD005	*D. fraxini*	*Fraxinus angustifolia*	Portugal	A. Deidda	KF307704	KF318751
CAD006	*D. fraxini*	*Fraxinus angustifolia*	Portugal	A. Deidda	KF307705	KF318752
CAD007	*D. fraxini*	*Fraxinus angustifolia*	Portugal	A. Deidda	KF307706	KF318753
CAD008	*D. fraxini*	*Fraxinus angustifolia*	Portugal	A. Deidda	KF307707	KF318754
CAD009	*D. fraxini*	*Fraxinus angustifolia*	Portugal	A. Deidda	KF307708	KF318755
CAD010	*D. fraxini*	*Fraxinus angustifolia*	Portugal	A. Deidda	KF307709	KF318756
CBS 112556	*D. intermedia*	*Malus sylvestris*	Portugal	A.J.L. Phillips	*AY259096*	*GQ923851*
CAA147	*D. intermedia*	*Malus domestica* (fruit rot)	Portugal	A. Alves	*GQ923857*	*GQ923825*
CBS 124130	*D. malorum*	*Malus sylvestris*	Portugal	A.J.L. Phillips	*GQ923865*	*GQ923833*
CBS 112554	*D. malorum*	*Malus sylvestris*	Portugal	A.J.L. Phillips	*AY259095*	*DQ458870*
BL126	*D. malorum*	*Populus alba*	Italy	B.T. Linaldeddu	KF307716	KF318763
BL127	*D. malorum*	*Populus alba*	Italy	B.T. Linaldeddu	KF307717	KF318764
CBS 302.36	*D. mutila* (as *P. mutila*)	unknown	unknown	N.E. Stevens		
CBS 112553	*D. mutila*	*Vitis vinifera*	Portugal	A.J.L. Phillips	*AY259093*	*AY573219*
CAA096	*D. mutila*	*Taxus baccata*	Portugal	A. Alves	*JX878523*	
CAA115	*D. mutila*	*Chamaecyparis lawsoniana*	Portugal	A. Alves	*JX878524*	
CBS 136014	*D. mutila*	*Populus alba*	Portugal	A. Alves		
CBS 136015	*D. mutila*	*Populus alba*	Portugal	A. Alves		
CBS 136016	*D. mutila*	*Fraxinus ornus*	Portugal	A. Alves		
CBS 136017	*D. mutila*	*Fraxinus ornus*	Portugal	A. Alves		
CBS 230.30	*D. mutila*	*Phoenix dactylifera*	USA	L. L. Huillier	*DQ458886*	*DQ458869*
BL98	*D. mutila*	*Vitis vinifera*	Italy	A. Deidda	KF307718	KF318765
PD46	*D. mutila*	unknown	USA	T.J. Michailides	*GU251116*	*GU251248*
PD61	*D. mutila*	*Persea americana*	USA	T.J. Michailides	*GU251117*	*GU251249*
PD73	*D. mutila*	*Ilex* sp.	USA	T.J. Michailides	*GU251118*	*GU251250*
PD75	*D. mutila*	*Ilex* sp.	USA	T.J. Michailides	*GU251119*	*GU251251*
STE-U5038	*D. mutila*	*Vitis vinifera*	Portugal	A.J.L. Phillips	*AY343484*	*AY343370*
STE-U5824	*D. mutila*	*Prunus salicina*	South Africa	U. Damm	*EF445346*	*EF445381*
UCD288Ma	*D. mutila*	*Vitis vinifera*	USA	J.R. Úrbez-Torres	*DQ008313*	*EU012411*
CBS 121887	*D. olivarum*	*Olea europaea*	Italy	S. Frisullo	*EU392302*	*EU392279*
CAP301	*D. olivarum*	*Ceratonia siliqua*	Italy	A. Sidoti	*GQ923873*	*GQ923841*
BL97	*D. olivarum*	*Quercus coccifera*	Tunisia	B.T. Linaldeddu	KF307719	KF318766
CBS 109725	*D. pinea*	*Pinus patula*	Indonesia	M.J. Wingfield	*DQ458896*	*DQ458881*

CBS 109727	*D. pinea*	*Pinus radiata*	South Africa	W.J. Swart	*DQ458897*	*DQ458882*
CBS 393.84	*D. pinea*	*Pinus nigra*	Netherlands	H.A. van der Aa	*DQ458895*	*DQ458880*
CBS 109943	*D. pinea*	*Pinus patula*	Indonesia	M.J. Wingfield	*DQ458898*	*DQ458883*
CBS 124906	*D. pseudoseriata*	*Blepharocalyx salicifolius*	Uruguay	C. Pérez	*EU080927*	*EU863181*
UY1263	*D. pseudoseriata*	*Myrciaria tenella*	Uruguay	C. Pérez	*EU080933*	*EU863182*
BL132	*D. pseudoseriata*	*Fraxinus angustifolia*	Italy	B.T. Linaldeddu	KF307720	KF318767
BL133	*D. pseudoseriata*	*Fraxinus angustifolia*	Italy	B.T. Linaldeddu	KF307721	KF318768
CBS 133852	*D. quercivora*	*Quercus canariensis*	Tunisia	B.T. Linaldeddu	*JX894205*	*JX894229*
CBS 133853	*D. quercivora*	*Quercus canariensis*	Tunisia	B.T. Linaldeddu	*JX894206*	*JX894230*
CBS 116470	*D. rosulata*	*Prunus africana*	Ethiopia	A. Gure	*EU430265*	*EU430267*
CBS 116472	*D. rosulata*	*Prunus africana*	Ethiopia	A. Gure	*EU430266*	*EU430268*
CBS 109944	*D. scrobiculata*	*Pinus greggii*	Mexico	M.J. Wingfield	*DQ458899*	*DQ458884*
CBS 113423	*D. scrobiculata*	*Pinus greggii*	Mexico	M.J. Wingfield	*DQ458900*	*DQ458885*
CAP163	*D. scrobiculata*	*Olea europaea*	Italy	S. Frisullo	*EU392283*	*EU392260*
BL5	*D. scrobiculata*	*Arbutus unedo*	Italy	B.T. Linaldeddu	*GU722102*	*JX894231*
CBS 112555	*D. seriata*	*Vitis vinifera*	Portugal	A.J.L. Phillips	*AY259093*	*AY573219*
CBS 119049	*D. seriata*	*Vitis vinifera*	Italy	L. Mugnai	*DQ458889*	*DQ458874*
CAA502	*D. seriata*	*Fraxinus ornus*	Portugal	A. Alves		
BL130	*D. seriata*	*Fraxinus angustifolia*	Italy	B.T. Linaldeddu	KF307723	KF318770
BL131	*D. seriata*	*Fraxinus angustifolia*	Italy	B.T. Linaldeddu	KF307724	KF318771
CBS 124131	*D. subglobosa*	*Fraxinus ornus*	Italy	A. Sidoti	*GQ923855*	*GQ923823*
CBS 124132	*D. subglobosa*	*Fraxinus excelsior*	Spain	J. Luque	*DQ458887*	*DQ458871*
CBS 124133	*D. subglobosa*	*Lonicera nigra*	Spain	J. Luque	*GQ923856*	*GQ923824*
CMW7776	*D. subglobosa*	*Fraxinus excelsior*	Italy	B. Slippers	*AY972106*	*DQ280420*
CBS 418.64	*D. tsugae*	*Tsuga heterophylla*	Canada	A. Funk	*DQ458888*	*DQ458873*
CBS 124.13	*L. theobromae*	unknown	USA	J.J. Taubenhaus	*DQ458890*	*DQ458875*
CBS 164.96	*L. theobromae*	Fruit along coral reef coast	New Guinea	A. Aptroot	*AY640255*	*AY640258*

[1]Acronyms of culture collections: BL – B.T. Linaldeddu, Università degli Studi di Sassari, Italy; CAA – A. Alves, Universidade de Aveiro, Portugal; CAD – A. Deidda, Università degli Studi di Sassari, Italy; CAP – A.J.L. Phillips, Universidade Nova de Lisboa, Portugal; CBS – Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CMW – M.J. Wingfield, FABI, University of Pretoria, South Africa; PD – Department of Plant Pathology, University of California, Davis; STE-U – University of Stellenbosch, South Africa; UCD – Phaff Yeast Culture Collection, Department of Food Science and Technology, University of California, Davis, USA; UCROK – Department of Plant Pathology and Microbiology, University of California, Riverside; UY – Department of Plant Pathology, University of Minnesota. Isolates in bold are cultures ex-type.

[2]Sequence numbers in italics were retrieved from GenBank. All others were obtained in the present study.

Phylogenetic analyses of sequence data were done using PAUP* v.4.0b10 (Swofford 2003) for Maximum-parsimony (MP) analyses and Mr Bayes v.3.0b4 (Ronquist and Huelsenbeck 2003) for Bayesian Inference (BI) analyses. The general time-reversible model of evolution (Rodriguez et al. 1990), including estimation of invariable sites and assuming a discrete gamma distribution with six rate categories (GTR+Γ+G) was used for BI analyses. Trees were visualized with TreeView (Page 1996).

Maximum-parsimony analyses were performed using the heuristic search option with 1,000 random taxon additions and tree bisection and reconnection (TBR) as the branch-swapping algorithm. All characters were unordered and of equal weight and gaps were treated as fifth character. Maxtrees were set to 500, branches of zero length were collapsed, and all multiple equally parsimonious trees were saved. The robustness of the most parsimonious trees was evaluated from 1,000 bootstrap replications (Hillis and Bull 1993). Other measures used were consistency index (CI), retention index (RI) and homoplasy index (HI). Bayesian analyses employing a Markov Chain Monte Carlo method were performed. Four MCMC chains were run simultaneously, starting from random trees for 1,000,000 generations. Trees were sampled every 100th generation for a total of 10,000 trees. The first 1,000 trees were discarded as the burn-in phase of each analysis. Posterior probabilities (Rannala and Yang 1996) were determined from a majority-rule consensus tree generated with the remaining 9,000 trees. This analysis was repeated three times starting from different random trees to ensure trees from the same tree space were sampled during each analysis. A comparison of highly supported clades (bootstrap support values≥70 %) among trees generated from MP analyses of individual data sets was performed in order to detect conflict between individual phylogenies (Alves et al. 2008).

Results

DNA phylogeny

Approximately 550 and 300 bases were determined for ITS and EF1-α respectively. New sequences were deposited in GenBank (Table 1) and the alignment in TreeBase (14460). Individual gene phylogenies revealed no major conflicts thus indicating that the two loci could

be combined. The combined ITS and EF1-α dataset consisted of 853 characters (including alignment gaps) for 87 ingroup and 2 outgroup taxa. Of the 853 characters 165 were excluded due to ambiguous alignment, 538 were constant and 9 were variable and parsimony-uninformative. Maximum parsimony analysis of the remaining 141 parsimony-informative characters resulted in 279 most parsimonious trees of 302 steps (CI=0.717, RI=0.945, HI=0.283) and one is shown in Fig. 1.

In the phylogenetic analysis four main clades (labeled 1 to 4) were resolved in the ingroup (Fig. 1). These clades are characterized by distinct conidial morphological features as explained in detail by Phillips et al. (2012, 2013).Within these four main clades, 19 sub-clades corresponding to species were recognized; of which 17 represent known *Diplodia* species (Fig. 1). The *D. mutila*-like isolates studied were distributed into 5 sub-clades within clade 1. Thus, two isolates from *P. alba* were identified as *Diplodia malorum* Fuckel; one isolate from *Q. coccifera* was identified as *Diplodia olivarum* A.J.L. Phillips, Frisullo & Lazzizera and a set of isolates from *P. alba*, *F. ornus* and *Vitis vinifera* L. (grapevine) clustered in a larger group identified as *D. mutila*. For one of the remaining two clades the name *Diplodia fraxini* (Fr. : Fr.) Fr. was deemed to be appropriate and this name is reinstated for a set of isolates obtained from *F. excelsior* and *F. angustifolia*. The second clade containing isolates from *F. excelsior*, *F. ornus* and *Lonicera nigra* L. (black-berried honeysuckle) represents a previously unrecognized species, which is described here as *Diplodia subglobosa* sp. nov.

The remaining isolates, morphologically distinct from *D. mutila*, were distributed amongst clades 2 and 3 and were identified as *Diplodia cupressi* A.J.L. Phillips & A. Alves (one isolate from *C. sempervirens*), *Diplodia seriata* De Not. (one isolate from *F. ornus* and two isolates from *F. excelsior*) and *Diplodia pseudoseriata* C.A. Pérez, Blanchette, Slippers & M.J. Wingf. (two isolates from *F. angustifolia*).

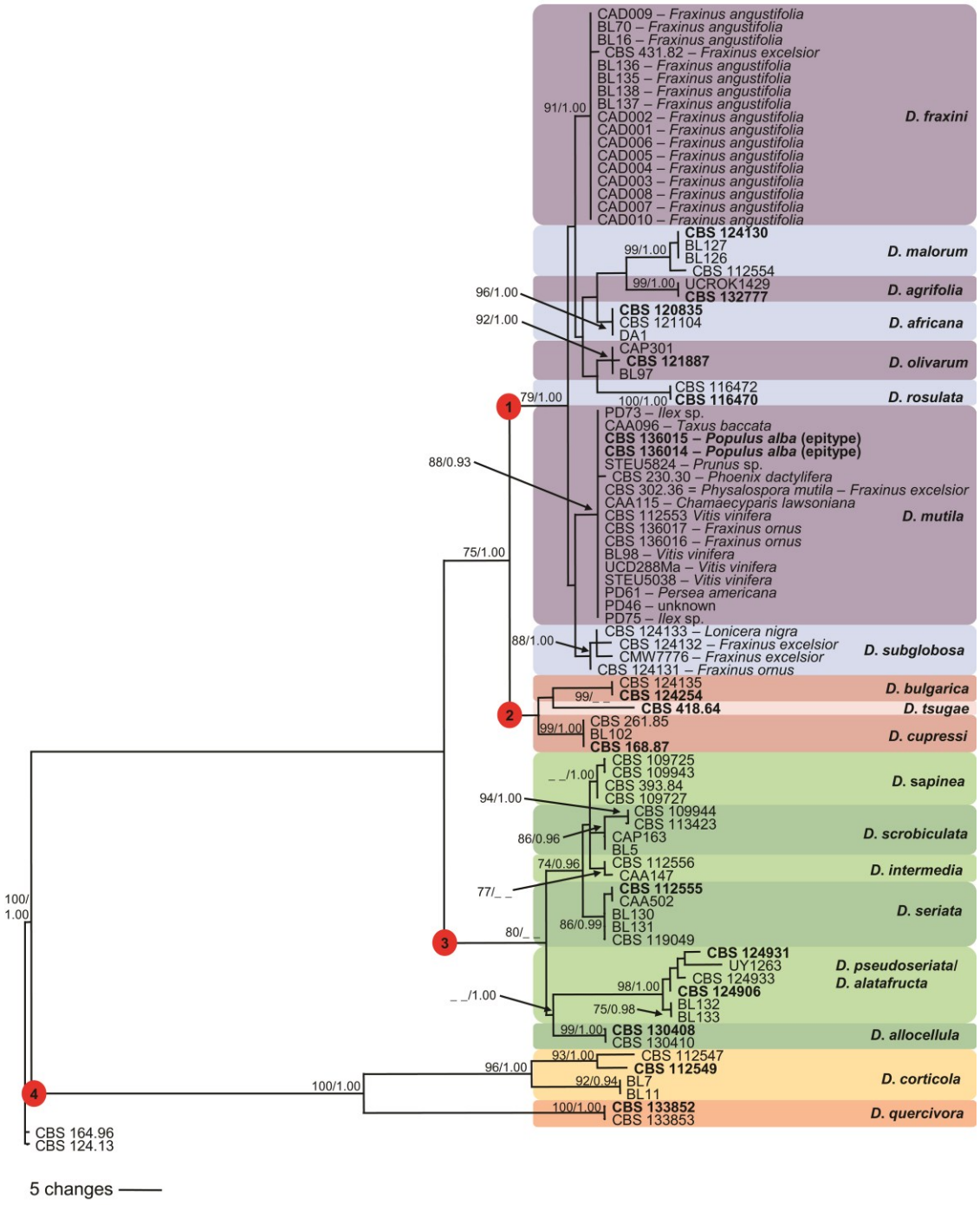

Figure 1 One of the 279 most parsimonious trees resulting from the combined analysis of ITS and EF1-α sequence data. Bootstrap support and posterior probability values are given at the nodes. The tree was rooted to *Lasiodiplodia theobromae*. Ex-type isolates are in bold. The bar shows five changes

Taxonomy

Diplodia Fr., in Montagne, Ann. Sci. Nat., Bot., 2e Sér., 1: 302. 1834.

MycoBank: MB8047

Diplodia fraxini (Fr. : Fr.) Fr., Summa Veg. Scand. 2: 417. 1849.

MycoBank: MB247549 (Fig. 2)

≡ *Sphaeria fraxini* Fr. : Fr., Syst. Mycol. 2: 493. 1823.

≡ *Botryodiplodia fraxini* (Fr. : Fr.) Sacc., Syll. Fung. 3: 378. 1884.

Ascomata not seen. Conidiomata stromatic, pycnidial, solitary to aggregated, immersed, partially erumpent when mature, dark brown to black, globose, up to 600 µm diam., wall composed of three layers, an outer of dark brown, thick-walled textura angularis, a middle layer of dark brown thin-walled cells, an inner layer of thin-walled hyaline cells. Ostiole central, circular, papillate. Conidiophores absent. Conidiogenous cells (11–)12–15(–16.5)×(2.5–)3.5–4.5(–5) µm, holoblastic, discrete, cylindrical, hyaline, smooth, indeterminate, proliferating at the same level giving rise to periclinal thickenings, or proliferating percurrently to form one or two indistinct annellations. Conidia hyaline, aseptate, smooth, thick-walled, oblong to ovoid, straight, both ends broadly rounded, becoming pale brown to brown and one-two septate with age.

Significant differences in conidial dimensions were observed among the *D. fraxini* isolates considered in this work. In most of the isolates the conidia were (23.5−)25–27(–30)×(11−)12.5–13.5(−15) µm, 95 % confidence limits = 25.72–26.45×12.87–13.33 µm (mean ± S.D. of 50 conidia = 26.08±1.31×13.10±0.83 µm, L/W ratio=2.00±0.14), which correspond to the conidial dimensions reported by Saccardo (1884) and for this reason these isolates are here considered as typical of the species.

Two isolates, one from Portugal and one from Sardinia, consistently produced longer conidia measuring (26.5−)29–31.5(–33)×(11−)12.5–14(−17.5) µm, 95 % confidence limits = 29.28–30.23×13.12–13.83 µm (mean ± S.D. of 50 conidia = 29.76±1.72×13.47±1.28 µm, L/W ratio = 2.22±0.19). Since these isolates are phylogenetically indistinguishable from the typical ones we regard these as a morphological variant and report them as *Diplodia fraxini* morphotype A.

Cultural characteristics: Colonies on PDA grew moderately, reaching 90 mm diameter or less in 7 days at 25 °C, the mycelium was sparsely to moderately aerial, surface white at first and later turned pale to dark grey and greyish to brown in reverse (Fig. 3). Colonies of morphotype A on PDA grey-brown with dense aerial mycelium.

Cardinal temperatures for growth: minimum 5 °C, maximum <35 °C and optimum 25 °C.

Habitat: On branches of *F. angustifolia* and *F. excelsior*.

Known distribution: Italy, Portugal and the Netherlands (this paper), Sweden, Germany, Italy, France (Saccardo 1884).

Specimens examined: PORTUGAL, Monte da Caparica, on dead twigs of *Fraxinus angustifolia*, 14 March 2013, Antonio Deidda (LISE 96134, neotype designated herein), MBT176183, culture ex-neotype CBS 136010 = CAD001. Cascais, on dead twigs of *F. angustifolia*, 13 April 2013, Antonio Deidda, designated morphotype A, culture CBS 136012 = CAD010. ITALY, Bortigiadas, isolated from a branch canker of *F. angustifolia*, 03 June 2011, Benedetto T. Linaldeddu, culture CBS 136011 = BL70. ITALY, Siliqua, isolated from a branch canker of *F. angustifolia*, 11 November 2009, Benedetto T. Linaldeddu, CBS 136013 = BL16 (morphotype A). Additional isolates are given in Table 1.

Notes: Fries (1849) did not refer specifically to any previous description and gave only a brief Latin comment "Vidi triloc." (= "I have seen trilocular [pycnidia]"). Nevertheless, the binomial *Diplodia fraxini* should be interpreted as a recombination based on *Sphaeria fraxini* as suggested by Saccardo (1884). The holotype of neither *S. fraxini* nor *D. fraxini* (on a branch of *Fraxinus* sp. collected in Sweden by Fries) could be located and are presumed lost. For this reason a neotype (LISE 96134) is designated here. All except two of the isolates studied here conformed morphologically to Saccardo's (1884) description of the species. The morphotype A isolates, with large conidia, were phylogenetically indistinguishable from typical *D. fraxini* isolates and thus were considered to be a morphological variant of *D. fraxini*.

Figure 2 *Diplodia fraxini*. a Colony of typical *D. fraxini* after 7 days growth at 25 °C on PDA. b colony morphology of *D. fraxini* morphotype A growing on PDA. c conidia oozing from picnidia. d–e conidia developing on conidiogenous cells. f–k conidiogenous cells with periclinal thickenings (arrowed). l hyaline conidia of *D. fraxini* morphotype A. m hyaline conidia of *D. fraxini*. n hyaline and one pale brown aseptate conidia. o pale brown one septate conidium. p mature, brown conidium showing two septa. Bars: c=1 mm; d–p=10 μm

Diplodia mutila (Fr. : Fr.) Fr., Summa Veg. Scand. 2: 417. 1849.

MycoBank: MB201741 (Fig. 3)

≡ *Sphaeria mutila* Fr. : Fr., Syst. Mycol. 2: 424. 1823.

≡ *Physalospora mutila* (Fr. : Fr.) N.E. Stevens, Mycologia 28: 333. 1936.

≡ *Botryosphaeria stevensii* Shoemaker, Can. J. Bot. 42: 1299. 1964.

Ascomata unilocular, solitary or clustered, immersed, partially erumpent when mature, globose, up to 300 μm diam., dark brown to black, thick-walled, wall composed of outer layers of thick-walled, dark-brown textura angularis, inner layers of thin-walled, hyaline, textura angularis. Ostiole central, circular, papillate, periphysate. Pseudoparaphyses hyaline, branched, septate, 2–3 μm wide, constricted at septa. Asci clavate, stipitate, bitunicate with a thick endotunica and well-developed apical chamber, 100–160×14–22 μm (including stipe), containing eight, biseriate ascospores. Ascospores (25–)28–35(–36)×(9.5–)10–12.5(–13.5) μm; 95 % confidence limits=30.8–32.1×11.2–11.7 μm (mean ± S.D. of 50 ascospores=31.5±2.3×11.4±0.9 μm) with L/W of 2.8±0.3, fusiform to oval, widest in the middle, both ends obtuse, hyaline, thin-walled, smooth, aseptate, rarely becoming light brown with age. Conidiomata solitary or aggregated in clusters of up to five or more, immersed, partially erumpent when mature, dark brown to black, more or less globose, up to 600 μm diam., wall composed of three layers, an outer of dark brown, thick-walled textura angularis, a middle layer of dark brown thin-walled cells, an inner layer of thin-walled hyaline cells. Ostiole central, circular, papillate. Conidiophores absent. Conidiogenous cells (11−)12–15×4–5 μm, holoblastic, discrete, cylindrical, hyaline, smooth, indeterminate, proliferating at the same level giving rise to periclinal thickenings, or proliferating percurrently to form one or two indistinct annellations. Conidia hyaline, aseptate, smooth, thick-walled, oblong to ovoid, straight, both ends broadly rounded, (20−)21.5–25.5(–27.5)×(9.5−)12–14(−15.5) μm, 95 % confidence limits=24.69–25.73×13.26–13.78 μm (mean ± S.D. of 50 conidia=25.4±1.0×13.4±0.5 μm, L/W ratio=1.9±0.1), rarely becoming pale brown and one-septate with age. Table 2 illustrate conidial sizes for all species of *Diplodia* belonging to clade 1.

Tab 2 Conidial sizes and L/W ratios

Species	Conidia		References
	Size (µm)	L/W ratio	
D. mutila Fr.	(23.5–)25.1–25.7(–27.4) (12.4–)13.2–13.5(–14.3)	1.9	Alves et al. (2004)
D. agrifolia S.C. Lynch & A. Eskalen	(21.3–)27.0–36.5 (12.1–)14.5–17.8	1.9	Lynch et al. (2013)
D. africana Damm & Crous	(17–)25.5–33(–34) (10–)12–14(–15)	2.2	Damm et al. (2007)
D. olivarum A.J.L. Phillips, Frisullo & Lazzizera	(21.5–)22–27.5(–28.5) × (10–)11–13.5(–14.5)	1.97	Lazzizera et al. (2008)
D. rosulata Gure, Slippers & Stenlid	(21–)25–32(–36) (10–)11–17.5(–19.5)	1.93	Gure et al. (2005)
D. malorum Fuckel	(24–)26–32(–36) × (12–)13–17.5(–18.5)	1.9	Phillips et al. (2012)
D. fraxini Fr.	20–25 × 10	2.25	Saccardo (1884)
D. fraxini Fr.	(23.5−)25–27(–30) × (11−)12.5–13.5(−15)	2.00	This study
D. fraxini Fr. morphotype A	(26.5−)29–31.5(–33) × (11−)12.5–14(−17.5)	2.22	This study
Diplodia subglobosa	(24.0−)24.5−27.0(−32) × (15.5−)16.5−19.0(−22)	1.5	This study

Habitat: While Farr and Rossman (2013) list 55 hosts for *D. mutila* it is now clear that many of the earlier reports of this fungus could be misidentifications (Alves et al. 2004, 2006; Lazzizera et al. 2008; Phillips et al. 2012). The following are confirmed hosts: *Chamaecyparis lawsoniana*, *Fraxinus* spp., *Malus* spp., *Populus* spp., *Taxus baccata*, *Vitis vinifera* (Phillips et al. 2013).

Known distribution: England, France, Italy, Portugal, South Africa, USA (California) (Phillips et al. 2013), New Zealand (Dingley 1969; Laundon 1973).

Specimens examined: *Diplodia mutila*: FRANCE, Ardenne, Sedan, on bark of *Populus nigra*, date unknown, Montagne, (K 99664, isotype). PORTUGAL, Alentejo, Montemor-o-Novo, *Vitis vinifera*, 1996, A.J.L. Phillips (CBS H-20187), living culture CBS 112553. PORTUGAL, Beira Litoral, Aveiro, *Populus alba*, 2012, A. Alves, (LISE 96136, epitype of *Diplodia mutila* designated herein), MBT176182, culture ex-epitype CBS 136014. *Sphaeria mutila*: Scler. Suec. 164 (STR); Scler. Suec. 385 (STR). *Physalospora mutila*: ENGLAND, Cornwall, Saltash, on bark of *Malus* sp., 22 Aug. 1935, N.E. Stevens (BPI 599153, lectotype); Surrey, Ranmore Common, on *Fraxinus* sp., 19 Apr. 1957, C. Booth (BPI 599150 ex IMI 69064).

Notes: Although Montagne (1834) indicated *Sphaeria mutila* as the type of the new genus *Diplodia*, the 1834 protologue did not make any definite association of "*mutila*" with "*Diplodia*", as required for a valid comb. nov. Therefore, the frequently cited date of 1834 for publication of the combination *Diplodia mutila* is incorrect. Fries (1823) described *Sphaeria mutila* and distributed two exsiccati under that name as Scler. Suec. 164 and 385. Stevens (1933) and Sutton (1980) reported that these two exsiccati in BPI and K had no spores. Alves et al. (2004) examined material of the same two exsiccati in STR and also found no spores. Montagne sent Fries a fungus that was identified as *S. mutila*. The record was listed under *S. mutila* Fr. by Montagne (1834) with the note that this would become the type of a new genus, *Diplodia*, later characterized by Fries (1849). Thus, the binomial *Diplodia mutila* was first introduced by Fries (1849). Montagne distributed this fungus in his exsiccatus No. 498. According to Alves et al. (2004) no material of this exsiccatus could be found in STR. Alves et al. (2004) examined Montagne's specimen of *D. mutila* in Kew, K(M) 99664 (presumed to

be an isotype) and found it to agree in all aspects with Stevens' (1933) account of Montagne's exs. 498. Stevens (1933) described *Physalospora mutila* as the teleomorph of *D. mutila* referring to BPI 599151, but this name was invalid because it lacked a Latin description. Alves et al. (2004) examined this specimen and could find no teleomorph, but they did find ample material of the teleomorph on BPI 599153, which is a specimen on apple collected by Stevens from the same locality at same time he collected BPI 599151. Shoemaker (1964) considered the teleomorph to be a species of *Botryosphaeria* and since the name *B. mutila* was already taken, he proposed the name *Botryosphaeria stevensii*. After Crous et al. (2006) revised *Botryosphaeria* reducing it to *B. dothidea* (Moug.) Ces. & De Not. and *B. corticis* (Demaree & Wilcox) Arx & E. Müll., the fungus known as *B. stevensii* was referred to only by its anamorphic name *D. mutila*. The epitype designated here conforms in all ways with the isotype of *D. mutila* and with the asexual morph on BPI 599153 as described by Alves et al. (2004).

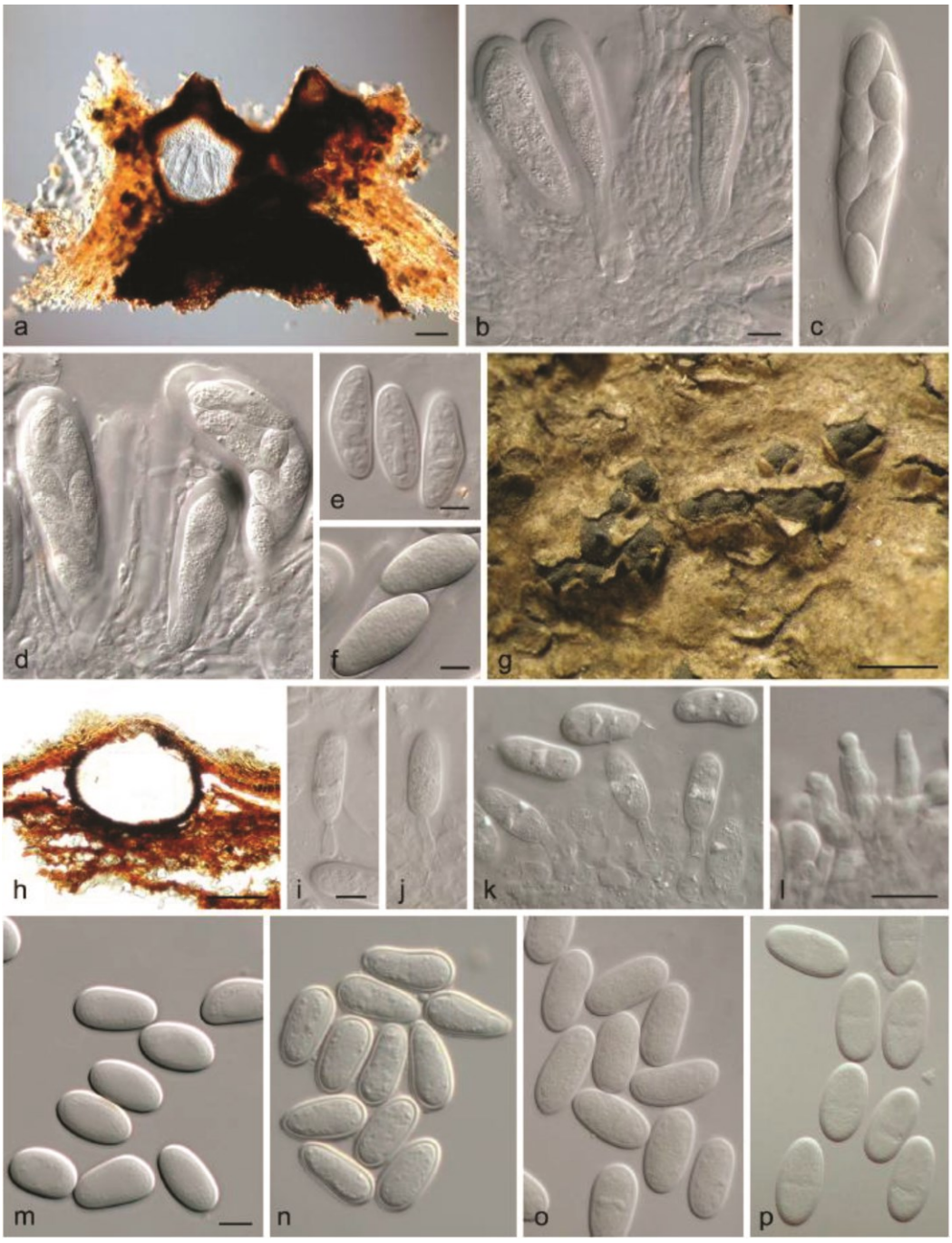

Figure 3 *Diplodia mutila*. a Sectioned ascoma. b Immature asci and pseudoparaphyses. c, d Asci with ascospores. e, f Ascospores. g Conidiomata partially erumpent through host. h Sectioned conidioma. i–l Conidiogenous cells. m–p Conidia. m Hyaline, aseptate conidia of CBS 112553. n Hyaline, aseptate conidia of CBS 136014. o Hyaline, aseptate conidia of BPI 599153. p Hyaline, aseptate conidia of K(M) 99664. Scale bars a=100 µm, b=10 µm, e, f=10 µm, g=500 µm, h= 100 µm, i, l=10 µm, m=10 µm. Scale bar in b applies to c, d. Scale bar in i applies to j, k. Scale bar in m applies to n, o, p

Diplodia subglobosa A.J.L. Phillips, Deidda & Linaldeddu sp. nov.

MycoBank: MB806049 (Fig. 4)

Etymology: Named for the sub-globose conidia.

Ascomata not seen. Conidiomata solitary, immersed in the host, dark brown to black, globose to ovoid, up to 560 µm diam and 400 µm high, wall composed of three layers, an outer of dark brown, thick-walled textura angularis, a middle layer of dark brown thin-walled cells, an inner layer of thin-walled hyaline cells. Ostiole central, circular, papillate. Conidiogenous cells 11–25×3–9 µm, holoblastic, discrete, cylindrical, hyaline, smooth, indeterminate, proliferating at the same level giving rise to periclinal thickenings, or proliferating percurrently to form one or two indistinct annellations. Conidia hyaline, aseptate, smooth, thick-walled, oblong to ovoid, straight, both ends broadly rounded, (24.0−)24.5–27.0(−32)×(15.5−)16.5–19.0(−22) µm, 95 % confidence limits=27.7–28.0×18.1–18.5 µm (mean ± S.D. of 150 conidia=27.7±1.8×18.3±1.2 µm, L/W ratio=1.5±0.1), becoming pale brown and septate when aged.

Habitat: Twigs and branches of *Fraxinus* spp. and *Lonicera nigra*.

Known geographic distribution: Italy and Spain.

Specimens examined: ITALY: Sicily, *Fraxinus ornus*, 2006, A. Sidoti (CBS 124131). SPAIN: Cataluña, *Fraxinus excelsior*, (no date), J. Luque (CBS 124132); *Lonicera nigra*, (no date), J. Luque Holotype LISE 96135 (culture ex-type CBS 124133). ITALY, *Fraxinus excelsior*, (no date) B. Slippers (CMW7776).

Notes: The relatively wide conidia and L/W ratio of 1.5 of this species are distinctive amongst *Diplodia* species with hyaline conidia belonging to clade 1.

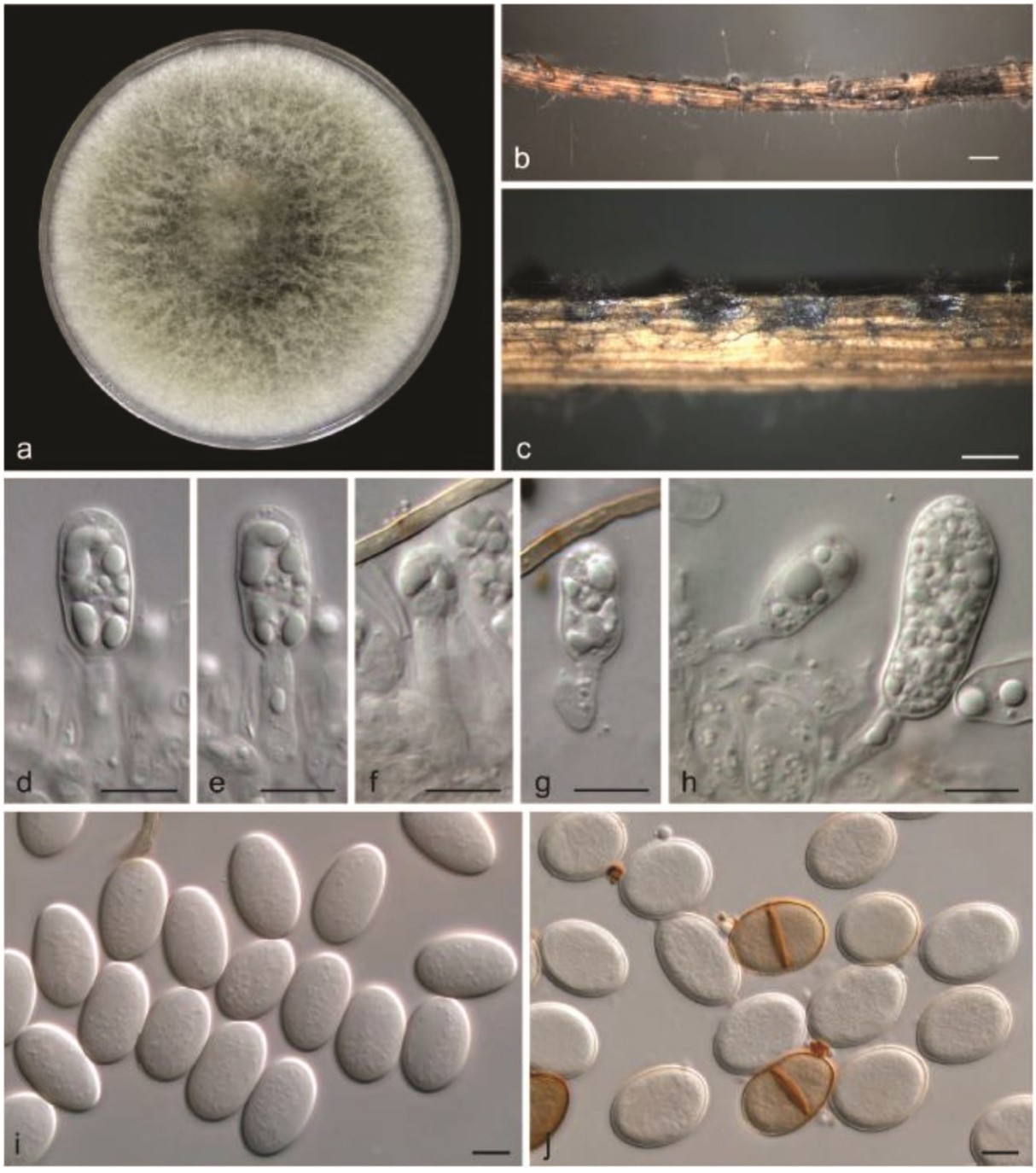

Figure 4 *Diplodia subglobosa*. a Colony on PDA after 7 days at 25 °C. b, c Conidiomata formed in culture on pine needles. d–h Conidiogenous cells with developing conidia. i Hyaline, aseptate conidia. j Hyaline, aseptate and coloured, 1-septate conidia. Bars: b, c=500 µm; d–j=10 µm

Discussion

Results of phylogenetic analyses provide robust evidence that the *D. mutila*-like isolates from *Fraxinus* spp. in Italy, the Netherlands, Portugal and Spain belong to three separate sub-clades corresponding to three distinct species within *Diplodia* clade 1. For one clade, which includes several isolates from *F. angustifolia* in Italy and Portugal, and an isolate from *F. excelsior* in the Netherlands (CBS 431.82), the name *D. fraxini* was considered appropriate. The type of neither *S. fraxini* nor *D. fraxini* could be located, but morphologically the isolates we studied agree in all ways with Saccardo's (1884) description of the species and therefore the name is re-instated and a neotype designated. Two isolates, one from Italy and one from Portugal, differed in cultural characteristics and conidia dimensions from typical isolates and since they were phylogenetically indistinguishable from the typical isolates they were considered to be morphological variants and are designated as morphotype A. The descriptions of distinct morphological forms has been reported for another species in *Diplodia*, namely *D. sapinea* (morphotype A and C) and the C morphotype is considered to be the most virulent (deWet et al. 2002).

The second clade includes isolates from *F. ornus* in Italy, *F. excelsior* in Italy and Spain and one isolate from *Lonicera nigra* in Spain and represents a previously unrecognized *Diplodia* species, which we describe here as *D. subglobosa* sp. nov. Conidia of this new species are sub-globose with an average L/W ratio of 1.5. In this respect it resembles the anamorph of "*Botryosphaeria*" *quercuum* (Shoemaker 1964) but it can be distinguished on account of its larger conidia (24.0−)24.5–27.0(−32)×(15.5−)16.5–19.0(−22) µm. In "*Botryosphaeria*" *quercuum* the conidia are (18–)21–24(–25)×(12–)15–16(–17).

The third group of isolates obtained from *F. ornus* in Portugal were considered to be *D. mutila*, the type species of the genus *Diplodia* (Montagne 1834; Fries 1849). Unfortunately, no live cultures linked to the holotype of *D. mutila* are extant and this has severely hampered studies on the taxonomy and phylogeny of *Diplodia*. Alves et al. (2004) provided a detailed description of this species based on one isolate from grapevines in Portugal (CBS 112553), an isotype of *D. mutila* (K99664) and one of Stevens' (1936) specimens of *Physalospora mutila* (BPI 99153). They showed that CBS 112553 correlated closely with the morphology of *D.*

mutila and this was confirmed in the present study. This culture has subsequently been cited as typical of *D. mutila* and has been regarded as a standard isolate for this species (Alves et al. 2006; Damm et al. 2007; Lazzizera et al. 2008; Phillips et al. 2012; Lynch et al. 2013). Although it is possible to use as epitype a specimen collected from a different host it is preferable, whenever possible, to obtain the epitype from the same host as the type specimen.

In an effort to obtain a suitable specimen that can be used as epitype for *D. mutila* we collected samples from *Populus* (type host of *D. mutila*) and *Fraxinus* (type host of the sexual morph). No sexual morph was found on either of the hosts, but several isolates with typical morphological features of *D. mutila* were obtained. Isolates obtained from *P. alba* and *F. ornus* in Portugal clustered with isolates from several other hosts including *V. vinifera*, *Persea americana* Mill., *Taxus baccata* L. and *Chamaecyparis lawsoniana* (A. Murray bis) Parl. CBS 302.36, deposited by N. E. Stevens as *Physalospora mutila* N.E. Stevens also clustered in this group. Since the culture is no longer sporulating, most likely due to the fact that it has been in culture for many years now, we could not study its morphology. This culture is not linked in any way to the type specimen but given that it was obtained by Stevens it can be regarded as representative of his concept of the sexual morph of *D. mutila*. Thus, this clade is regarded as representing true *D. mutila*. The isolates from *P. alba* and *F. ornus* conformed in all ways with the morphological characters of the isotype of *D. mutila* (K99664), the asexual morph of *P. mutila* on BPI 599153 (Alves et al. 2004) and the isolate from grapevines in Portugal (CBS 112553) that has been used as a standard. Furthermore, the type host of *D. mutila* is a *Populus* species. Therefore, the specimen on *P. alba* is herein designated as epitype for *D. mutila*. The data presented here supports the plurivorous nature of this pathogen since isolates from several different hosts clustered together in the clade corresponding to this species.

Diplodia mutila clustered in the *Diplodia* clade 1, which includes species with hyaline conidia that become brown and one-septate sometime after discharge from the pycnidia. This clade comprises eight species that are morphologically similar and can be difficult to separate on morphology alone. Nonetheless, they can be differentiated on slight differences in conidial dimensions (Table 2). Thus, conidia of *Diplodia africana* Damm & Crous are the largest in the clade 1, while *D. olivarum* has the smallest conidia. Although conidia of *D. malorum* are

morphologically similar to those of *Diplodia rosulata* Gure, Slippers & Stenlid this last species forms distinctive rosulate colonies (Gure et al. 2005). *Diplodia agrifolia* S.C. Lynch & A. Eskalen differs from *D. mutila* by its longer and wider conidia. Moreover, conidia of *D. agrifolia* are hyaline and aseptate, but most become dark brown and one-septate before discharge from pycnidia (Lynch et al. 2013).

Another group of isolates obtained in this study from cankered branches of declining *P. alba* trees in Italy clustered in another clade together with *D. malorum*. The name *D. malorum* was reinstated by Phillips et al. (2012) for isolates obtained from *Malus* spp. Although this species is morphologically similar to *D. mutila* it can be distinguished by its larger conidia and from DNA sequence data (Phillips et al. 2012, 2013). Until now *D. malorum* has been reported only from *Malus* spp. and other *Rosaceae* and this represents the first report of the species in a different host (*Populus*) and a different family. In addition, it is reported for the first time in Italy.

A single isolate obtained from a cankered branch of *Quercus coccifera* in Tunisia clustered within the *D. olivarum* clade. This species was initially described from *Olea europaea* L. (olive tree) in Italy where it was associated with diseased olive drupes (Lazzizera et al. 2008). Since then, it has been found associated with cankers on Ceratonia siliqua L. (carob tree) in Italy (Granata et al. 2011) and trunk disease of *Prunus dulcis* (Mill.) D.A. Webb (almond) in Spain (Gramaje et al. 2012). This represents the first report of the species in Tunisia and first report on *Q. coccifera*.

Additional *Diplodia* isolates morphologically different from *D. mutila* were included in this study. One isolate from *F. ornus* in Portugal and two isolates from *F. angustifolia* in Italy clustered within *D. seriata*. This is the first report of the species on *Fraxinus* spp. in Europe. The only other known report is on *Fraxinus americana* L. (green ash) in the USA under the name *Physalospora obtusa* (=*Botryosphaeria obtusa*) (Farr et al. 2013) which is the sexual morph of *D. seriata* (Phillips et al. 2007). This species is well known by its cosmopolitan and plurivorous nature (Punithalingam and Walker 1973; Phillips et al. 2007).

Another two isolates from *F. angustifolia* in Italy clustered with *D. pseudoseriata* and *D. alatafructa*. *Diplodia pseudoseriata* was described from several species of native *Myrtaceae*

trees in Uruguay (Pérez et al. 2010) while *Diplodia alatafructa* Mehl & Slippers was first reported on *Pterocarpus angolensis* DC. (wild teak) in South Africa (Mehl et al. 2011). Interestingly, the two Italian isolates form a distinct sub-clade within *D. pseudoseriata*/*D. alatafructa* clade with 75 % bootstrap support. Further morphological and phylogenetic analysis are currently in progress to clarify the status of these two isolates.

In this study we have shown that the name *D. mutila* has been applied to a number of cryptic species. In order to stabilize the name and allow its unambiguous application an epitype specimen with associated ex-epitype cultures was selected. At the same time the name *D. fraxini* is re-instated and a neotype designated. In the future more studies should be done in order to verify the pathogenicity of *D. mutila*, *D. fraxini* and *D. subglobosa* on *Fraxinus* spp. and establish their role in the etiology of ash dieback.

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Chapter 5

Neofusicoccum australe, the main pathogen associated with cankers and decline of *Eucalyptus camaldulensis* trees in Sardinia (Italy)

Abstract Due to their rapid growth and adaptability to different environmental conditions, *Eucalyptus* species have been widely introduced in both hemispheres for pulpwood production. In Sardinia (Italy), eucalypt plantations were established in the 20th century primarily in areas reclaimed from marshland and subsequently all over the island where they are currently cultivated as ornamental plants, windbreaks and for honey production. In recent years, a severe and unusual disease of unknown aetiology has been observed in several artificially established plantations of *Eucalyptus camaldulensis* throughout the island. The affected plants showed leaf chlorosis, crown thinning, shoot and branch dieback, cankers on branches and trunk, epicormic shoots and exudations of Kino. Since there is no information about this unusual disease and given the high ecological and economic relevance of these ecosystems, in 2013 a survey was carried out to establish the causal agents involved in the aetiology of the disease. Isolations from 510 symptomatic woody samples yielded a total of 489 fungal isolates belonging to 9 distinct species namely *Diaporthe foeniculina*, *Neofusicoccum australe*, *N. luteum*, *N. mediterraneum*, *N. parvum*, *N. vitifusiforme*, *Valsa fabianae* and two unidentified *Cytospora* species. *Neofusicoccum australe* was the only species recovered in all surveyed sites and its isolation frequencies ranged from 51 to 95 %. Pathogenicity trials on *E. camaldulensis* trees showed that only the *Neofusicoccum* species, with the exception of *N. vitifusiforme*, are pathogenic on this host.

Keywords timber industries - exotic species - biosecurity

Introduction

Eucalyptus L'Hér. is a large genus in the family *Myrtaceae* which includes evergreen trees and shrubs, mostly native to Australia (Brooker 2000; McKinnon et al. 2008). Due to their rapid growth and their adaptability to different environmental conditions, species of eucalypts were widely introduced, particularly in the tropics and in the Southern Hemisphere, providing

one of the most important sources of structural timber, pulp and fuel-wood worldwide (Flynn 2010).

In Italy the total area covered by eucalypt plantations now reaches 72,000 hectares. The most common species introduced were *Eucalyptus camaldulensis*, *E. globulus* and *E. occidentalis*. The massive introduction of these exotic species in the country started in the first half of the 20th century, both to protect areas interested by soil erosion and to obtain pulpwood productions for the paper industry (Mercurio and Minotta 2000; Boggia 1987). In Sardinia eucalypt plantations cover an area of 8,700 hectares. They were established initially in areas reclaimed from marshland and have subsequently spread all over the island where they are currently cultivated as windbreaks, for pulpwood, biomass and construction timber, as ornamentals and more recently for honey production (Mercurio and Minotta 2000).

Over the last decades, several pathogens have threatened *Eucalyptus* spp. in both native and introduced areas worldwide (Crous and Wingfield 1997; Wingfield et al. 2008, Branco et al. 2014). Among the most serious diseases affecting eucalypt trees worldwide, Mycosphaerella Leaf Disease (MLD) represents one of the most important limiting factors for commercial plantations (Crous et al. 2007; Perez et al. 2009; Silva et al. 2012; Wingfield et al. 2008; Branco et al. 2014). The predominant symptoms of MLD consist in leaf spot of variable size that often enlarge and coalesce to form larger blotches across the leaf surface causing leaf blight, premature defoliation and shoot dieback, leading to reduced growth and resulting in significant economic losses (Lundquist and Purnell 1987; Dungey et al. 1997). Many fungal species belonging to the genus *Mycosphaerella sensu lato* have been associated with MLD worldwide but recent studies employing DNA sequence data for the large subunit (LSU) region of the rRNA operon have shown that *Mycosphaerella* is polyphyletic, therefore various species occurring on eucalypts can be more appropriately accommodated in the genus *Teratosphaeria* (Crous et al. 2007, Hunter et al. 2011). In particular, one of the most virulent species of *Teratosphaeria* involved in the aetiology of MLD on eucalypts is *Teratosphaeria nubilosa* (= *Mycosphaerella nubilosa*).

Other severe diseases such as Eucalyptus rust caused by *Puccinia psidii* G. Winter, Ceratocystis wilt caused by *Ceratocystis* species and some canker-causing agents such as

species of *Botryosphaeriaceae* Theiss. & P. Syd. and *Chrysoporthe cubensis* (Bruner) Hodges (= *Cryphonectria cubensis*) pose a threat to *Eucalyptus* plantations worldwide (Gryzenhout et al. 2004; Adams et al. 2005; Slippers et al. 2009).

Because of their wide distribution and virulence, botryosphaeriaceous fungi are currently regarded amongst the pathogens having the greatest negative impact on eucalypt trees both in native and introduced areas (Smith et al. 1994; Slippers et al. 2004; Burgess et al. 2005; Mohali et al. 2007). Several studies have revealed the involvement of some species belonging to the genera *Botryosphaeria*, *Lasiodiplodia* and *Neofusicoccum* in the decline and mortality of *Eucalyptus* spp. In particular, *Botryosphaeria dothidea* (Moug.: Fr.) Ces. & De Not. and *Neofusicoccum ribis* (Slippers, Crous & M.J. Wingf.) Crous, Slippers & A.J.L. Phillips have been commonly associated with different disease symptoms affecting *Eucalyptus* spp. in temperate climates (Webb 1983; Barnard et al. 1987; Shearer et al. 1987; Crous et al. 1989; Old et al. 1990; Fisher et al. 1993; Smith et al. 1994), while *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl. was the species most frequently reported in tropical areas (Sharma et al. 1984; Sankaran et al. 1995; Roux et al. 2000, 2001; Mohali et al., 2005). However, recent studies based on comparison and analysis of DNA sequence data have allowed us to ascertain that several strains previously identified as *B. dothidea* and *N. ribis* are in fact *Neofusicoccum parvum* (Slippers et al. 2004a, b; Burgess et al. 2005; Rodas et al. 2009). At the same time, the occurrence of several cryptic species has been demonstrated within the *L. theobromae* species complex and currently four species, namely *L. crassispora* T.I. Burgess & Barber, *L. pseudotheobromae* A.J.L. Phillips, A. Alves & Crous, *L. rubropurpurea* Burgess, Barber & Pegg and *L. theobromae* are known to infect eucalypt trees (Burgess et al. 2006; Alves et al. 2008; Slippers et al. 2009).

Unlike other countries, until 2010 eucalypt plantations in Sardinia were not affected by serious diseases, but since then an unusual and widespread decline and mortality has been observed on young and mature *E. camaldulensis* trees in plantations as well as along windbreaks and in urban and periurban areas. Given the economic importance of eucalypt plantations for the regional economy and the potential losses caused by this emerging disease, the main aims of this work were: 1) to clarify the symptomatology and aetiology of the decline

events occurring in eucalypt ecosystems in Sardinia; 2) to assess the virulence of the main fungal species associated with declining trees.

Materials and Methods

Field surveys and sampling procedure

Field surveys were carried out in spring, summer and autumn 2013, and interested 12 eucalypt growing areas throughout Sardinia (Table 1). Given that stem and branch cankers constituted the most frequent symptoms, in order to ascertain the causing agents at each site 5 declining trees were selected *at random* and samples from branches were collected from the margin of three active cankers per tree, for a total of 510 samples.

Tab 1 Site characteristics of Eucalyptus plantations investigated

Sites	Locality	Latitude	Longitude	Elevation (m a.s.l.)	Type of plantation
1	Ottana	40°13'56.82432" N	9°1'54.02496" E	185 m	Windbreak
2	Arborea	39°48'6.43212" N	8°37'47.20044" E	7 m	Plantation
3	Serramanna	39°24'48.37356" N	8°51'46.14336" E	30 m	Windbreak
4	Uta	39°15'24.83748" N	8°55'50.54412" E	6 m	Plantation
5	Siliqua	39°16'16.31568" N	8°48'52.57080" E	66 m	Plantation
6	San Vito	39°20'3.84216" N	9°31'51.34800" E	13 m	Windbreak
7	Arbatax	39°55'42.06900" N	9°42'30.31416" E	13 m	Ornamental
8	Siniscola	40°35'27.69684" N	9°42'34.51644" E	39 m	Plantation
9	Olbia	40°52'14.61432" N	9°30'55.80540" E	15 m	Windbreak
10	Ozieri	40°36'48.17988" N	8°55'49.95300" E	390 m	Windbreak
11	S. Maria La Palma	40°39'9.62460" N	8°17'25.72944" E	30 m	Windbreak
12	Alghero	40°35'37.91040" N	8°17'25.11852" E	7 m	Ornamental

Isolation and identification of canker-causing agents

Eucalypt samples were brought to the laboratory to be inspected and then the outer bark surface tissue was cut away with a scalpel. Longitudinal and transversal cuts from symptomatic branch samples were made to observe any internal symptom. Isolations were made from chips of inner bark and xylem tissues, approx. 5 mm^2, cut by a sterile scalpel from the margin of necrotic lesions. All chips were cultured on potato dextrose agar (PDA, Oxoid Ltd.) in Petri dishes. After incubation at 25 °C for 5-7 days in the dark, fungal colonies were sub-cultured onto half-strength PDA (1/2 PDA) supplemented with autoclaved holm oak twigs and kept on the laboratory bench with natural daylight at about 20-25 °C until the differentiation of reproductive structures. For microscopy, the contents of conidiomata were dissected out, mounted in 100% lactic acid and observed at x400 magnification. Fungal isolates were grouped on the basis of morphological features and, for each group, representative isolates were selected and used in molecular and pathogenicity studies.

DNA extraction, PCR amplification and sequencing

Following morphological identification, a subset of isolates of each species obtained in this study was selected for DNA sequence analysis (Table 2). Instagene Matrix (BioRad Laboratories, Hercules, CA) was used to extract genomic DNA from 5-day-old cultures grown on PDA and incubated at 25 °C. The entire Internal Transcribed Spacer region (ITS) of the rDNA, including the 5.8S gene, was amplified and sequenced with primers ITS1 and ITS4 (White *et al*., 1990), while the primers EF446f and EF1035r (Inderbitzin *et al*., 2005, 2010) were used to amplify and sequence part of the translation Elongation Factor 1-alpha (EF-1α) gene. Polymerase chain reaction (PCR) mixtures and amplification conditions were conducted as described by Linaldeddu et al. (2013). The PCR products were purified using the EUROGOLD gel extraction kit (EuroClone S.p.A.) following the manufacturer's instructions. Both strands were sequenced by the BMR Genomics DNA sequencing service (www.bmr-genomics.it). The nucleotide sequences were read and edited with FinchTV 1.4.0 (Geospiza, Inc.; http://www.geospiza.com/finchtv) and compared with sequences deposited in GenBank using the BLAST software (http://blast.ncbi.nlm.nih.gov).

Tab 2 *Neofusicoccum* isolates included in this study

Isolate number[1]	Species	Host	Locality	Collector	GenBank	
					ITS[2]	EF
CBS 117453	*N. andinum*	*Eucalyptus* sp.	Venezuela	S. Mohali	*AY693976*	*AY693977*
CBS 117452	*N. andinum*	*Eucalyptus* sp.	Venezuela	S. Mohali	*DQ306263*	*DQ306264*
CBS 116131	*N. arbuti*	*A. menziesii*	USA	M. Elliot	*AY819720*	*KF531792*
CBS 117090	*N. arbuti*	*A. menziesii*	USA	M. Elliot	*AY819724*	*KF531791*
CAD028*	*N. australe*	*E. camaldulensis*	Italy	A. Deidda	KP142944	KP142975
CAD040*	*N. australe*	*E. camaldulensis*	Italy	A. Deidda	KP142945	ns
CAD041*	*N. australe*	*E. camaldulensis*	Italy	A. Deidda	KP142946	ns
CAD042*	*N. australe*	*E. camaldulensis*	Italy	A. Deidda	KP142947	ns
CAD043*	*N. australe*	*E. camaldulensis*	Italy	A. Deidda	KP142948	ns
CMW6837	*N. australe*	*Acacia* sp.	Australia	M.J. Wingfield	*AY339262*	*AY339270*
CMW6853	*N. australe*	*Sequoiadendron* sp.	Australia	M.J. Wingfield	*AY339263*	*AY339271*
CBS 124924	*N. batangarum*	*T. catappa*	Africa	Begoude & Roux	*FJ900607*	*FJ900653*
CBS 124923	*N. batangarum*	*T. catappa*	Africa	Begoude & Roux	*FJ900608*	*FJ900654*
CBS 123634	*N. cordaticola*	*S. cordatum*	South Africa	D. Pavlic	*EU821898*	*EU821868*
CBS 123635	*N. cordaticola*	*S. cordatum*	South Africa	D. Pavlic	*EU821903*	*EU821873*
CBS 122813	*N. cryptoaustrale*	*Eucalyptus* sp.	South Africa	H.M. Maleme	*FJ752742*	*FJ752713*
CAD023	*N. cryptoaustrale*	*V. vinifera*	Sardinia, Italy	A. Deidda	*KJ638328*	*KJ638346*
CMW20738	*N. cryptoaustrale*	*Eucalyptus* sp.	South Africa	H.M. Maleme	*FJ752740*	*FJ752710*
CBS 115679	*N. eucalypticola*	*E. rossii*	Australia	M.J. Wingfield	*AY615141*	*AY615133*
CBS 115766	*N. eucalypticola*	*E. rossii*	Australia	M.J. Wingfield	*AY615143*	*AY615135*
CBS 115791	*N. eucalyptorum*	*E. grandis*	South Africa	H. Smith	*AF283686*	*AY236891*
CMW10126	*N. eucalyptorum*	*E. grandis*	South Africa	H. Smith	*AF283687*	*AY236892*
CBS 123639	*N. kwambonambiense*	*S. cordatum*	South Africa	D. Pavlic	*EU821900*	*EU821870*

CBS 123641	*N. kwambonambiense*	*S. cordatum*	South Africa	D. Pavlic	*EU821919*	*EU821889*
CAD029*	*N. luteum*	*E. camaldulensis*	Italy	A. Deidda	KP142949	KP142976
CAD044*	*N. luteum*	*E. camaldulensis*	Italy	A. Deidda	KP142950	ns
CAD045*	*N. luteum*	*E. camaldulensis*	Italy	A. Deidda	KP142951	ns
CAD046*	*N. luteum*	*E. camaldulensis*	Italy	A. Deidda	KP142952	ns
CAD047*	*N. luteum*	*E. camaldulensis*	Italy	A. Deidda	KP142953	ns
CBS 110299	*N. luteum*	*V. vinifera*	Portugal	A.J.L. Phillips	*AY259091*	*AY573217*
CBS 110497	*N. luteum*	*V. vinifera*	Portugal	A.J.L. Phillips	*EU673311*	*EU673277*
CBS 118223	*N. macroclavatum*	*E. globulus*	Australia	T.I. Burgess	*DQ093196*	*DQ093217*
WAC12445	*N. macroclavatum*	*E. globulus*	Australia	T.I. Burgess	*DQ093197*	*DQ093218*
CBS 118531	*N. mangiferae*	*M. indica*	Australia	G.I. Johnson	*AY615185*	*DQ093221*
CBS 118532	*N. mangiferae*	*M. indica*	Australia	G.I. Johnson	*AY615186*	*DQ093220*
CAD026*	*N. mediterraneum*	*E. camaldulensis*	Italy	A. Deidda	KP142954	KP142977
CAD032*	*N. mediterraneum*	*E. camaldulensis*	Italy	A. Deidda	KP142955	ns
CAD033*	*N. mediterraneum*	*E. camaldulensis*	Italy	A. Deidda	KP142956	ns
CAD034*	*N. mediterraneum*	*E. camaldulensis*	Italy	A. Deidda	KP142957	ns
CAD035*	*N. mediterraneum*	*E. camaldulensis*	Italy	A. Deidda	KP142958	ns
CBS 121718	*N. mediterraneum*	*Eucalyptus* sp.	Greece	Crous, Wingfield, Phillips	*GU251176*	*GU251308*
CBS 121558	*N. mediterraneum*	*O. europaea*	Italy	C. Lazzizzera	*GU799463*	*GU799462*
CBS 126655	*N. nonquaesitum*	*U. californica*	USA	F.P. Trouillas	*GU251163*	*GU251295*
PD301	*N. nonquaesitum*	*V. corymbosum*	Chile	Briceño et al.	*GU251164*	*GU251296*
CBS 128008	*N. occulatum*	*E. grandis hybrid*	Australia	T.I. Burgess	*EU301030*	*EU339509*
MUCC286	*N. occulatum*	*E. pellita*	Australia	T.I. Burgess	*EU736947*	*EU339511*
CAD025*	*N. parvum*	*E. camaldulensis*	Italy	A. Deidda	KP142959	KP142978
CAD030*	*N. parvum*	*E. camaldulensis*	Italy	A. Deidda	KP142960	ns
CAD031*	*N. parvum*	*E. camaldulensis*	Italy	A. Deidda	KP142961	ns

CMW9081	*N. parvum*	*P. nigra*	New Zealand	G.J. Samuels	*AY236943*	*AY236888*
CBS 110301	*N. parvum*	*V. vinifera*	Portugal	A.J.L. Phillips	*AY259098*	*AY573221*
WAC13153	*N. pennatisporum*	*A. fraseriana*	Australia	K.M. Taylor	*EF591925*	*EF591976*
CBS 115475	*N. ribis*	*Ribes* sp.	USA	B. Slippers	*AY236935*	*AY236877*
CBS 121.26	*N. ribis*	*R. rubrum*	USA	N.E. Stevens	*AF241177*	*AY236879*
CBS 123645	*N. umdonicola*	*S. cordatum*	South Africa	D. Pavlic	*EU821904*	*EU821874*
CBS 123646	*N. umdonicola*	*S. cordatum*	South Africa	D. Pavlic	*EU821905*	*EU821875*
CBS 122811	*N. ursorum*	*E. arboretum*	South Africa	H.M. Maleme	*FJ752746*	*FJ752709*
CMW23790	*N. ursorum*	*E. arboretum*	South Africa	H.M. Maleme	*FJ752745*	*FJ752708*
CBS 112878	*N. viticlavatum*	*V. vinifera*	South Africa	F. Halleen	*AY343381*	*AY343342*
CBS 112977	*N. viticlavatum*	*V. vinifera*	South Africa	F. Halleen	*AY343380*	*AY343341*
CAD027*	*N. vitifusiforme*	*E. camaldulensis*	Italy	A. Deidda	KP142962	KP142979
CAD036*	*N. vitifusiforme*	*E. camaldulensis*	Italy	A. Deidda	KP142963	ns
CAD037*	*N. vitifusiforme*	*E. camaldulensis*	Italy	A. Deidda	KP142964	ns
CAD038*	*N. vitifusiforme*	*E. camaldulensis*	Italy	A. Deidda	KP142965	ns
CAD039*	*N. vitifusiforme*	*E. camaldulensis*	Italy	A. Deidda	KP142966	ns
CBS 110887	*N. vitifusiforme*	*V. vinifera*	South Africa	J. Van Niekerk	*AY343383*	*AY343343*
CBS 110880	*N. vitifusiforme*	*V. vinifera*	South Africa	J. Van Niekerk	*AY343382*	*AY343344*
CMW23791	*N. vitifusiforme*	*Eucalyptus* sp.	South Africa	H.M. Maleme	*FJ752738*	*FJ752705*
CMW24571	*N. vitifusiforme*	*E. paniculata*	South Africa	H.M. Maleme	*FJ752739*	*FJ752707*
CBS 119047	*B. corticis*	*V. corymbosum*	USA	P.V. Oudemans	*DQ299245*	*EU017539*
CBS115476	*B. dothidea*	*Prunus* sp.	Switzerland	B. Slippers	*AY236949*	*AY236898*

[1]Acronyms of culture collections: CAD – A. Deidda, Università degli Studi di Sassari, Italy; CBS – CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands; CMW – Tree Pathology Co-operative Program, Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa; PD – Culture collection, University of California, Davis, USA; WAC – Department of Agriculture, Western Australia Plant Pathogen Collection, South Perth, Western Australia. MUCC – Culture Collection, Laboratory of Plant Pathology, Mie University, Tsu, Mie prefecture, Japan. Isolates in bold are cultures ex-type

[2]Sequence numbers in italics were retrieved from GenBank. All others were obtained in the present study

*The asterisk indicates the isolates obtained in this work

Phylogenetic analysis

Given that *Neofusicoccum* was the dominant genus, some representative isolates of each species were used in the phylogenetic analysis. The ITS and EF1-α sequences were combined and the dataset including sequences of other *Neofusicoccum* species downloaded from GenBank was compiled with the outgroups *Botryosphaeria corticis* (Demaree & Wilcox) Arx & E. Müll. and *B. dothidea* (Moug.) Ces. & De Not. Sequences were aligned with ClustalX 1.83 (Thompson et al. 1997), using the following parameters: pairwise alignment parameters (gap opening=10, gap extension=0.1) and multiple alignment parameters (gap opening=10, gap extension=0.2, transition weight=0.5, delay divergent sequences=25 %). A partition homogeneity test was carried out to determine the possibility of combining the ITS and EF1-α datasets (Farris et al. 1995, Huelsenbeck et al. 1996).

Maximum parsimony analysis was performed with PAUP 4.0b10 using the heuristic search option with 1000 random taxon additions and tree bisection and reconnection as the branch-swapping algorithm. All characters were unordered and of equal weight, and gaps were treated as missing data. Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. The robustness of the most parsimonious trees was evaluated by 1000 bootstrap replications (Hillis and Bull 1993). Other measures used were consistency index (CI), retention index (RI) and homoplasy index (HI).

Bayesian analyses were done with Mr Bayes v.3.0b4 (Ronquist and Huelsenbeck 2003) employing a Markov Chain Monte Carlo (MCMC) method. The general time-reversible model of evolution (Rodriguez et al. 1990), including estimation of invariable sites and assuming a discrete gamma distribution with six rate categories was used. Four MCMC chains were run simultaneously, starting from random trees for 106 generations. Trees were sampled every 100th generation for a total of 10^4 trees. The first 10^3 trees were discarded as the burn-in phase of each analysis. Posterior probabilities (Rannala and Yang 1996) were determined from a 50 % majority-rule consensus tree generated with the remaining 9,000 trees. This analysis was repeated three times starting from different random trees to ensure trees from the same tree space were sampled during each analysis. Trees were visualized with TreeView (Page 1996).

Pathogenicity tests

To verify the pathogenicity of each species obtained in this study, a field inoculation trial was conducted in May 2014 on asymptomatic *E. camaldulensis* trees located in a plantation in the south of Sardinia [Serramanna: 39°41′96″N, 8°92′71″E]. During the experimental period, daily mean air temperatures ranged from 14.6 to 32.3°C and total rainfall was 0.4 mm. Six plants were inoculated at breast height with a representative isolate of each fungal species and six plants were used as control. The region of the trunk to be wounded was surface-disinfected with 70% ethyl alcohol and then a piece of bark was removed with a flamed cork borer and replaced with an agar-mycelium plug taken from the margin of an actively growing colony on PDA. The original bark plug was put on top of the agar disk and the inoculation site was covered with cotton-wool soaked in sterile water and wrapped with a piece of aluminium foil secured with masking tape. Inoculation of controls consisted of a sterile PDA plug applied as described above. After one month, the outer bark was carefully removed with a chisel and the area of the necrotic lesion for each inoculation was determined by placing transparent paper over the top of the lesion and drawing around the perimeter. The image of each lesion was scanned and the area was determined with Assess 2.0 software (Lamari 2002).

Re-isolation of inoculated species was attempted by transferring onto PDA 10 pieces of inner bark and wood taken around the margin of each lesion. Cultures were grown at 25 °C in daylight and room temperature until full colony development.

Data analysis.

Data from pathogenicity assays were checked for normality, then subjected to analysis of variance (ANOVA). Significant differences among mean values were determined with LSD multiple range test ($P = 0.05$) after one-way ANOVA. Statistical analyses were performed with XLSTAT software (Addinsoft, France).

Results

Field surveys

Eucalyptus camaldulensis trees affected by decline and mortality occurred with epidemic proportion in all monitored sites. Both young and old trees exhibited different symptoms on the canopy including a progressive dieback of twigs and branches associated with an abnormal emission of epicormic shoots on stems and branches (Fig. 1). All declining trees showed extensive sunken cankers on branch and trunk, often with copious exudation of kino. After removing the outer bark in correspondence of these cankers, dark brown necrotic lesions of xylematic tissues were visible. When branch and trunk cankers were cross-sectioned, internal wood symptoms included characteristic wedge-shaped necrosis which often involved a large part of the section (Fig. 1). Cankers and bark necrosis on branches and trunks developed from the top downwards. In correspondence of necrotic tissues a number of dark pycnidia erupting from the bark were usually visible. The high frequency of trees showing cankers on stem and branches in different stages and fresh exudates suggests that the epidemic phase of the disease had not yet been completed.

Isolation and identification of canker-causing agents

Isolations from symptomatic woody samples yielded a total of 489 fungal isolates belonging to three distinct families, namely *Botryosphaeriaceae* Theiss. & P. Syd., *Diaporthaceae* Höhn. ex Wehm. and *Valsaceae* Tul. & C. Tul. The ITS region was sequenced for 55 representative isolates and relative sequences were compared to sequences available in GenBank using a BLAST search. Botryosphaeriaceous fungi were the dominant component obtained in this study with 391 isolates. On the basis of morphological features and DNA sequence data (ITS), five distinct species namely *Neofusicoccum parvum* (6 isolates), *N. vitifusiforme* (12), *N. luteum* (14), *N. mediterraneum* (20) and *N. australe* (339) were identified. For all species BLAST searches in GenBank showed 99-100 % similarity with reference sequences of representative strains including those of ex-type cultures.

Figure 1. Main disease symptoms observed on investigated eucalypt trees: Crown thinning, dieback of twigs and branches associated with an abnormal emission of epicormic shoots along stems and branches (a-e); necrotic bark lesion in correspondence of a canker (f); typical sunken canker observed on branches (g); necrotic lesion visible after bark removal (h); cross section of a branch showing a characteristic wedge shaped necrotic sector (i); colonies of *Neofusicoccum australe* obtained from isolations on PDA (l).

Identification was also confirmed by DNA phylogeny analysis. Alignment of the combined ITS and EF1-α sequences of *Neofusicoccum* taxa gave rise to a data set of 857 characters (including alignment gaps). Of these characters 518 were constant, 50 were variable and parsimony uninformative and 210 were parsimony informative. A heuristic search of the 210 parsimony informative characters generated 22 most parsimonious trees (TL=398; CI=0.789; RI=0.931; HI=0.211) each with similar clade topologies, and one is presented in Fig. 2.

The phylogenetic analyses of Maximum-parsimony (MP) and Bayesian methods (BM) generated trees with essentially the same topology. In the MP analysis 22 clades were resolved corresponding to species known from culture and for which molecular data are available (Fig. 2). The *Neofusicoccum* isolates obtained in this study were distributed into 5 clades corresponding to the species identified on the basis of ITS sequence data.

N. australe was the only species recovered in all surveyed sites and its isolation frequencies ranging from 51 to 95% (Tab. 3). The other four species of *Neofusicoccum* were isolated with a lower frequency and often only in a few sites. With regard to the sampling period, *N. australe* was isolated in all seasons of samplings. Interestingly, its isolation frequency showed a decreasing trend from May to October, while *N. mediterraneum* showed an opposite trend. The other species were isolated mainly in spring.

According to a BLAST search on GenBank and morphological data, the other 98 isolates were found to belong to 4 distinct species: *Diaporthe foeniculina* (3 isolates) (accession no. KP142970), two unidentified species *Cytospora* sp.1 (7 isolates) (accession no. KP142967-KP142968) and *Cytospora* sp.2 (5 isolates) (accession no. KP142969) and *Valsa fabianae* (83 isolates) (accession no. KP142971-KP142974).

Valsa fabianae was the second most frequently isolated fungus after *N. australe*. It was isolated from 11 sites and showed increasing values of isolation frequency from the spring to the autumn sampling. The other three species were isolated with a relatively low frequency only in one or a few surveyed sites and only in the spring sampling (Tab. 3).

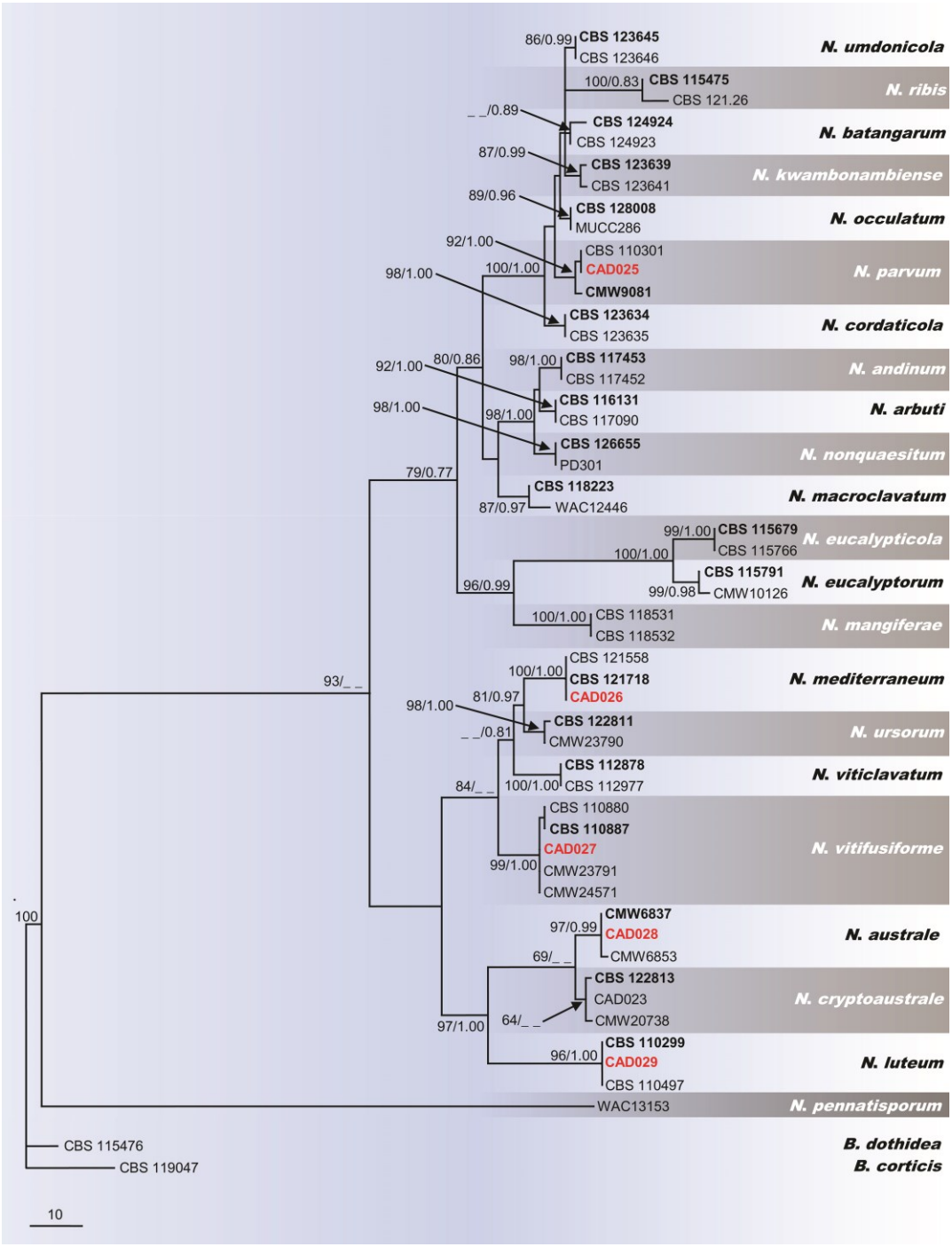

Figure 2 One of the most parsimonious trees resulting from the combined analysis of ITS and EF1-α sequence data. MP Bootstrap support values and Bayesian posterior probability scores are given at the nodes. The tree was rooted to *B. dothidea* and *B. corticis*. Ex-type isolates are in bold. Isolates obtained in this study are in red. The scale bar represents 10 changes

Table 3. Isolation frequency of fungal species at each site.

Fungal species	Sampling period	Site											
		1	2	3	4	5	6	7	8	9	10	11	12
Cytospora sp.1	Spring	-	6.7	-	26.7	-	13.3	-	-	-	-	-	-
	Summer	-	-	-	-	-	-	-	-	-	-	-	ns*
	Autumn	-	-	-	-	-	-	-	-	-	-	-	ns
Cytospora sp.2	Spring	-	-	-	-	13.3	-	20	-	-	-	-	-
	Summer	-	-	-	-	-	-	-	-	-	-	-	ns
	Autumn	-	-	-	-	-	-	-	-	-	-	-	ns
Diaporthe foeniculina	Spring	-	-	-	-	-	-	-	-	-	-	-	20
	Summer	-	-	-	-	-	-	-	-	-	-	-	ns
	Autumn	-	-	-	-	-	-	-	-	-	-	-	ns
Neofusicoccum australe	Spring	46.7	93.3	80	46.7	66.7	46.7	53.3	100	80	80	100	80
	Summer	66.7	40	40	86.7	60	73.3	53.3	93.3	80	80	93.3	ns
	Autumn	53.3	26.7	66.7	46.7	80	46.7	46.7	93.3	26.7	40	93.3	ns
Neofusicoccum luteum	Spring	-	-	-	-	-	-	-	-	-	-	-	-
	Summer	-	26.7	-	-	-	-	-	-	20	-	-	ns
	Autumn	-	6.7	-	6.7		6.7		6.7	6.7	6.7	6.7	ns
Neofusicoccum mediterraneum	Spring	-	-	-	-	6.7	-	-	-	-	20	-	-
	Summer	20	-	-	-	-	7	-	-	-	20	-	ns
	Autumn	-	-	-	40	-	20	-	-	-	-	-	ns
Neofusicoccum parvum	Spring	-	-	-	-	-	-	-	-	-	-	-	-
	Summer	6.77	-	-	-	26.7	-	-	-	-	-	-	ns
	Autumn	-	-	-	-	6.7	-	-	-	-	-	-	ns
Neofusicoccum vitifusiforme	Spring	40	-	-	26.7	-	-	-	-	-	-	-	-
	Summer	-	-	-	13.3	-	-	-	-	-	-	-	ns
	Autumn	-	-	-	-	-	-	-	-	-	-	-	ns
Valsa fabianae	Spring	-	-	-	-	-	13.3	-	-	-	-	-	-
	Summer	-	46.7	60	-	-	20	46.7	6.7	-	-	6.7	ns
	Autumn	46.7	66.7	33.3	6.7	13.3	26.7	53.3	-	66.7	53.3	-	ns

*ns= not sampled

Pathogenicity tests

Data from the pathogenicity tests highlighted significant differences in aggressiveness among the 9 species obtained from branch cankers (Fig. 3). All *Neofusicoccum* species, except *N. vitifusiforme*, proved to be aggressive pathogens on *Eucalyptus camaldulensis*. In particular, at the end of the experimental period, all trees inoculated with *N. australe*, *N. luteum*, *N. mediterraneum* and *N. parvum* displayed dark brown bark lesions, which developed both upward and downward from the inoculation point. The lesion developed from bark tissues toward the wood where it penetrated a few millimeters. Average size of necrotic lesions caused by *Neofusicoccum* spp. differed significantly. In particular, mean lesion size caused by *N. mediterraneum* (25.3 cm^2) was significantly larger than those caused by *N. luteum* (18.6 cm^2), *N. parvum* (11.1 cm^2) and *N. australe* (8.9 cm^2). All the other fungal species assayed caused small necrotic lesions confined to the inoculation point that did not differ statistically from the control. All inoculated fungi were successfully re-isolated from symptomatic tissues (wood and inner bark) of all trees.

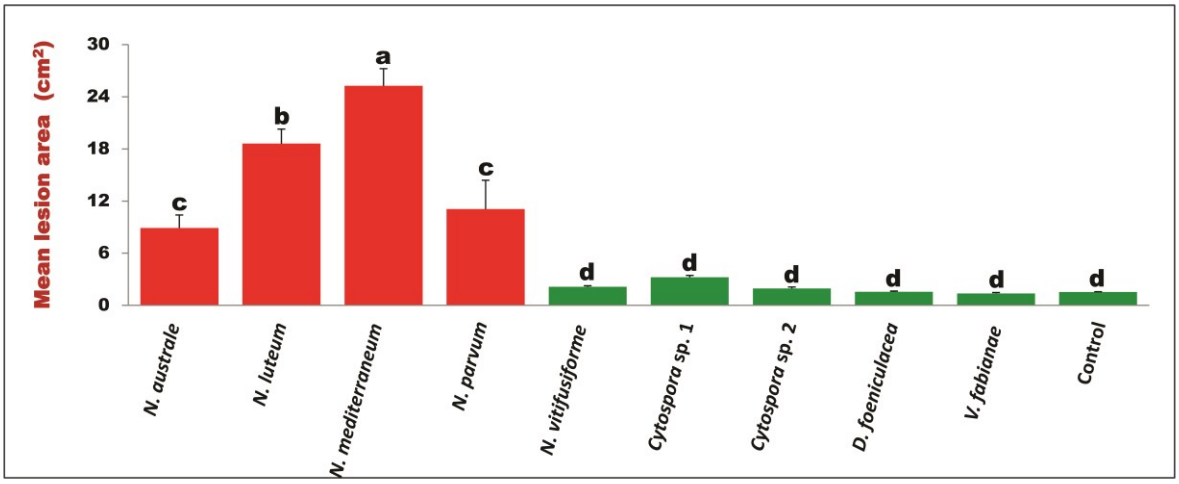

Figure 3 Mean lesion length (cm) caused by isolates of *Neofusicoccum australe*, *Neofusicoccum luteum*, *Neofusicoccum mediterraneum, Neofusicoccum parvum, Neofusicoccum vitifusiforme, Cytospora* sp.1*, Cytospora* sp. 2, *Diaporthe foeniculina, Valsa fabianae* on eucalypt trees. Error bars represent the standard deviations from the mean. Values with the same letter above the bar do not differ significantly at $P = 0.05$ according to LSD multiple range test.

Discussion

This study represents the first survey of diseases to threaten *E. camaldulensis* plantations in Sardinia. Results obtained have allowed us to clarify both symptomatology and aetiology related to severe decline affecting eucalypt trees. On the basis of morphological studies and DNA sequence analyses, nine different fungal species were identified from V-shaped cankers, including *Diaporthe foeniculina*, *Neofusicoccum australe*, *N. luteum*, *N. mediterraneum*, *N. parvum*, *N. vitifusiforme*, *Valsa fabianae* and two putative new species of *Cytospora*.

Eighty percent of the isolates obtained belonged to the genus *Neofusicoccum*, making this by far the most common genus associated with branch cankers of declining *E. camaldulensis* trees in Sardinia. All *Neofusicoccum* species identified in this study, except *N. luteum* which is reported for the first time on eucalypt, were previously detected on *Eucalyptus* spp. worldwide (Gezahgne et al. 2004; Slippers et al. 2004; Barber et al. 2005; Burgess et al. 2005; Crous et al. 2007; Taylor et al. 2009).

Neofusicoccum australe was the most frequently isolated species, 339 of the 510 cankers processed yielded colonies of this pathogen. Field survey and pathogenicity tests have clearly shown that *N. australe* is the major cause of stem and branch canker in Sardinian eucalypt plantations. The high frequency with which *N. australe* was isolated was concordant with results obtained in other investigations conducted in native eucalypt forests where this pathogen was proved to be the dominant species, leading the authors to hypothesize that this species is native to the southern hemisphere (Burgess et al. 2005, 2006; Taylor et al. 2009). As well as on eucalypts, *N. australe* has been reported as a pathogen of pistachio in Spain (Armengol et al. 2008), blueberry in Chile (Espinoza et al. 2009), citrus in California (Adesemoye and Eskalen 2011) and pedunculate oak in Portugal (Barradas et al. 2013). In Italy *N. australe* was previously reported in association with drupe rot of olives and declining grapevine plants (Lazzizzera et al. 2008; Linaldeddu et al. 2010). Interestingly, Italian isolates showed 1 bp difference in ITS sequences from the ex-type isolate of *N. australe* (CMW6837), placing them within the haplotype H4 proposed by Sakalidis et al. (2011). Recently, Crous et al. (2013) in a taxonomic revaluation of *N. australe* isolates demonstrated that isolates belonging to haplotype H4 represent in fact a new cryptic species named *N. cryptoaustrale*

Pavlic, Maleme, Slippers & M.J. Wingf. More recently Andolfi et al. (2012) studying a large collection of *N. australe* isolates obtained from declining juniper trees and Linaldeddu et al. (2014) studying the botryosphaeriaceous species associated with declining grapevine plants, found as both *N. australe* and *N. cryptoaustrale* occurring on agricultural and forestry ecosystems in Sardinia.

The other four species of *Neofusicoccum* were isolated with a lower frequency and in only a few sites. *Neofusicoccum luteum* was previously reported on eucalypt in Australia by Smith and Stanosz (2001). However, it is now well-accepted that in the past there has been great confusion in the identification of some species of *Botryosphaeriaceae* and it is likely that the name *N. luteum* over the years has been applied for more than one species. After a BLAST search of the ITS sequence of the isolate (96-130 = ATCC56125) studied by Smith and Stanosz (2001) we found 100% similarity with sequences of the ex-type culture of *N. australe* (AY339262) suggesting that the isolate (96-130 = ATCC56125)) is in fact *N. australe*. Although little information is available on the importance of this species as an eucalypt pathogen, *N. luteum* is a well-known pathogen of a wide range of fruit and forest trees worldwide (McDonald et a., 2009; Adesemoye et al., 2013; Barradas et al., 2013) and recently it has also been reported in Sardinia associated with declining tree heath shrubs in a natural area (Linaldeddu et al. 2014c).

Neofusicoccum mediterraneum was first described on *Eucalyptus* sp. in Greece (Crous et al. 2007). Subsequently, it was reported as an aggressive pathogen on olive drupes in southern Italy, grapevine in California and Spain and English walnut in California (Lazzizzera et al. 2008; Trouillas et al. 2010; Úrbez-Torres et al. 2010; Martin et al. 2011). This is the first report of this pathogen in Sardinia. In the pathogenicity tests *N. mediterraneum* has proven to be the most aggressive species. However, since its occurrence was limited to only a few sites the role of this pathogen appears to be secondary in relation to that of *N. australe*.

Neofusicoccum parvum is emerging as a common and cosmopolitan species on a wide variety of hosts. It is now recognised as an aggressive pathogen of grapevine (Úrbez-Torres 2001), mango (Marques et al. 2013), walnut (Cheon et al. 2013). At the same time, this fungus has been reported on several species of eucalypt including: *Eucalyptus citriodora*, *E. globulus*,

E. grandis, *E. saligna* (Gezahgne et al. 2004), *E. pellita* (Barber et al. 2005), *E. urophylla* (Mohali et al. 2007). In particular, Gezahgne et al. (2004) have found that *N. parvum* is the major cause of Botryosphaeria canker in Ethiopian *Eucalyptus* plantations. In this study *N. parvum* was isolated only sporadically suggesting a marginal role played by this pathogen in the aetiology of the eucalypt disease observed in Sardinia. However, it is interesting to report that only the eucalypt trees artificially inoculated with *N. parvum* showed copious exudation of kino at the end of the experimental period.

Neofusicoccum vitifusiforme was originally thought to be restricted to *Vitis vinifera* (van Niekerk et al. 2004), but it was later isolated from *Olea europaea* (Lazzizera et al. 2008, Úrbez-Torres et al. 2013), *Prunus persica* and *P. salicina* (Damm et al. 2007) and *Vaccinium corymbosum* (Kong et al. 2010). Furthermore, both Lazzizera et al. (2008) and Phillips et al. (2013) showed that *N. vitifusiforme* is phylogenetically indistinguishable from *Dichomera eucalypti* (G. Winter) B. Sutton, and have suggested that *D. eucalypti* is the synanamorph of *N. vitifusiforme*, which was confirmed in the present study based on ITS and EF1-α sequence data. Therefore, *Eucalyptus* spp. can be regarded as additional hosts for *N. vitifusiforme*. Although the plurivorous nature of *N. vitifusiforme* has now been established, there are still conflicting reports regarding its pathogenicity. In particular, it has been reported as an aggressive pathogen on grapevine and on olive drupes in Italy, on apple in South Africa and blueberry in China (Lazzizzera et al. 2008; Kong et al. 2010; Cloete et al. 2011; Mondello et al. 2013), as a weak pathogen on grapevine in South Africa and *Eucalyptus globulus* in Australia (van Niekerk et al. 2004; Burgess et al. 2005) and as not pathogenic on grapevine and pear trees in South Africa by Cloete et al. (2011). In this study only four of the nine species isolated were pathogenic on *E. camaldulensis* causing lesions statistically different from the control. Thus, on the basis of pathogenicity tests it is possible to assume that *N. vitifusiforme*, together with *Diaporthe foeniculina*, *Valsa fabianae* and the two unidentified *Cytospora* species represent a part of the endophytic eucalypt mycoflora.

The genus *Diaporthe* (teleomorph of *Phomopsis*) includes a great number of endophytic, saprobic and plant pathogenic fungi from a wide range of woody and herbaceous hosts (Gomes et al. 2013). Santos and Phillips (2009) in an attempt to resolve the complex of

Diaporthe species occurring on *Foeniculum vulgare* in Portugal introduced the species *D. neotheicola* A.J.L. Phillips & J.M. Santos as teleomorph of *Phomopsis theicola* Curzi and stated that *P. theicola* is not the same as *D. theicola*. Udayanga et al. (2014), studying species of *Diaporthe* causing melanose and stem end rot diseases of *Citrus* spp., revised the taxonomy of this group and introduced the new combination *D. foeniculina* to include ex-type isolates of *D. neotheicola*. In this study, *D. foeniculina* was isolated with a relatively low frequency at a single survey site and only during the spring sampling.

Eucalyptus spp. are known to host a large number of species of *Cytospora* and their *Valsa* teleomorphs, some of which are reported as agents of stem cankers. The taxonomic history of these genera has long been confused since species identification was based solely on the analysis of morphological features. The recent introduction of molecular techniques and phylogenetic analyses of different genomic regions has contributed to the clarification of the taxonomic position of many species within these genera (Adams et al. 2005). In this work two species of *Cytospora*, here reported as *Cytospora* sp. 1 and *Cytospora* sp. 2, were isolated both with low frequency values and only in the spring sampling at three and two investigated sites, respectively. We refrain from giving species names in this study because further investigations are required to allow unequivocal identification of these taxa. *Valsa fabianae* (anamorph *Cytospora eucalypticola* van der Westh.) was recently described from cankered and dead branches of several species of *Eucalyptus* in Australia, Uganda and South Africa (Adams et al. 2005). More recently this species was isolated from symptomatic and asymptomatic leaves of *E. globulus* trees affected by Mycosphaerella leaf disease in Spain (Sánchez Márquez et al. 2010). During the present survey *V. fabianae* was isolated from 11 sites and showed increasing values of isolation frequency from the spring to the autumn. Seasonal variations in the isolation frequency is a frequently-occurring characteristic in endophytic fungi (Collado et al. 1999; Linaldeddu et al. 2011).

In conclusion, this work represents the first attempt to identify the fungal pathogens which threaten *E. camaldulensis* plantations in Sardinia. Our results have shown that *N. australe* is the primary pathogen associated with V-shaped cankers and branch dieback. Abiotic factors that may have contributed to the outbreak of *N. australe* infections still remain unknown, as

well as its possible synergistic interaction with the other *Neofusicoccum* species. Data presented here, together with the results of previous studies on the aetiology of decline affecting holm oak, narrow-leaved ash and Phoenician juniper (Linaldeddu et al. 2011, 2014a; Alves et al. 2014), emphasize how species in the family *Botryosphaeriaceae* represent a growing threat to forest ecosystems in Sardinia.

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Chapter 6

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A new disease of *Erica arborea* in Italy caused by *Neofusicoccum luteum*

Abstract Shoot blight was observed on *Erica arborea* L., in a natural growing area on Caprera Island (Italy), during 2011 and 2012. Fungal isolates obtained from 324 symptomatic shoots were identified as *Neofusicoccum luteum* by analysis of morphological and cultural characteristics as well as DNA sequence data of the Internal Transcribed Spacer (ITS) of the ribosomal DNA and part of the translation Elongation Factor 1-alpha (EF1-α) gene. Pathogenicity was verified by stem inoculation of 3-year-old seedlings of *E. arborea*. This is the first report of *N. luteum* as a pathogen of *E. arborea*.

Keywords *Botryosphaeriaceae* - shoot blight - Caprera Island

Introduction

Tree heath (*Erica arborea* L.) in the family *Ericaceae*, is one of the main components of the Mediterranean "maquis" biome and a valuable ornamental plant for gardening and landscaping that is increasingly cultivated in commercial nurseries. In Sardinia (Italy) and in the surrounding minor islands belonging to the La Maddalena Archipelago, tree heath grows preferably on siliceous substrates and in mixed stands with cork oak, holm oak and strawberry tree (Camarda and Valsecchi 2008). Since 2011, an unusual disease on tree heath has been observed in a natural area located in the centre of Caprera Island. Given that there was no information about this disease, a survey was carried out to establish the causal agent and characterize the disease.

Materials and methods

Field surveys were carried out in autumn 2011 and spring 2012 at the major tree heath growing formations on Caprera Island [41°12′N, 9°28′E]. A network of 6 monitoring plots (MP, diameter 10 m) was established inside the tree heath stands and the geographic coordinate recorded by a portable GPS (eTrex, Garmin). The plots were randomly selected throughout the centre of the island where the disease was observed for the first time. At each

MP the number of tree heath shrubs present was detected and incidence of the disease estimated on the basis of number of symptomatic plants compared to the total plant number. At each plot 9 symptomatic shoots were collected from 3 tree heath shrubs chosen *at random*. Collected samples were processed within 48 hours.

After a preliminary microscopic examination aimed at checking the presence and nature of fungal reproductive structures, the samples were surface disinfected with 70% ethanol for 30 s, rinsed in sterile water and then placed to dry in aseptic conditions. Fungal isolation was made from fragments of inner bark and xylem tissues measuring approx. 3–5 mm, aseptically cut from the margin of necrotic lesions. All samples were cultured in Petri dishes containing potato dextrose agar (PDA; Oxoid Ltd). After incubation at 25°C for 1 week, fungal colonies were sub-cultured onto PDA and kept on the laboratory bench in natural daylight at room temperature (20–26°C). To induce sporulation, fungal isolates were also sub-cultured on PDA with sterile twigs of holm oak.

Conidia oozing from pycnidia were mounted in lactophenol for microscopic examination. Conidial dimensions were recorded using an Optika™ Vision Pro version 2.7 digital camera connected to a Leitz Diaplan (Leitz, Wetzelar, Germany) microscope. The colony appearance of cultures growing on PDA at 25°C in the dark for 1 week was recorded. Given that only a botryosphaeriaceous fungus was consistently isolated, six isolates were used as representative cultures for further molecular studies and stored on PDA slants under oil in the culture collection of the "Sez. di Patologia vegetale ed Entomologia, Dipartimento di Agraria" at the University of Sassari, as BL141-BL146.

Genomic DNA was extracted from cultures grown on PDA for 5 days at 25°C following the CTAB method (Doyle and Doyle 1987). The Internal Transcribed Spacer (ITS) of the ribosomal DNA was amplified and sequenced with primers ITS1 and ITS4 (White *et al*., 1990), while the primers EF446f and EF1035r (Inderbitzin et al. 2010) were used to amplify and sequence part of the translation Elongation Factor 1-alpha (EF-1α) gene. Polymerase chain reaction (PCR) mixtures and amplification conditions were conducted as described by Linaldeddu et al. (2013). The PCR products were purified using the EUROGOLD gel extraction kit (EuroClone S.p.A.) following the manufacturer's instructions. Both strands were

sequenced by the BMR Genomics DNA sequencing service (www.bmr-genomics.it). The nucleotide sequences were read and edited with FinchTV 1.4.0 (Geospiza, Inc.; http://www.geospiza.com/finchtv) and compared with sequences deposited in GenBank using the BLAST software (http://blast.ncbi.nlm.nih.gov).

Pathogenicity was verified by shoot and stem inoculation on 3-year-old seedlings of tree heath. Shoots of seven seedlings were inoculated by placing a mycelial plug (3 to 4 mm^2) taken from the margin of an actively growing colony on PDA into a shallow wound (~3 mm) made by a sterile scalpel. A further seven seedlings were inoculated at the base of the stem using the same technique described above. The inoculation point was covered with cotton wool soaked in sterile water and wrapped with Parafilm®. Fourteen control seedlings were inoculated (seven on shoots and seven on the stem) with a sterile PDA plug. Inoculated seedlings were kept in the laboratory at 18 to 26°C in natural daylight for 2 months.

Results and discussion

Symptomatology: typical symptoms of this unusual disease appear in late spring with the sudden wilting of new shoots (Fig. 1a). The infection spreads within the shoot tissues, rarely into the secondary xylem tissues of the branches, causing the shoot tip to curl and form a crook (Fig. 1b). An abnormal mass of new epicormic shoots grows under infected shoots, with the resulting structure resembling a witch's broom (Fig. 1c). Leaves on affected shoots turn yellow, then dull red and finally brown. They often remain attached for some time after the death of the shoots. All diseased plants showed the same symptoms. Disease incidence: field surveys conducted on 120 tree heath shrubs occurring inside the 6 MP showed that 112 of them were symptomatic and that the disease affects all tree heath formations located in the central part of the island. In particular, values of disease incidence among the 6 monitoring plots ranged from 45 to 100%.

Aetiology: all 324 symptomatic shoot samples processed, yielded colonies of a single species of *Botryosphaeriaceae*. On PDA at 25°C, all isolates developed a moderate aerial mycelium, with a light yellow pigment diffusing into the medium that became pale grey after 5 to 6 days on the reverse side of Petri dishes (Fig. 1d). Conidiomata were formed from the

centre of colonies within 3–4 weeks. All isolates produced hyaline, aseptate and fusiform to elliptical conidia (average of 50 conidia: 18.1 × 6.3 µm, l/w 2.9) (Fig. 1e), and sporadically elliptical microconidia. On the basis of morphological and cultural features the strains were identified as *Neofusicoccum luteum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips. Identification was confirmed by analysis of the ITS and EF1-α sequences. BLAST searches in GenBank showed 100% similarity with reference sequences of *N. luteum* including the ex-type isolate (CBS110299). New sequences were deposited in GenBank (ITS accession numbers: KJ406187-KJ406192; EF1-α accession numbers: KJ943360-KJ943365).

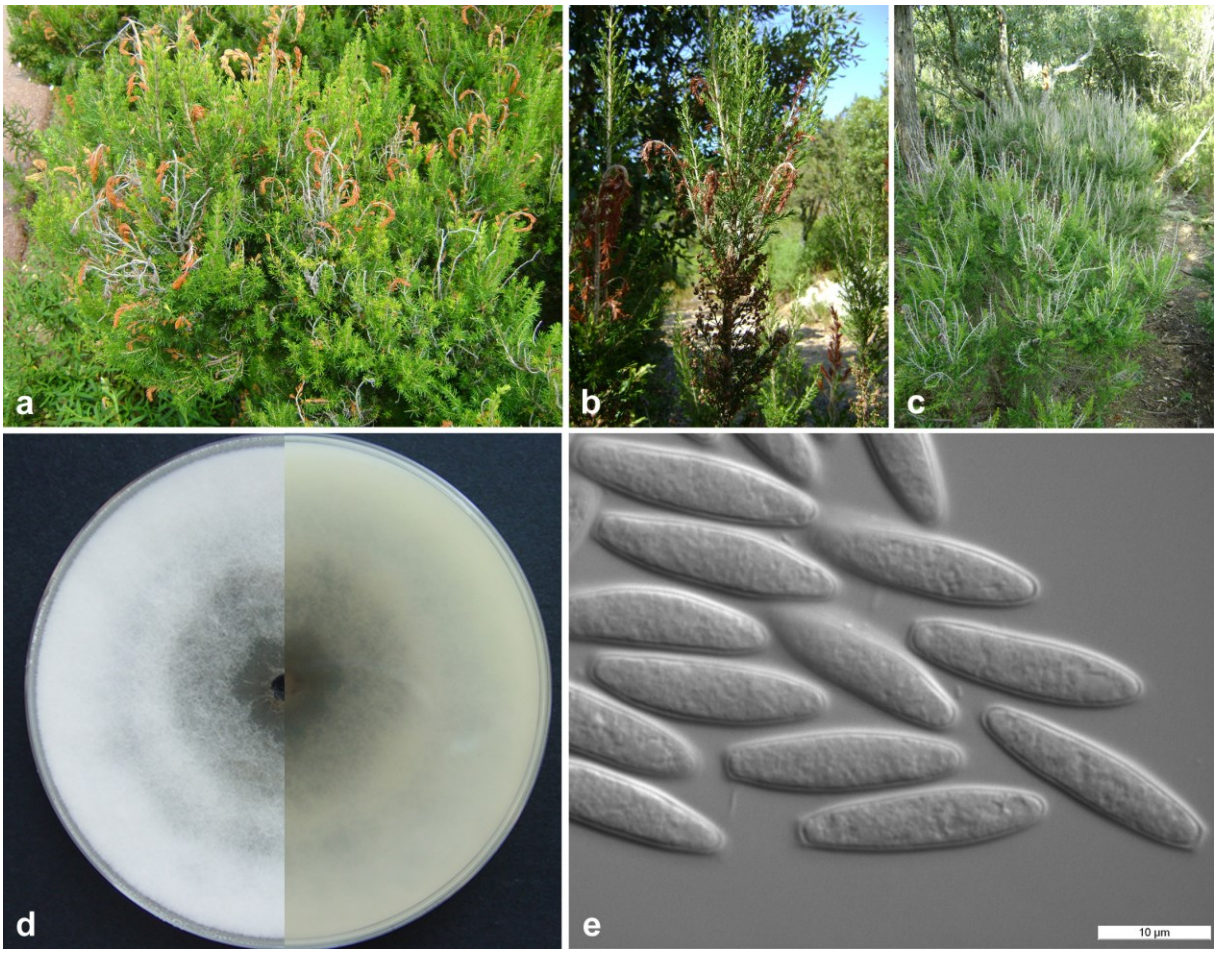

Figure 1. Early dieback symptoms on shoots of tree heath (a). Particular of shoots wilted with brown leaves still attached during the summer (b). Extensive twig and branch dieback (c). Colonies of *Neofusicoccum luteum* on PDA after 7 days at 25°C (left upper, right reverse) (d). Hyaline and aseptate conidia (e).

Pathogenicity: two months after inoculation, all seedlings inoculated with the strain BL142 obtained in this study showed dark brown necrosis at the inoculation point. However, on stems the mean lesion length produced by *N. luteum* was not significantly different from that of the control (Fig. 2). Whereas, on shoots *N. luteum* caused extensive necrotic lesions significantly different from those of the controls and the symptoms were identical to those observed in the field. The pathogen was successfully re-isolated from all shoots and stem samples processed, thus fulfilling Koch's postulates.

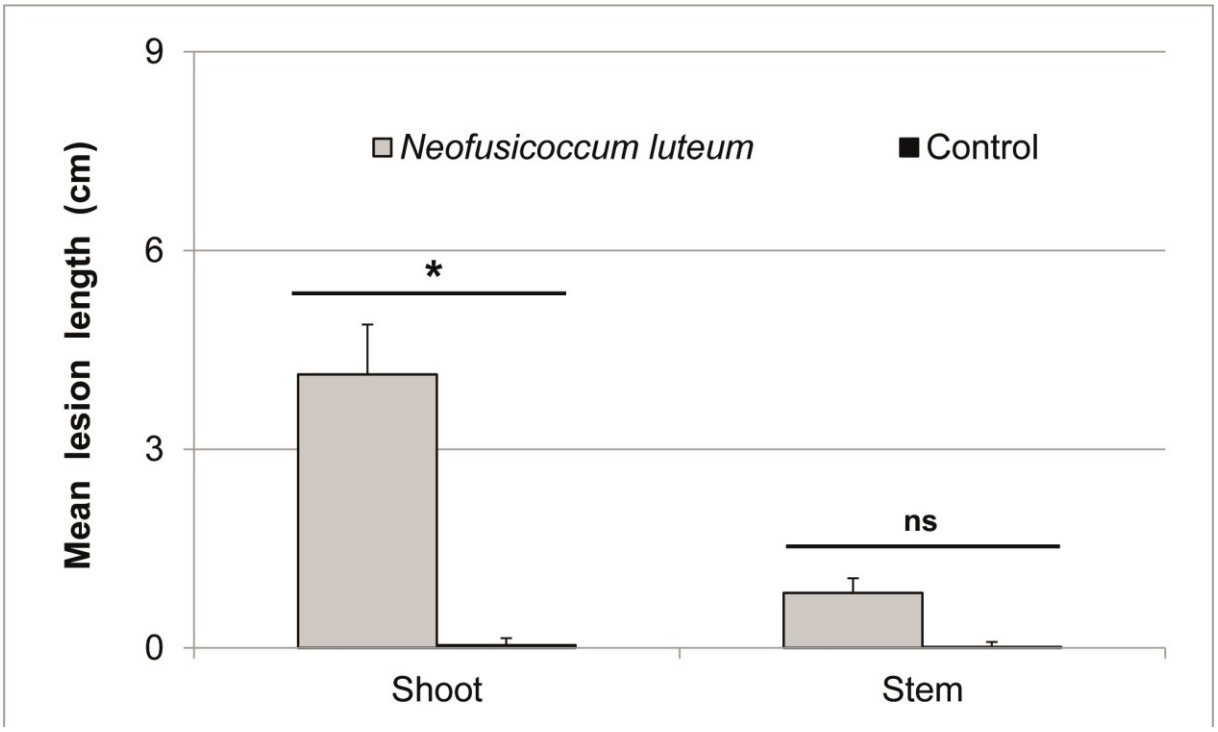

Figure 2. Mean lesion length caused by *Neofusicoccum luteum* on tree heath seedlings. Error bars represent the standard deviations from the mean. The data are plotted as average ± SD. Significant differences among mean values were determined with the Student's *t* test: * = $P < 0.05$, ns = not significant, using XLSTAT software (Addinsoft, France).

Neofusicoccum luteum was first isolated from lesions on ripe fruit of *Actinidia deliciosa* (A.Chev.) C.F. Liang & A.R. Ferguson in New Zealand (Pennycook and Samuels 1985), and more recently was found to be pathogenic to a wide range of woody hosts of agricultural and forestry importance such as *Crataegus mexicana* Moc. & Sessé ex DC., *Olea europaea* L., *Persea americana* Mill., *Quercus robur* L., *Rhododendron* spp., *Syzygium cordatum* Hochst. ex C. Krauss and *Vitis vinifera* L., in different countries around the world (Pavlic et al. 2007, McDonald et al. 2009, Sergeeva et al. 2009; Pintos Varela et al. 2011; Amponsah et al. 2012; Adesemoye et al. 2013; Barradas et al. 2013).

To our knowledge, this is the first report of *N. luteum* as a pathogen of tree heath. Despite the ecological importance of Mediterranean "maquis" the diseases affecting these plants have not yet been adequately studied. Given the high ecological relevance of this outbreak and the abundance of Mediterranean "maquis" ecosystems in Sardinia, further investigations are underway to monitor their health status.

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Concluding remarks

Research conducted as part of this thesis has contributed to the clarification of both the symptomatology and aetiology of some of the major emerging diseases affecting crops and forest trees in Sardinia. In particular, the results obtained showed that species belonging to the *Botryosphaeriaceae* family represent key pathogens of the patho-systems studied and can strongly affect the quality and quantity of the produce yielded as well as the integrity of natural ecosystems.

Many of the species studied are characterised by a high polyphagia and invasiveness and some were reported for the first time in Sardinia. Furthermore, three species (*Diplodia subglobosa*, *Lasiodiplodia exigua* and *Lasiodiplodia mediterranea*) have been isolated, characterised and described as new species, which has helped broaden scientific knowledge about taxonomy and phylogeny of this important group of plant pathogenic fungi.

Finally, new knowledge acquired regarding the complex patho-systems studied may have important practical applications. It can serve as a model for the assessment of the environmental risks associated with the diffusion of invasive pathogens in Mediterranean ecosystems and to develop new guidelines for integrated pest management strategies.

Abstract

Species of *Botryosphaeriaceae* have a cosmopolitan distribution and are known to cause serious diseases with dramatic economic implications in both agricultural and forestry ecosystems. In recent years, epidemic attacks of *Diplodia* and *Neofusicoccum* species have gradually increased in Sardinia on different hosts such as holm oak, cork oak and Juniper. Considering the plurivorous nature of many *Botryosphaeriaceae* species and the little information available on their geographic distribution, host range and genetic variability in Sardinia, it was considered appropriate in this PhD thesis to expand knowledge on five patho-systems by studying taxonomy, morphology, phylogeny and pathogenicity of the main species associated with plant cankers and dieback. Results obtained highlight that more than twenty *Botryosphaeriaceae* species occur on declining plants in both agricultural and forestry ecosystems. Among these, three species *Diplodia subglobosa*, *Lasiodiplodia exigua* and *Lasiodiplodia mediterranea*, morphologically and phylogenetically (ITS and EF1-α sequences data) distinct from all other known species, are described and illustrated herein. Finally, this study showed that species belonging to the *Botryosphaeriaceae* family currently represent a serious threat to the biodiversity of Sardinian natural ecosystems and can strongly affect the quality and quantity of the produce yielded.