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Botryosphaeriaceae species associated with lentisk dieback in Italy and description of *Diplodia insularis* sp. nov.

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Abstract

Lentisk (*Pistacia lentiscus* L.) is an evergreen shrub that is widespread throughout the Mediterranean region. Since spring 2012, a severe and unusual disease of unknown aetiology has been observed on lentisk in six islands of the La Maddalena archipelago (Italy). The affected plants showed leaf chlorosis, crown thinning, branch dieback and sunken cankers. When branches with sunken cankers were cross-sectioned, internal wood symptoms included characteristic V-shaped necrotic sectors. Frequently, the necrotic lesions girdled the branches resulting in death of the upper crowns. Since there is no information about the aetiology of this disease and given the high ecological importance of these natural ecosystems, from spring 2012 to summer 2014, 37 samples of twigs and branches of lentisk showing sunken cankers were collected and processed. Symptomatic woody samples yielded fungal isolates representing two distinct genera in the *Botryosphaeriaceae*. On the basis of morphological features and DNA sequence data three distinct species: *Diplodia olivarum*, *Neofusicoccum cryptoaustrale* and *N. luteum* were identified. In addition, another *Diplodia* species morphologically distinct from all known species was isolated. Phylogenetic analyses based on nucleotide sequences of ITS and *tef1*-α regions showed that this new *Diplodia* species is most closely related to *D. pseudoseriata* and *D. alatafructa*. Pathogenicity trials carried out in field conditions on asymptomatic branches of lentisk showed that all four species are aggressive pathogens on this host and therefore directly involved in the severe dieback that is currently threatening this typical shrub of the Mediterranean maquis.

Key words – *Diplodia* – Mediterranean maquis – *Neofusicoccum* – pathogenicity – phylogeny

Introduction

The La Maddalena archipelago (north-eastern Sardinia, Italy) is considered a hot spot of biodiversity characterized by a large number of endemic species and multiple habitats typical of the Mediterranean climate (Bocchieri 1992). The archipelago comprises 7 major islands and more than 50 small islets. The natural vegetation of the main islands is characterized by evergreen forest trees such as *Juniperus phoenicea*, *Olea europaea* var. *sylvestris*, *Quercus ilex*, *Salix atrocinerea* and *Tamarix africana*, mixed with species typical of the Mediterranean maquis such as *Arbutus unedo*,

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Erica arborea, *Myrtus communis*, *Phillyrea angustifolia*, *Pistacia lentiscus* and *Rhamnus alaternus* (Biondi & Bagella 2005).

Since 2008, a severe and widespread decline and mortality affecting several of these Mediterranean species has been recognized on Caprera island. Results of etiological studies carried out to establish the cause of these phenomena have revealed the primary role played by some species belonging to the genera *Diplodia* and *Neofusicoccum*. In particular, *Diplodia corticola* and *Neofusicoccum parvum* have been detected as the species most directly involved in the aetiology of decline affecting *Q. ilex* trees (Linaldeddu et al. 2014), *Diplodia africana*, *Neofusicoccum australe* and *N. cryptoaustrale* have been consistently associated with different disease symptoms on branches of *J. phoenicea* (Linaldeddu et al. 2011, Andolfi et al. 2012), while *Neofusicoccum luteum* has been recognized as the causal agent of a new disease of *E. arborea* (Linaldeddu et al. 2015b).

Furthermore, since spring 2012, a new disease of unknown aetiology has been observed on *P. lentiscus* (lentisk) in six of the main islands belonging to the La Maddalena archipelago. Since there is no information about the cause of this disease and given the high ecological importance of these natural ecosystems, the aim of this study was to clarify the aetiology of this new disease and determine the virulence of the major fungal species associated.

Materials & Methods

Field surveys and sampling procedure

Field surveys were carried out from spring 2012 to summer 2014 in six of the main islands belonging to the La Maddalena archipelago (Budelli, Caprera, Mortorio, Santa Maria, Santo Stefano and Spargi). A circular monitoring plot (MP, diameter 10 m) was established in each island (two in Caprera), and the geographic coordinates of plots were recorded by a portable GPS (*e*Trex, Garmin). The location of plots was based on the results of a preliminary survey about occurrence and distribution of symptomatic plants. At each MP, the number of trees was detected and their health status was assessed based on the occurrence of symptoms such as dieback, exudates, cankers and epicormic shoots. From each symptomatic plant a branch showing an active canker was collected. In total, 37 plants were sampled (Table 1).

Isolation and morphological characterization of canker-associated fungi

Symptomatic branch samples were taken to the laboratory and the outer bark surface tissue was cut away with a scalpel. Longitudinal and transversal cuts from symptomatic branch samples revealed internal symptoms. Fungi were isolated from chips of inner bark and xylem tissue (~5 mm^2) removed from the margin of necrotic lesions with a sterile scalpel. All chips were cultured on potato dextrose agar (PDA, Oxoid Ltd., Basingstoke, UK) in Petri dishes. After incubation at 25°C for 5–7 days in the dark, fungal colonies were sub-cultured onto half-strength PDA supplemented with autoclaved *Q. ilex* and *P. lentiscus* twigs and incubated at room temperature under natural daylight until pycnidia developed.

For each fungal species, colony growth characteristics including surface and reverse colony appearance were observed and recorded after 7 days of incubation at 25°C in the dark on PDA. Identification of isolates to species level was based on colony and conidial morphology as described by Phillips et al. (2013).

For novel species, cardinal temperatures for growth were determined on plates of PDA incubated at 5, 10, 15, 20, 25, 30 and 35°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 and mounted in 100% lactic acid. Measurements of conidiogenous cells and conidia were made with the Leica IM 500 measurement module from images recorded with the ×100 objective on a Leica DFC 320 digital camera. Spore dimensions are presented as mean values of 50 conidia with extreme values in parentheses. Dimensions of other structures are given as means of at least 20 measurements.

Table 1 Characteristics of the seven monitoring plots and number of *Pistacia lentiscus* plants sampled.

Island	Plot	Latitude	Longitude	Number of plants	
				Total	Sampled
Budelli	1	41°16'48"N	09°21'22"E	3	3
Caprera	2	41°12'51"N	09°27'01"E	12	10
	3	41°12'36"N	09°27'42"E	8	8
Mortorio	4	41°04'28"N	09°36'10"E	13	6
Santa Maria	5	41°17'47"N	09°22'25"E	5	3
Santo Stefano	6	41°11'56"N	09°24'38"E	5	5
Spargi	7	41°14'04"N	09°21'11"E	2	2

Table 2 *Diplodia* isolates included in the phylogenetic analyses. The newly generated sequences are indicated in italics and ex-type strains in bold face.

Isolate number	Species	Host	Country	GenBank accession number	
				ITS	*tef1-α*
CBS 120835	*D. africana*	*Prunus persica*	South Africa	EF445343	EF445382
CBS 121104	*D. africana*	*P. persica*	South Africa	EF445344	EF445383
CBS 132777	*D. agrifolia*	*Quercus agrifolia*	USA	JN693507	JQ517317
UCROK1429	*D. agrifolia*	*Q. agrifolia*	USA	JQ411412	JQ512121
CBS 124931	*D. alatafructa*	*Pterocarpus angolensis*	South Africa	FJ888460	FJ888444
CBS 130408	*D. allocellula*	*Acacia karroo*	South Africa	JQ239397	JQ239384
CBS 130410	*D. allocellula*	*A. karroo*	South Africa	JQ239399	JQ239386
CBS 124254	*D. bulgarica*	*Malus sylvestris*	Bulgaria	GQ923853	GQ923821
CBS 124135	*D. bulgarica*	*M. sylvestris*	Bulgaria	GQ923852	GQ923820
CBS 112549	*D. corticola*	*Quercus suber*	Portugal	AY259100	AY573227
BL7	*D. corticola*	*Quercus afares*	Tunisia	JX894190	JX894209
BL10	*D. corticola*	*Quercus ilex*	Italy	JX894191	JX894210
BL11	*D. corticola*	*Q. ilex*	Italy	JX894192	JX894211
MFLUCC 15-0648	*D. crataegicola*	*Crataegus* sp.	Italy	KT290244	KT290248
CBS 168.87	*D. cupressi*	*Cupressus sempervirens*	Israel	DQ458893	DQ458878
BL102	*D. cupressi*	*C. sempervirens*	Tunisia	KF307722	KF318769
CBS 140851	*D. eriobotryicola*	*Eriobotrya japonica*	Spain	KT240355	KT240193
CBS 136010	*D. fraxini*	*Fraxinus angustifolia*	Portugal	KF307700	KF318747
CBS 136013	*D. fraxini*	*F. angustifolia*	Italy	KF307710	KF318757
MFLUCC 15-0647	*D. galiicola*	*Galium* sp.	Italy	KT290245	KT290249
CBS 129750	*D. guayanensis*	*Acacia mangium*	Venezuela	JX545108	JX545126
CBS 129749	*D. guayanensis*	*A. mangium*	Venezuela	JX545106	JX545128
GUCC 0922-1	*D. huaxii*	*Platanus* sp.	China	KU848201	-
MFLUCC 14-1007	*D. italica*	*Crataegus* sp	Italy	KU848202	-
CBS 124462	*D. intermedia*	*M. sylvestris*	Portugal	GQ923858	GQ923826
CBS 112556	*D. intermedia*	*M. sylvestris*	Portugal	AY259096	GQ923851
CBS 140350	*D. insularis*	*Pistacia lentiscus*	Italy	*KX833072*	*KX833073*
BL99	*D. insularis*	*P. lentiscus*	Italy	*KX833074*	*KX833075*
BL183	*D. insularis*	*P. lentiscus*	Italy	*KX833076*	*KX833077*
BL132	*D. insularis*	*F. angustifolia*	Italy	KF307720	KF318767
BL133	*D. insularis*	*F. angustifolia*	Italy	KF307721	KF318768
BN-55	*D. insularis*	*E. japonica*	Spain	KT240361	KT240275

Isolate number	Species	Host	Country	GenBank accession number	
				ITS	*tef1-α*
CBS 124130	*D. malorum*	*M. sylvestris*	Portugal	GQ923865	GQ923833
BL127	*D. malorum*	*Populus alba*	Italy	KF307717	KF318764
CBS 136014	*D. mutila*	*P. alba*	Portugal	KJ361837	KJ361829
CBS122553	*D. mutila*	*Vitis vinifera*	Portugal	AY259093	AY573219
CPC 22753	*D. neojuniperi*	*Juniperus chinensis*	Thailand	KM006431	KM006462
CPC 22754	*D. neojuniperi*	*J. chinensis*	Thailand	KM006432	KM006463
CBS 121887	*D. olivarum*	*Olea europaea*	Italy	EU392302	EU392279
BL96	*D. olivarum*	*P. lentiscus*	Italy	*KX833078*	*KX833079*
CBS 124906	*D. pseudoseriata*	*B. salicifolius*	Uruguay	EU080927	EU863181
GUCC G603-1	*D. pseudoplatani*	*Platanus* sp.	China	KU848200	-
CBS 133852	*D. quercivora*	*Quercus canariensis*	Tunisia	JX894205	JX894229
CBS 133853	*D. quercivora*	*Q. canariensis*	Tunisia	JX894206	JX894230
CBS 116470	*D. rosulata*	*Prunus africana*	Ethiopia	EU430265	EU430267
CBS 116472	*D. rosulata*	*P. africana*	Ethiopia	EU430266	EU430268
CBS 393.84	*D. sapinea*	*Pinus nigra*	Netherlands	DQ458895	DQ458880
CBS 109725	*D. sapinea*	*Pinus patula*	Indonesia	DQ458896	DQ458881
CBS 109943	*D. sapinea*	*P. patula*	Indonesia	DQ458898	DQ458883
BL103	*D. sapinea*	*Retama raetam*	Tunisia	*KX833080*	*KX833081*
BL189	*D. sapinea*	*Corylus avellana*	Italy	*KX833082*	*KX833083*
CBS 118110	*D. scrobiculata*	*Pinus banksiana*	USA	KF766160	KF766399
CBS 109944	*D. scrobiculata*	*Pinus greggii*	Mexico	DQ458899	DQ458884
CBS 113423	*D. scrobiculata*	*P. greggii*	Mexico	DQ458900	DQ458885
CAP163	*D. scrobiculata*	*O. europaea*	Italy	EU392283	EU392260
BL5	*D. scrobiculata*	*Arbutus unedo*	Italy	GU722102	JX894231
CBS 112555	*D. seriata*	*V. vinifera*	Portugal	AY259094	AY573220
CBS 119049	*D. seriata*	*V. vinifera*	Italy	DQ458889	DQ458874
CAA502	*D. seriata*	*Fraxinus ornus*	Portugal	KJ361842	KJ361836
BL130	*D. seriata*	*F. angustifolia*	Italy	KF307723	KF318770
CBS 124133	*D. subglobosa*	*Lonicera nigra*	Spain	GQ923856	GQ923824
CBS 124131	*D. subglobosa*	*F. ornus*	Italy	GQ923855	GQ923823
CBS 418.64	*D. tsugae*	*Tsuga heterophylla*	Canada	DQ458888	DQ458873

Acronyms of culture collections: BL: B.T. Linaldeddu culture collection housed at Dipartimento di Agraria, Università di Sassari, Italy; CAA: Collection of Artur Alves housed at Department of Biology, University of Aveiro, Portugal; CAP, A.J.L. Phillips, Universidade Nova de Lisboa, Portugal; CBS: Centraalbureau voor Schimmelcultures, The Netherlands; CPC: Collection of Pedro Crous housed at CBS; UCROK, Department of Plant Pathology and Microbiology, University of California, Riverside; GUCC: Guizhou University Culture Collection (GUCC); MFLUCC: Mae Fah Luang University Culture Collection.

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. A representative culture of the new *Diplodia* species was also deposited at the Centraalbureau voor Schimmelcultures (CBS), Utrecht, the Netherlands and nomenclatural data in MycoBank (Crous et al. 2004) and Faces of Fungi (Jayasiri et al. 2015) databases. The holotype was lodged with the herbarium of Instituto Nacional de Investigação Agrária e Veterinária I.P., Oeiras, Portugal (LISE).

DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted from 5-day-old cultures grown on PDA at 25°C using Instagene Matrix (BioRad Laboratories, Hercules, California, USA). The internal transcribed

spacer (ITS) region of the ribosomal DNA was amplified and sequenced using primers ITS1 and ITS4 (White et al. 1990), while part of the translation elongation factor 1 alpha gene (*tef1*-α) was amplified and sequenced with primers EF446f and EF1035r (Inderbitzin et al. 2010). PCR amplification was carried out as described by Linaldeddu et al. (2013) and the products were purified using the EUROGOLD gel extraction kit according to the manufacturer's instructions (EuroClone S.p.A., Pero, Italy). Both strands were sequenced by the BMR Genomics DNA sequencing service. Sequences were edited with FinchTV v1.4.0 (Geospiza, Inc., Seattle, Washington, USA) and compared with sequences deposited in GenBank using the BLAST algorithm. New sequences were deposited in GenBank (Table 2 & Table 4) and alignments in TreeBase (S19828).

Phylogenetic analyses

ITS and *tef1-α* sequences of the isolates obtained in this study were combined and aligned with sequences retrieved from GenBank, representing all 28 *Diplodia* species known from culture. Alignments were done 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 made if necessary using BioEdit v. 7.2.5 (Hall 1999). Maximum likelihood (ML) analyses were done using MEGA6 (Tamura et al. 2013) using the best fitting DNA evolution model determined by the program. ML analyses were performed on a Neighbour-Joining starting tree automatically generated by the software. Nearest-Neighbour-Interchange (NNI) was used as the heuristic method for tree inference and 1000 bootstrap replicates were performed. The robustness of the trees was evaluated by 1000 bootstrap replications. Trees were visualized with TreeView v. 1.6.6 (Page 1996).

An initial ITS only phylogenetic analysis was carried out because for the species *Diplodia huaxii*, *D. italica* and *D. pseudoplatani* there are no *tef1-α* sequences available. However, since ITS alone cannot resolve all species in *Diplodia* an additional ITS plus *tef1-α* phylogenetic analysis was performed with all *Diplodia* species excluding the previously mentioned *D. huaxii, D. italica* and *D. pseudoplatani*.

Pathogenicity test

To verify the pathogenicity of each species investigated in this study, a field inoculation trial was conducted in August 2015 on asymptomatic *P. lentiscus* plants located on Caprera island (41°12'26"N, 09°27'54"E). During the experimental period, the daily mean air temperature was 20.3–31.5°C. Six branches (1–2.5 cm diameter) were inoculated with a representative isolate of each fungal species, and six uninoculated branches were used as controls. The inoculated region of the branch was surface-disinfected with 70% ethanol and a piece of outer and inner bark was removed with a flamed scalpel and replaced with an agar-mycelium plug taken from the margin of an actively growing colony on PDA. The inoculation site was covered with cotton wool soaked in sterile water and wrapped in a piece of aluminium foil secured with masking tape. Controls were inoculated with a sterile PDA plug applied as described above. After 1 month, the outer bark was carefully removed with a scalpel and the length of necrotic lesion surrounding each inoculation site was measured.

Re-isolation of inoculated species was attempted by transferring onto PDA 10 pieces of inner bark and wood taken from around the margin of each lesion. Cultures were grown in daylight and room temperature until fungal colonies developed.

Statistical analyses

Pathogenicity assay data were checked for normality, then subjected to analysis of variance (ANOVA). Significant differences among mean values were determined using Fisher's least significant differences multiple range test (P = 0.05) after one-way ANOVA using *XL*STAT software (Addinsoft, Paris, France).

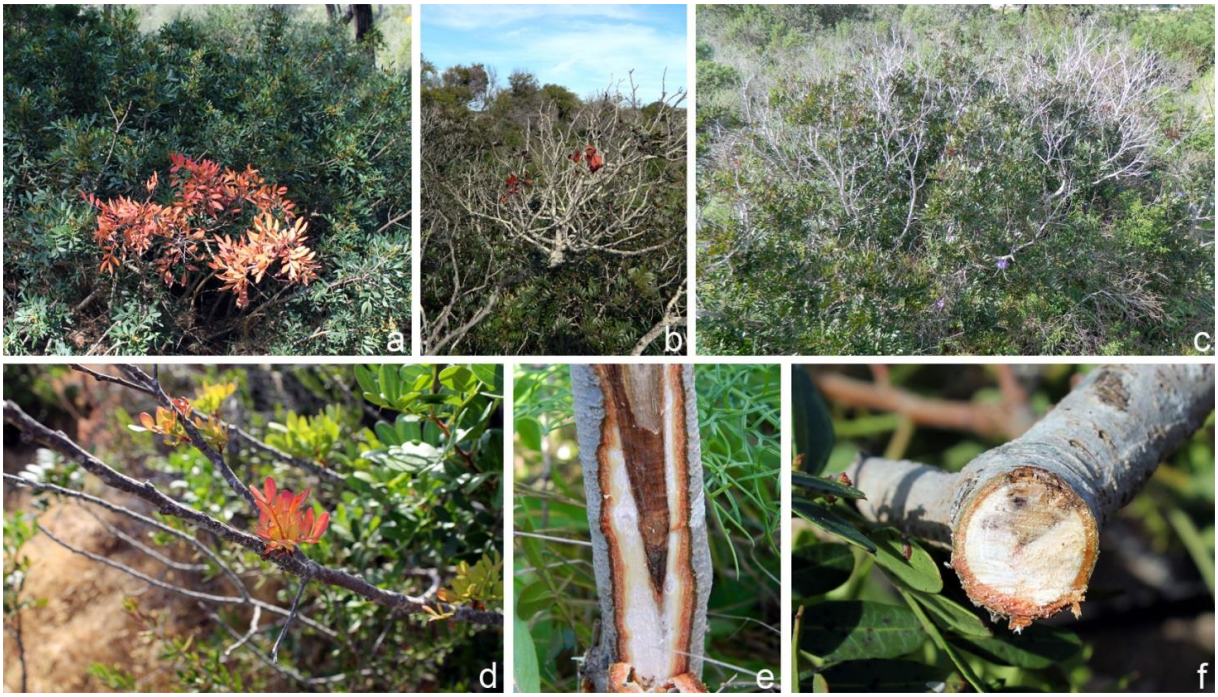

Fig. 1 – Disease symptoms observed in *Pistacia lentiscus* plants. **a–c**. Progressive dieback of twigs and branches. **d**. epicormic shoots along branches. **e**. wood necrosis visible after bark removal at level of a sunken cankers. **f**. cross section of a branch showing a characteristic wedge-shaped necrotic sector in the wood.

Table 3 Number of samples examined for each plot and associated fungi isolated.

Island	**Plot**	**Fungal species**			
		D. insularis	*D. olivarum*	*N. cryptoaustrale*	*N. luteum*
Budelli	1	-	-	2/3	1/3
Caprera	2	10/10	2/10	-	-
	3	5/8	3/8	-	-
Mortorio	4	-	1/6	2/6	1/6
Santa Maria	5	3/3	-	-	-
Santo Stefano	6	-	2/5	2/5	-
Spargi	7	-	2/2	-	-

Results

Field surveys

Out of 48 *P. lentiscus* plants investigated 37 displayed canopy disease symptoms, including the progressive dieback of twigs and branches associated with the abnormal growth of epicormic shoots (Fig. 1). Furthermore, symptomatic branches showed sunken cankers often associated with reddish brown exudations. After removing the outer and inner bark from cankers, dark brown necrotic lesions of variable size were visible on the xylem tissue. In cross-section, necrotic lesions appeared with the characteristic wedge-shaped aspect typical of *Botryosphaeriaceae* infections (Fig. 1).

Fungal isolation and identification

Isolation carried out from 37 cankered branch samples yielded a total of 36 fungal colonies belonging to the *Botryosphaeriaceae*. On the basis of morphological features and DNA sequence

data (ITS and *tef1*-α), three distinct species were identified: *Diplodia olivarum* (10 isolates), *N. cryptoaustrale* (6 isolates) and *N. luteum* (2 isolates) (Table 3). For each species BLAST searches against GenBank showed 99–100% identity to reference sequences of representative strains including those of ex-type isolates. In addition, eighteen *Diplodia*-like isolates on the basis of morphological features and DNA sequence data could not be assigned to any formerly described species. Although all processed samples showed the same symptoms the assemblages of pathogens associated was very different among the islands (Table 3). *Diplodia olivarum* was the species more widespread as it was isolated from plants in four of the six islands surveyed.

Phylogenetic analyses

The ITS ML analysis, as expected, did not resolve all species, especially within the clade containing *D. sapinea*, *D. scrobiculata* and other closely related species (Fig. 2). Within this large clade, the *Diplodia*-like isolates from lentisk (CBS 140350, BL99 and BL183) obtained in this study formed a separated and well-supported (89% bootstrap) clade containing also isolates from narrowed-leaved ash (BL132 and BL133) and loquat (BN-55) from previous studies. This clade clustered with the clade containing *D. alatafructa* and *D. pseudoseriata*, which contained also the ex-type strain of *D. pseudoplatani* GUCC G603-1 that could not be separated from *D. pseudoseriata* CBS124906. Additionally, the ex-type strains of *D. crataegicola* MFLUCC 15-0648 and *D. italica* MFLUCC 14-1007 clustered together while the ex-type strains of *D. huaxii* MFLUCC 15-0648 and *D. galiicola* MFLUCC 15-0647 clustered with isolates previously identified as *D. seriata* including the ex-epitype strain CBS112555.

Combined ITS plus *tef1-α* ML analysis resolved all *Diplodia* species although not all species clades received high (> 90%) bootstrap support (Fig. 3). The only exceptions were *D. scrobiculata*/*D. guayanensis* and *D. seriata/D. galiicola* that did not form separate clades. The *Diplodia*-like isolates from lentisk (CBS 140350, BL99 and BL183) formed a separate and highly supported (98% bootstrap) clade clustering closely to the *D. alatafructa* and *D. pseudoseriata* clade with 100% bootstrap support.

Taxonomy

Diplodia insularis Linaldeddu, A. Alves & A.J.L. Phillips, **sp. nov.** Fig. 4

MycoBank: MB 818231

Facesoffungi number: FoF 02607

Etymology – the epithet refers to the insular environments, where the species was originally found.

Sexual morph not seen. *Conidiomata* pycnidial, produced on *Pistacia lentiscus* twigs on ½ strength PDA within 2–4 weeks, solitary or aggregated, black, globose and uniloculate. *Conidiophores* absent. *Conidiogenous cells* hyaline, smooth, cylindrical, sometimes slightly swollen at the base, holoblastic, proliferating percurrently to form two or three distinct annellations or proliferating internally giving rise to periclinal thickenings, average of 20 conidiogenous cells 10.1 × 3.9 µm. *Conidia* initially hyaline becoming pigmented even while still attached to the conidiogenous cell, dark brown when mature, unicellular, rarely septate, ellipsoid to ovoid, wall finely roughened on inner surface 18.2–(22.6)–25.9 × 9.1–(11.7)–14.4 µm, ± S.D. = 22.6 ± 1.4 × 11.7 ± 0.6 µm, L/W 1.9 ± 0.2; n = 50).

Culture characteristics – Colonies on PDA attained 90 mm diameter before 7 days in the dark at 25 °C, the mycelium was moderately aerial, surface white at first and later turned pale grey to dark and dark in reverse.

Cardinal temperatures – Min. ≥ 5 °C, max. ≤ 35 °C, opt. 25 °C. All isolates failed to grow at 35 °C, but growth resumed when plates were moved to 25 °C.

Known distribution – Caprera, Santa Maria, Sardinia (Italy) and Castellón (Spain).

Habitat – On cankered branches of *Eriobotrya japonica*, *Fraxinus angustifolia* and *Pistacia lentiscus*.

Material examined – Italy, Santa Maria island, isolated from a branch canker of *Pistacia lentiscus*, 7 November 2013, Benedetto T. Linaldeddu, HOLOTYPE LISE 96309, a dried culture sporulating on *Pistacia lentiscus* twigs, culture ex-holotype CBS 140350 = BL140. Other isolates examined are listed in Table 2.

Notes – *Diplodia insularis* is phylogenetically closely related to *D. alatafructa* and *D. pseudoseriata*. However, the latter two species can be distinguished from *D. insularis* by the size and shape of conidia as expressed as the L/W ratio of about 1.9 for *D. insularis* and about 2.2 for both *D. alatafructa* and *D. pseudoseriata*.

Diplodia scrobiculata J. de Wet, Slippers & M.J. Wingf., Mycol. Res. 107: 562. 2003. MycoBank MB372427.
Synonym: *Diplodia guayanensis* F. Castro-Medina, J.R. Úrbez-Torres, S.R. Mohali & W.D. Gubler, *Plant Disease* (http://dx.doi.org/10.1094/PDIS-05-16-0612-RE). MycoBank MB812480

Notes – According to Úrbez-Torres et al. (2016) *D. guayanensis* is closely related phylogenetically to *D. scrobiculata* but can be distinguished by its larger conidia and the presence of up to 4 septa in conidia while in *D. scrobiculata* conidia up to 3 septa may be present. Here we show that both species are indistinguishable in phylogenetic analyses. Also, morphological variability is common in these fungi making it unreliable for species differentiation (Phillips et al. 2013).

Diplodia seriata De Not., Micr. Ital. Dec. 4: 6. 1942. MycoBank MB180468.
Synonym: *Diplodia galiicola* Dissanayake, Camporesi & K.D. Hyde, *Fungal Diversity* 75: 54. 2015, Facesoffungi: FoF00884.

Notes – According to Ariyawansa et al. (2015) *D. galiicola* is phylogenetically most closely related to *D. seriata* but can be distinguished by the shorter conidia. Here we show that both species are indistinguishable in phylogenetic analyses. Also, as already mentioned above morphological variability is common in these fungi making it unreliable for species differentiation (Phillips et al. 2013).

Pathogenicity test

All four *Botryosphaeriaceae* species proved to be pathogenic on *P. lentiscus*. At the end of the experimental period, all branches inoculated with *D. insularis*, *D. olivarum*, *N. cryptoaustrale* and *N. luteum* displayed dark brown bark lesions that spread up and down from the inoculation site (Fig. 5). The average lesion length differed significantly between species ($F_{3, 20} = 14.013$, $P < 0.001$; Tab. 4), e.g. the lesions caused by *N. cryptoaustrale* (mean = 7 cm) were significantly larger than those caused by *D. insularis* (mean = 3.8 cm), *D. olivarum* (mean = 2.6 cm) and *N. luteum* (mean = 2.4 cm). In addition, the branches inoculated with *D. insularis* and *N. cryptoaustrale* displayed wilting symptoms and a wedge-shaped necrotic sector in cross section congruent with field observations.

Control branches inoculated with sterile PDA plugs remained symptomless. All four fungal species were successfully re-isolated from symptomatic wood and inner bark tissues from all inoculated plants, thus fulfilling Koch's postulates (Tab. 4).

Discussion

This study represents the most comprehensive investigation of canker-causing agents of *P. lentiscus* to date in the Mediterranean region. For the first time the direct involvement of *Botryosphaeriaceae* species in the aetiology of canker and dieback symptoms of this important component of Mediterranean maquis was ascertained in various natural ecosystems. In particular, three fungal species belonging to two different genera were isolated and identified by means of morphological characters and DNA sequence data. These species included *D. olivarum*, *N. cryptoaustrale* and *N. luteum*. In addition, a novel species here described as *Diplodia insularis* was isolated.

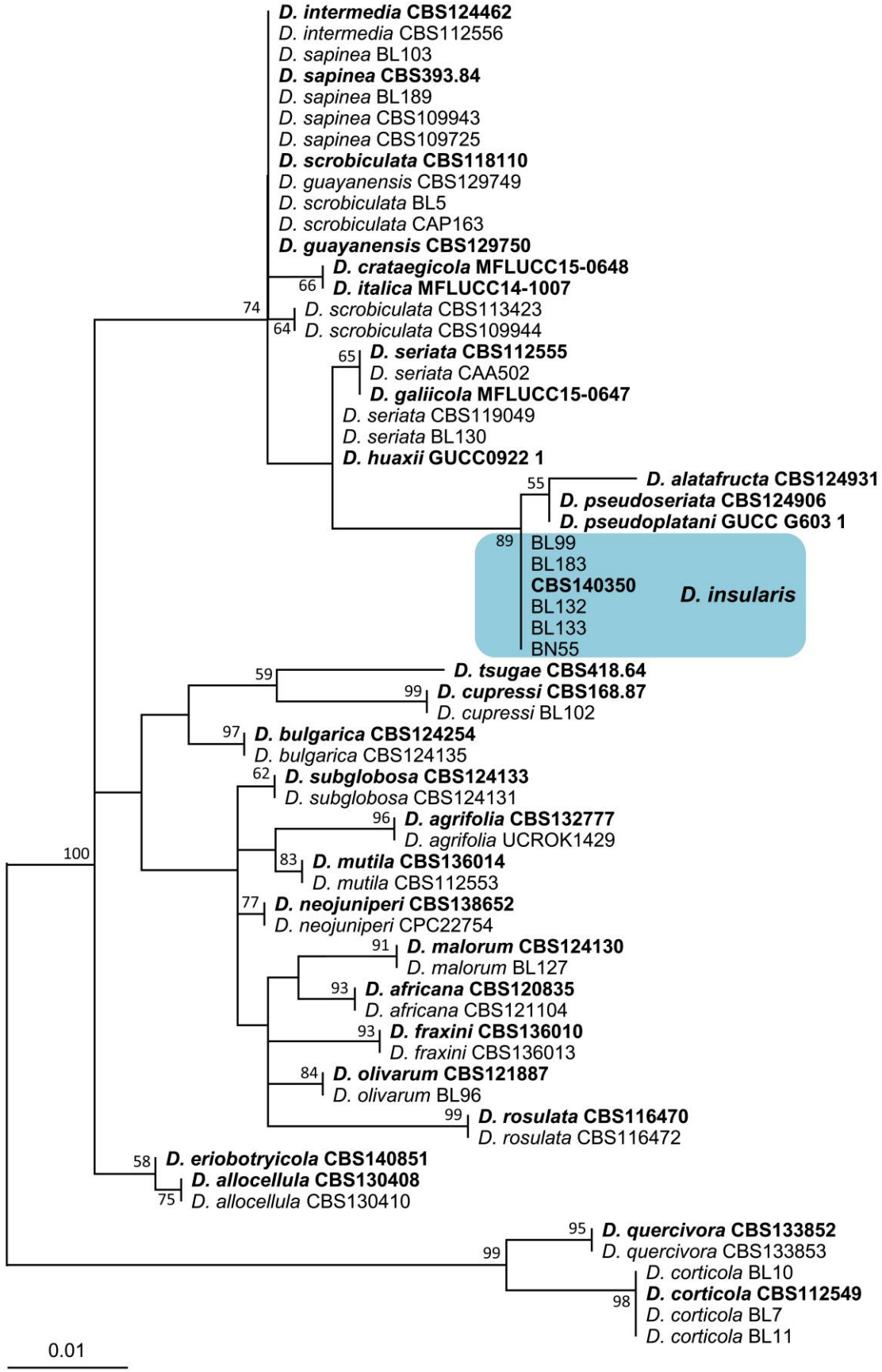

Fig.2 – Maximum Likelihood tree obtained from ITS sequence data and based on the Tamura 3-parameter model. The rate variation model allowed for some sites to be evolutionarily invariable. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Bootstrap support values in percentage (1000 replicates) are given at the nodes.

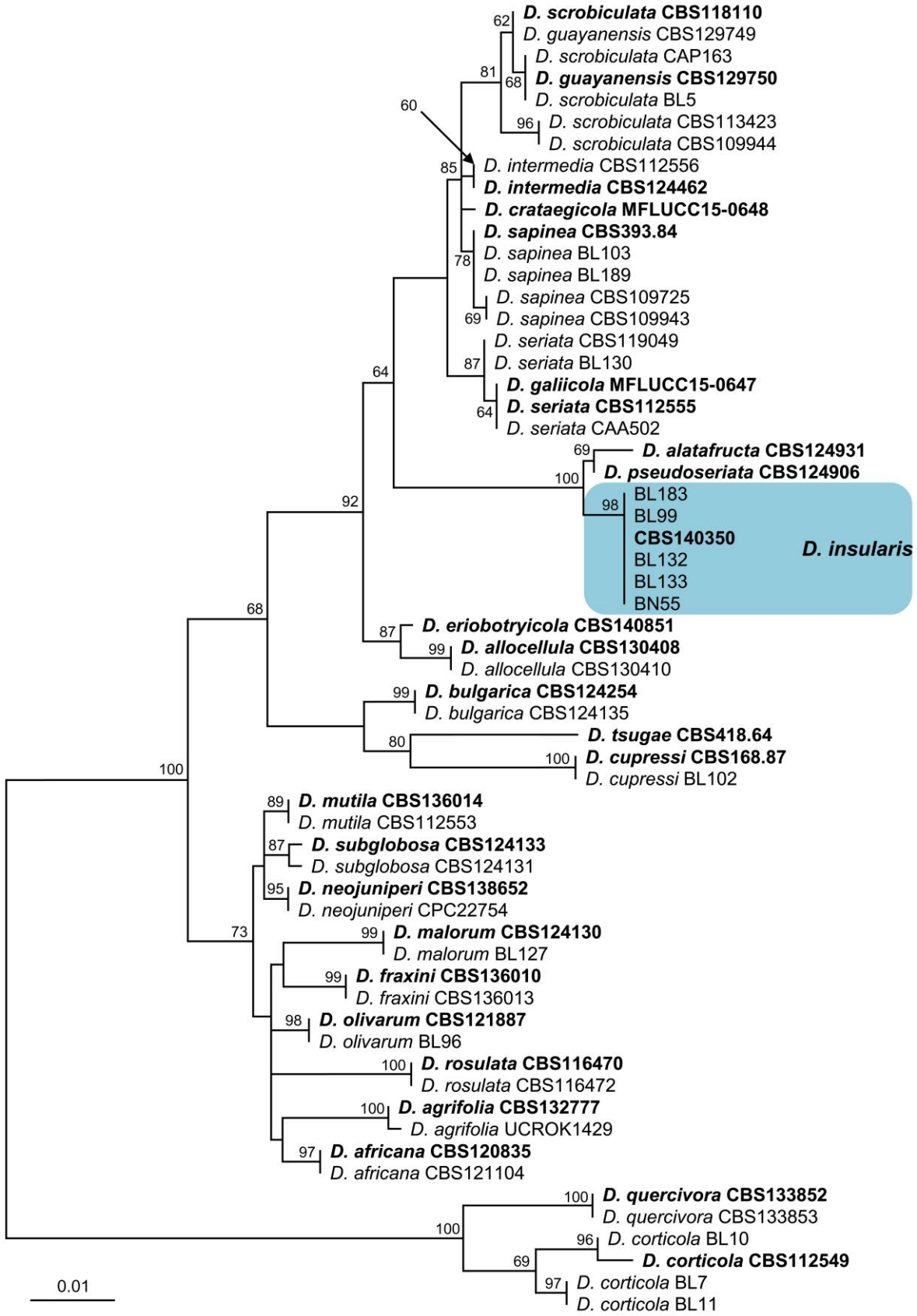

Fig. 3 – Maximum Likelihood tree obtained from combined ITS + *tef1-α* sequence data and based on the Hasegawa-Kishino-Yano model. A discrete Gamma distribution was used to model evolutionary rate differences among sites. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Bootstrap support values in percentage (1000 replicates) are given at the nodes.

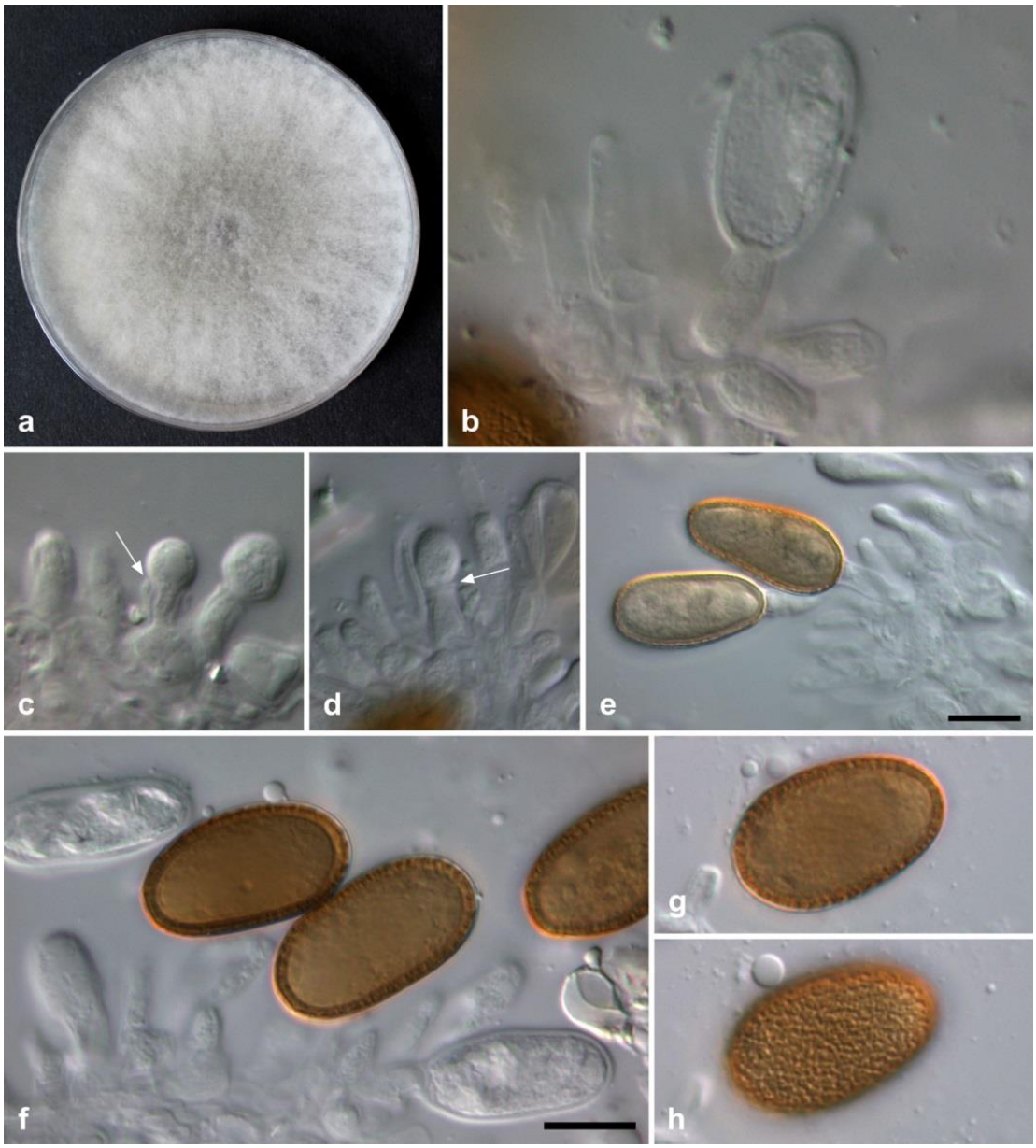

Fig. 4 – **a**. Colony morphology of *Diplodia insularis* after 7 days growth at 25 °C on PDA. **b–d**. hyaline immature conidia developing on conidiogenous cells. **e**. brown conidia still attached to the conidiogenous cell. **f**. mature conidia. **g, h**. two different focal planes to show the roughened inner surface of the conidium wall. Bars = 10 µm.

All four species are reported here for the first time on *P. lentiscus*. Strains of the new *Diplodia* species were first collected from declining *Fraxinus angustifolia* trees in Sardinia by Alves et al. (2014), but they did not introduce a new species to accommodate these strains, although the existence of important morphological and genetic differences between the fungal isolates from *F. angustifolia* and those belonging to the complex *Diplodia alatafructa*/*Diplodia pseudoseriata* were detected. These differences have been confirmed in the current study and the isolates obtained from *F. angustifolia* and *P. lentiscus* in Italy as well as a strain recently isolated from *E. japonica* in Spain by González-Domínguez et al. (2016), were considered representative of *D. insularis*. Morphologically, it is interesting to remark that in some species belonging to this *Diplodia* subclade, such as *D. allocellula*, *D. eriobotryicola* and *D. insularis*, conidia become pigmented while still attached to the conidiogenous cell, similar to what is seen in species of *Dothiorella* and *Spencermartinsia*. This has also been reported for *D. seriata*, *D. pinea*, *D. intermedia* and *D. scrobiculata* (Phillips et al. 2013).

Fig. 5 – Symptoms observed on *Pistacia lentiscus* branches 30 days after inoculation with, **a–c** *Diplodia insularis*. **d–f**. *Diplodia olivarum*. **g–i**. *Neofusicoccum cryptoaustrale*. **j–l**. *Neofusicoccum luteum*. **m, n**. Control branches.

Table 4 Mean lesion length ± standard deviation caused by fungal species on branches of *Pistacia lentiscus*.

Fungal species	Details of strains inoculated			Mean lesion length (cm)	Re-isolation frequency %
	Code	ITS	*tef1-α*		
Diplodia insularis	BL140	KX833072	KX833073	3.8±0.7b	100
Diplodia olivarum	BL96	KX833078	KX833079	2.6±0.6b	100
Neofusicoccum cryptoaustrale	BL94	KX833084	KX833085	7.0±2.5a	100
Neofusicoccum luteum	BL95	KX833086	KX833087	2.4±0.7b	100
Control				0.00	
LSD critical value				2.79	

Phylogenetic analysis based on the ITS region raised some doubts about the status of the recently described species *D. huaxii*, *D. italica* and *D. pseudoplatani* (Wijayawardene et al. 2016). However, the ITS region is known to be insufficient to accurately discriminate species in *Diplodia*. Thus, this issue can only be clarified when *tef1-α* sequences are available for these species. In ITS plus *tef1-α* phylogenetic analysis the ex-type of *D. galiicola* clustered with the ex-epitype of *D. seriata* and both species could not be separated. A comparison of the sequences from both species showed that the differences reported between them are located at the start of the *tef1-α* sequence of *D. galiicola*. This part of the *tef1-α* sequence, although located in a very conserved region, was highly divergent compared to all other *Diplodia* species, which suggests sequencing errors and lack of editing. For this reason, it was excluded from the alignments and coded as missing data.

Additionally, combined ITS and *tef1-α* phylogeny showed that the recently described *D. guayanensis* and *D. scrobiculata* (Úrbez-Torres et al. 2016) cannot be differentiated. A careful examination of sequence alignments showed that there are no differences in the ITS sequence and no fixed polymorphisms in the *tef1-α* sequences of *D. guayanensis* and the ex-type isolate of *D. scrobiculata* CBS118110 (= CMW189). Apparently these two species differ in a single nucleotide in the sequence of the β-tubulin gene. The differences between our results and those reported by Úrbez-Torres et al. (2016) arise from the fact that those authors used in their phylogenetic analyses older sequences for *D. scrobiculata* (ITS: AY253292 and *tef1-α*: AY624253) while we used more recent ones (ITS: KF766160 and EF: KF766399). Comparing the sequences it is evident that the older ones are shorter and contain sequencing errors. Thus, for the reasons explained above, *D. guayanensis* is considered as a synonym of *D. scrobiculata* and *D. galiicola* a synonym of *D. seriata*.

The pathogenicity of all *Botryosphaeriaceae* species obtained in this study was confirmed through an inoculation experiment in the field. All isolates tested were able to cause necrotic lesions and the isolate of *N. cryptoaustrale* proved to be highly aggressive. *Neofusicoccum cryptoaustrale* has previously been reported as an aggressive pathogen on other woody hosts in Sardinia such as *Vitis vinifera* and *Juniperus phoenicea* (Linaldeddu et al. 2010, Andolfi et al. 2012). The high degree of aggressiveness observed in this study for *D. insularis* is in agreement with the results obtained by González-Domínguez et al. (2016) on *E. japonica* in Spain.

Diplodia olivarum was first reported from rotting olive drupes and cankered branches of *Ceratonia siliqua* in Italy (Lazzizera et al. 2008, Granata et al. 2011). Subsequently it was reported as associated with declining *Prunus dulcis* trees in Spain (Gramaje et al. 2012) and cankered branches of *Quercus coccifera* in Tunisia (Alves et al. 2014).

Neofusicoccum luteum is emerging as a common and cosmopolitan species on diverse host plants, and it is now recognized as an aggressive pathogen of *Crataegus mexicana*, *Eucalyptus camaldulensis*, *Olea europaea*, *Persea americana*, *Quercus robur*, *Rhododendron* spp., *Syzygium cordatum* and *V. vinifera* (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, Deidda et al. 2016). Furthermore, *N. luteum* has recently been reported as a pathogen of *E. arborea* on Caprera island (Linaldeddu et al. 2015b). *Erica arborea* represents a "sporulation host" for *N. luteum*, large numbers of conidiomata and ascomata develop on infected shoots throughout the year, which serve as an important inoculum source (Linaldeddu unpublished data).

Members of *Botryosphaeriaceae* family represent a growing threat to agricultural crops, urban and natural forest ecosystems in Sardinia (Linaldeddu et al. 2014, 2015a, 2016). In particular, over the last few years there has been an exponential increase in the occurrence of diseases caused by species of this family in natural areas on the east coast of Sardinia, in particular on the islands of the La Maddalena archipelago, where in a limited geographic area of about 5,134 hectares, the involvement of 16 different species in the aetiology of new or unusual diseases of species of Mediterranean maquis have been detected (Linaldeddu unpublished data). This finding raises questions about the origin, introduction and pathway of these fungi as well as underlining the need to develop suitable actions to limit their further spread.

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