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Short Notes

First report of *Diplodia africana* on *Grevillea robusta*

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Summary. Branch cankers and dieback were observed on silky-oak trees (*Grevillea robusta*) along some streets of Palermo (Sicily, Italy). Fungi isolated from symptomatic branches were identified as *Diplodia africana* and *D. seriata* by morphological characters and phylogenetic analyses of combined ITS and translation elongation factor 1-α sequences. Pathogenicity was verified by inoculating twigs of 3-y-old silky-oak plants. This is the first report of *D. africana* on *G. robusta* and the first record of *D. seriata* on this host in the northern hemisphere.

Keywords. *Botryosphaeriaceae*, silky-oak, *D. seriata*, dieback, canker.

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INTRODUCTION

Grevillea robusta A. Cunn. ex R.Br. (Proteaceae), commonly known as silky-oak, is an evergreen tree native to the subtropical coastal regions of northern New South Wales and southern Queensland, Australia. The plant was introduced by the British to the Ligurian Riviera of Italy, and some shrubby species of *Grevillea* are common in Italian gardens. *G. robusta* is widely used as an ornamental tree along streets and in public and private gardens. This tree is well suited to the climates of southern Italy and the major islands of Sicily and Sardinia, as it prefers high atmospheric humidity (Raimondo *et al*., 1995; Venturella *et al*., 1990). In the USDA fungus-host database, 110 taxa are listed as associated with *G. robusta* (Farr & Rossman, 2019). Among these fungi there are three Botryosphaeriaceae species associated with branch cankers and dieback of *G. robusta* in Africa. and all three were shown to be pathogenic (Toljander *et al*., 2007; Njuguna *et al*., 2011). Members of the Botryosphaeriaceae have cosmopolitan distribution, occur on a very wide range of hosts, and several are known to cause dieback of woody hosts (Úrbez-Torres, 2011).

Symptoms of branch cankers and dieback similar to those previously reported in Africa (Toljander *et al*., 2007; Njuguna *et al*., 2011) were recently observed on silky-oak trees along some urban streets in Palermo, Sicily. The aims of the research reported here was to identify the Botryosphaeriaceae

species associated with diseased silky-oak trees, using a combination of morphological and DNA sequence data, and to evaluate the pathogenicity of the identified species to silky-oak plants.

MATERIALS AND METHODS

In September 2016 and in March 2017, 96 trees of *G. robusta* of approximate age 13 y, and cultivated as ornamental plants in Roccazzo Street in Palermo (38°07'18.1"N, 13°18'28.1"E) were examined for disease symptoms. The symptomatic plants showed branch dieback, lesions and cankers on branches and stems, dead shoots and presence of orange to red-brown resin exudation on the stems and branches. After preliminary observation, the trees with cankers were counted to determine disease incidence. In both surveys, samples of symptomatic branches were collected from nine randomly selected trees and transferred to the laboratory for analyses. Wood portions were flame sterilized, and 70 small fragments, cut from the margins of necrotic lesions, were transferred to plates of potato dextrose agar (PDA Oxoid Ltd) supplemented with 0.5 g L^{-1} of streptomycin sulphate (Sigma-Aldrich). After incubation at 25±1°C in the dark for 4 d, the percentage isolation frequency (IF) of fungi was determined, as the number of tissue portions infected by a given fungus divided by the total number of tissue segments incubated. Hyphal tips taken from colony margins were sub-cultured on PDA. Sporulation was induced by culturing the isolates on quarter-strength PDA (¼ PDA) supplemented with double-autoclaved pine needles for 2 months at 23°C. After sporulation, pycnidia were mounted in water, and 50 conidia were measured using a light microscope (Axioskop; Zeiss) at ×400 magnification, and images were captured using the Axio-Vision 4.6 software (Zeiss). Conidium dimensions are presented here as 5th and 95th percentiles with extreme values in parentheses, L/W ratios, means and standard deviations (S.D.).

One isolate of each species was used for determination of the optimum temperature for growth and for pathogenicity tests, and morphology of colonies grown on PDA at 25°C in the dark for 4 d was recorded. Cardinal temperatures for growth of one selected isolate for each species was determined by incubating the culture on PDA in the dark at temperatures from 5 to 40°C at 5°C intervals, with three replicates per temperature. Colony diameters were measured along two perpendicular axes when the colony reached at least two-thirds of the plate diameters, and the data were converted to daily radial growth rates)mm d^{-1}). Plates incubated at temperatures in which there was no growth were then incubated at 25°C to determine if these temperatures were fungistatic or fungicidal.

Genomic DNA was isolated from 1-week-old fungal cultures grown on PDA at 25°C in the dark, using a standard CTAB-based protocol (O'Donnell *et al*., 1998). The internal transcribed spacer (ITS) region of the ribosomal DNA and part of the translation elongation factor 1 alpha gene (tef1-α) were amplified and sequenced, respectively, with primers ITS1/ITS4 (White *et al*., 1990) and EF1-728F/EF1-986R (Carbone and Kohn, 1999). PCR amplification and sequencing of amplicons was carried out as described by Phillips *et al*. (2013). Sequences were edited with Sequencher v 4.7 (Gene Codes Corporation), and compared with sequences deposited in GenBank through BLASTn searches. New sequences were deposited in GenBank (Table 1). Phylogenetic analyses was performed as described by Giambra *et al*. (2016). Alignments were made using ClustalX v. 1.83 (Thompson *et al*. 1997), and when necessary these were manually edited using MEGA6 (Tamura *et al*. 2013). Maximum likelihood (ML) analyses were performed on a Neighbour-Joining starting tree automatically generated by MEGA6. Nearest-Neighbour-Interchange (NNI) was used as the heuristic method for tree inference, and 1,000 bootstrap replicates were performed.

Pathogenicity was tested by twig inoculation of one isolate for each species in July 2018, on 3-year-old silky-oak seedlings. For each inoculation, an agar plug, cut from the margin of a 6-d-old colony grown on PDA at 25°C in the dark, was placed in a wound (≅4 mm^2) made with a scalpel on the twig of each seedling, after bark disinfection with 70% ethanol. The plug was then covered with Parafilm®. Non-colonized agar plugs were applied to similar wounds on control plants. Five seedlings were inoculated with each isolate and five seedlings for controls were used in a randomized block experimental design. The inoculated plants were kept outdoors under natural environmental conditions. The lengths of vascular discoloration were measured 3 months after inoculation, and these data were checked for normality using Shapiro-Wilk test and were subjected to ANOVA.

RESULTS AND DISCUSSION

Surveys carried out on silky-oaks in an urban street revealed branch dieback, lesions and cankers on branches and stems, dead shoots, and the presence of orange to red-brown resin exudation on stems and branches (Figure 1a, b, c). In some cases, the disease caused the death of the affected trees. Disease incidence increased from

Table 1. Isolates included in the phylogenetic study. The newly generated sequences are indicated in italics and ex-type strains are indicated in bold font.

Species	Isolate number	Host	Country	GenBank	
				ITS	*tef1*-α
Diplodia africana	**CBS 120835**	*Prunus persica*	South Africa	EF445343	EF445382
	CBS 121104	*Prunus persica*	South Africa	EF445344	EF445383
	DA1	*Juniperus phoenicea*	Italy	JF302648	JN157807
	GB34	*Grevillea robusta*	Italy	KY486864	KY486895
	GB35	*Grevillea robusta*	Italy	*KY486865*	*KY486896*
	GB36	*Grevillea robusta*	Italy	*KY486866*	*KY486897*
	GB37	*Grevillea robusta*	Italy	*KY486867*	*KY486898*
	GB38	*Grevillea robusta*	Italy	*KY486869*	*KY486899*
	GB39	*Grevillea robusta*	Italy	*KY486870*	*KY486900*
	GB40	*Grevillea robusta*	Italy	*KY486871*	*KY486901*
	GB41	*Grevillea robusta*	Italy	*KY486872*	*KY486902*
	GB42	*Grevillea robusta*	Italy	*KY486873*	*KY486903*
	GB43	*Grevillea robusta*	Italy	*KY486874*	*KY486904*
	GB44	*Grevillea robusta*	Italy	KY486875	KY486905
	GB45	*Grevillea robusta*	Italy	*KY486876*	*KY486906*
	GB46	*Grevillea robusta*	Italy	*KY486877*	*KY486907*
D. agrifolia	**CBS 132777**	*Quercus agrifolia*	California	JN693507	JQ517317
	UCROK 1429	*Quercus agrifolia*	California	JQ411412	JQ512121
D. alatafructa	**CBS 124931**	*Pterocarpus angolensis*	South Africa	FJ888460	FJ888444
D. allocellula	**CBS 130408**	*Acacia karroo*	South Africa	JQ239397	JQ239384
	CBS 130410	*Acacia karroo*	South Africa	JQ239399	JQ239386
D. bulgarica	**CBS 124254**	*Malus sylvestris*	Bulgaria	GQ923853	GQ923821
	CBS 124135	*Malus sylvestris*	Bulgaria	GQ923852	GQ923820
D. corticola	**CBS 112549**	*Quercus suber*	Portugal	AY259100	AY573227
	BL10	*Quercus ilex*	Italy	JX894191	JX894210
D. cupressi	**CBS 168.87**	*Cupressus sempervirens*	Israel	DQ458893	DQ458878
	BL102	*Cupressus sempervirens*	Tunisia	DQ458894	DQ458879
D. fraxini	**CBS 136010**	*Fraxinus angustifolia*	Portugal	KF307700	KF318747
	CBS 136013	*Fraxinus angustifolia*	Italy	KF307710	KF318757
D. intermedia	**CBS 124462**	*Malus sylvestris*	Portugal	GQ923858	GQ923826
	CBS 112556	*Malus sylvestris*	Portugal	AY259096	GQ923851
D. malorum	**CBS 124130**	*Malus sylvestris*	Portugal	GQ923865	GQ923833
	BL127	*Populus alba*	Italy	KF307717	KF318764
D. mutila	**CBS 136014**	*Populus alba*	Portugal	KJ361837	KJ361829
	CBS 112553	*Vitis vinifera*	Portugal	AY259093	AY573219
D. neojuniperi	**CBS 138652**	*Juniperus chinensis*	Thailand	KM006431	KM006462
	CPC 22754	*Juniperus chinensis*	Thailand	KM006432	KM006463
D. olivarum	**CBS 121887**	*Olea europaea*	Italy	EU392302	EU392279
	CAP 257	*Olea europaea*	Italy	GQ923874	GQ923842
D. pseudoseriata	**CBS 124906**	*Blepharocalyx salicifolius*	Uruguay	EU080927	EU863181
D. quercivora	**CBS 133852**	*Quercus canariensis*	Tunisia	JX894205	JX894229
	CBS 133853	*Quercus canariensis*	Tunisia	JX894206	JX894230
D. rosacearum	**CBS 141915**	*Eriobotrya japonica*	Italy	KT956270	KU378605
	NB8	*Eriobotrya japonica*	Italy	KT956271	KU378606
D. rosulata	**CBS 116470**	*Prunus africana*	Ethiopia	EU430265	EU430267
	CBS 116472	*Prunus africana*	Ethiopia	EU430266	EU430268

(Continued)

Table 1. (Continued).

Species	Isolate number	Host	Country	GenBank	
				ITS	*tef1*-α
D. sapinea	**CBS 393.84**	*Pinus nigra*	Netherlands	DQ458895	DQ458880
	CBS 109725	*Pinus patula*	Indonesia	DQ458896	DQ458881
D. scrobiculata	**CBS 118110**	*Pinus banksiana*	Wisconsin	KF766160	KF766399
	CBS 109944	*Pinus greggii*	Mexico	DQ458899	DQ458884
	CBS 113423	*Pinus greggii*	Mexico	DQ458900	DQ458885
D. seriata	**CBS 112555**	*Vitis vinifera*	Portugal	AY259094	AY573220
	CBS 119049	*Vitis vinifera*	Italy	DQ458889	DQ458874
	NB4	*Eriobotrya japonica*	Italy	KT956267	KU310680
	GB1	*Grevillea robusta*	Italy	*KY486863*	*KY486893*
	GB2	*Grevillea robusta*	Italy	*KY486868*	*KY486894*
D. tsugae	**CBS 418.64**	*Tsuga heterophylla*	Canada	DQ458888	DQ458873

Acronyms of culture collections: BL: B.T. Linaldeddu culture collection housed at Dipartimento di Agraria, Università di Sassari, Italy; 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.

Figure 1. a, b: Symptoms of branch dieback and stem cankers, on *Grevillea robusta*. c: resin exudation on trunk.

61% in September 2016 to 83% in March 2017, indicating rapid development of the disease. Samples collected from diseased trees showed brown to black vascular longitudinal discoloration under the bark, and wedge-shaped necrotic sectors visible in cross sections.

Fifteen *Diplodia* isolates were obtained from the collected samples (Table 1). Identification based on morphological characters revealed two distinct species: 13 isolates of *Diplodia africana* Damm & Crous and two isolates of *Diplodia seriata* De Not., showing different isolation frequency (26% for *D. africana* and 4.0% for *D. seriata*).

Colonies of *D. africana* isolates on PDA at 25°C were initially white and became dark grey-olivaceous after 6 d, with moderate amounts of aerial mycelium, and the colonies reached the edges of Petri dishes within 5 d (Figure 2a). Colonies produced conidia that were hyaline, thick-walled, aseptate, smooth, cylindrical to

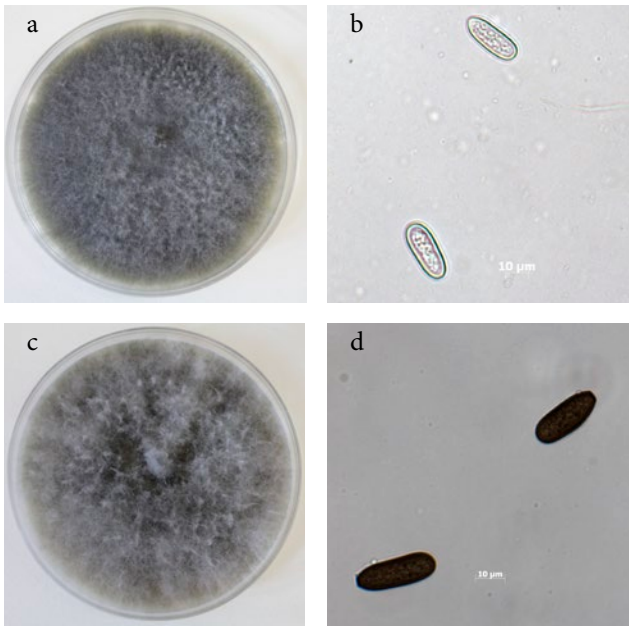

Figure 2. Colonies and conidia on PDA after 7 d at 25°C: (a, b) *Diplodia africana;* (c, d) *Diplodia seriata*.

oblong elliptical, with rounded apices. The conidium dimensions were (25.3–)26.3–32.3(–34.3) × (9.5–)11.9–15.6(–16.3) µm, 29.7 ± 1.9 × 14.1 ± 1.1 µm, with L/W ratio = 2.1 (Figure 2b). The optimum temperature for colony growth of *D. africana* was 25°C with a temperature range of 5–30°C. None of the tested temperatures were fungicidal. Conidium morphology and cultural features of the isolates agreed with the description of *D. africana* by Damm *et al*. (2007). Nevertheless, they reported a maximum temperature for growth of 35°C and optimum of 20°C (Damm *et al*., 2007).

Colonies of *D. seriata* on PDA at 25°C filled Petri dishes before 6 d, and had compact aerial mycelium, which was initially white, and becoming dark gray after 5 d (Figure 2c). Conidia were initially hyaline, becoming dark brown, aseptate, moderately thick-walled, wall externally smooth, and were ovoid, with obtuse apices. They measured (20.0–)21.3– 27.7(–28.3) × (8.7–)9.3–11.0(–11.8) µm, 24.9 ± 1.9 × 10.2 ± 0.6 µm, with L/W = 2.4 (Figure 2d). Isolates of *D. seriata* had optimum growth temperature of 26°C, with a temperature range of 5–35°C. Cultures incubated at 40°C did not grow when returned to 25°C. Optimum temperature, conidium dimensions and morphology determined in this study corresponded to those reported by Phillips *et al*. (2013).

Identification was confirmed by analysis of the ITS and *tef1*-α sequences. The phylogenetic tree (Figure 3) differentiated the *Diplodia* species, and bootstrap values showed strong support for many branches. Thirteen isolates were included in a clade with the ex-type isolate of *D. africana*, and two isolates clustered in a clade with the ex-epitype of *D. seriata*.

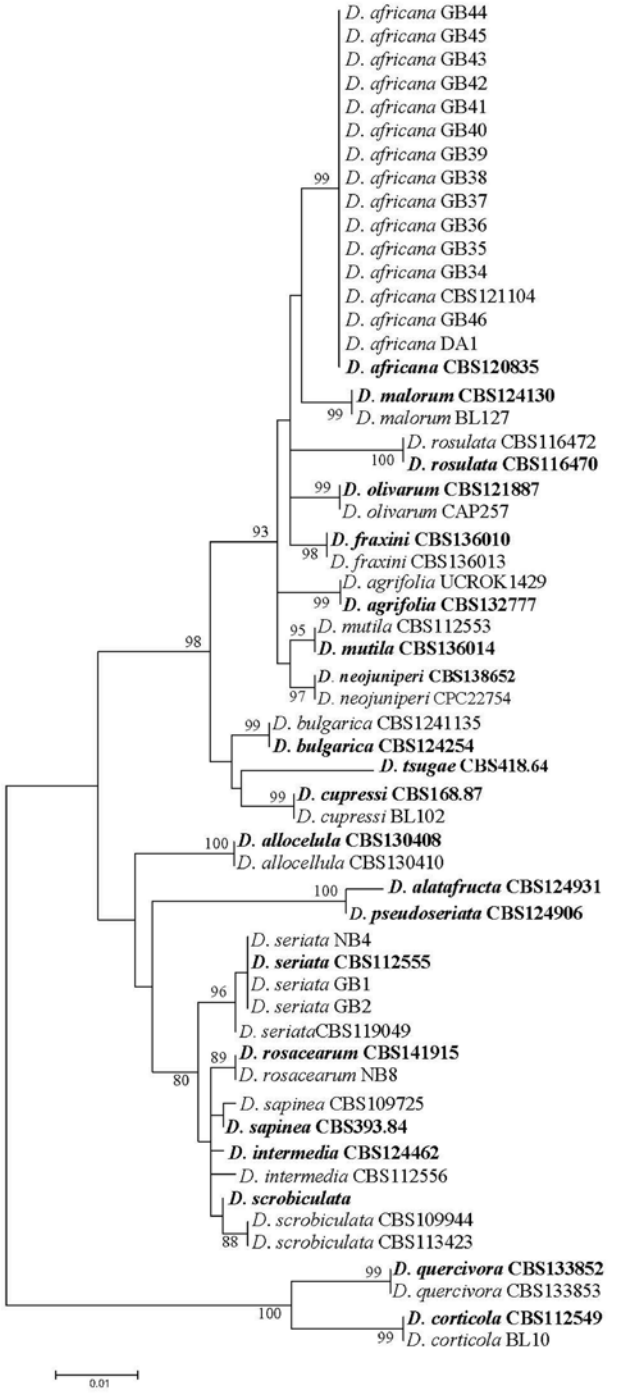

Figure 3. Maximum Likelihood tree of combined ITS and *tef1-α* sequence data for *Diplodia* species. The tree was drawn to scale, with branch lengths measured for the number of substitutions per site. Bootstrap support values ≥ 70% (1,000 replicates) are given at the nodes. The type species are in bold.

Figure 4. Subcortical dark-brown discolorations spreading from the inoculation points on *Grevillea robusta* twigs, 3 months after artificial inoculation with *Diplodia africana* (a), *Diplodia seriata* (b) and agar plugs not inoculated with a fungus (c).

Three months after inoculation, twigs of test plants displayed necrotic bark lesions spreading upward and downward from the inoculation points (Figure 4a, b). Slight sub-cortical discoloration was observed in the lesions caused by *D. seriata* while infections caused by *D. africana* developed from the bark tissues toward the wood where they penetrated a few millimeters, and the bark surface was longitudinally fissured (Figure 4a, b). No discoloration was observed in the stems of control plants (Figure 4c). *Diplodia africana* induced lesions with a mean length of 18.2±1.3 mm, while mean lesion lengths caused by *D. seriata* were 15.5±1.4 mm. Differences between the two species were not statistically significant ($F_{1, 6}$ = 1.98; P = 0.21). Both fungus species tested were successfully re-isolated from inoculated plants, thus fulfilling Koch's postulates. No fungal pathogens were isolated from the control plants.

The results reported here indicate that *D. africana* and *D. seriata* were the etiological agents for branch cankers and dieback of *G. robusta* trees in Sicily. This is the first report of *D. africana* on *G. robusta*, and also the first record of *D. seriata* on *G. robusta*, in the northern hemisphere. Similar disease symptoms were observed in Africa on *G. robusta*, and the causal agents were shown to be *Neofusicoccum parvum* (Pennycook and Samuels) Crous, Slippers and Phillips, *Lasiodiplodia theobromae* (Pat.) Griffon and Maubl. and, *D. seriata* (Toljander *et al*., 2007; Njuguna *et al*., 2011). Pathogenicity tests showed that *N. parvum* and *L. theobromae* were highly pathogenic on *G. robusta*, whereas *D. seriata* was moderately or weakly pathogenic (Toljander *et al*., 2007; Njuguna *et al*., 2011). *Diplodia seriata* is a cosmopolitan botryosphaeriaceous fungus and is known to be polyphagous, occurring on many native or introduced plants (Phillips *et al*. 2013), while *D. africana* has a limited reported host range geographical occurrence. *Diplodia africana* was associated for the first time with disease symptoms on *Prunus persica* (L.) Batsch in South Africa (Damm *et al*., 2007). The occurrence of *D. africana* on *Juniperus phoenicea* L. and *J. oxycedrus* L. in Italy (Linaldeddu *et al*., 2011) shows the ability of this species to colonize different hosts.

Many authors have shown that diseases caused by fungi in the Botryosphaeriaceae are usually linked to environmental stress factors acting on the host (Úrbez-Torres, 2011). Desprez-Loustau *et al*. (2006) highlighted that environmental stresses predispose plants to infection by endophytes or opportunistic pathogens, indicating that host stress may trigger shifts from latent to pathogenic phases of some endophytic fungi, including the Botryosphaeriaceae. In the present case, environmental stresses caused by poor soil aeration and low water content, due to the road pavement, could have introduced sufficient stress to predispose *G. robusta* to infection by the *Diplodia* pathogens.

Further studies should be undertaken to assess the distribution of the disease, and to determine the roles of biotic and abiotic factors in the development of dieback. It would also be of interest to study the possible interaction between *D. africana* and *D. seriata* in the disease.

LITERATURE CITED

Carbone I., Kohn L.M., 1999. A method for designing primer sets for speciation studies in filamentous ascomycetes. *Mycologia* 91: 553–556.

Damm U., Crous P.W., Fourie P.H., 2007. *Botryosphaeriaceae* as potential pathogens of *Prunus* species in South Africa, with descriptions of *Diplodia africana* and *Lasiodiplodia plurivora* sp. nov. *Mycologia* 99: 664–680.

Desprez-Loustau M.L., Marçais B., Nageleisen M.L., Piou D., A. Vannini, 2006. Interactive effects of drought and pathogens in forest trees. *Annals of Forest Science* 63: 597–612.

Farr D.F., Rossman A.Y., 2019. Fungal Databases, U.S. National Fungus Collections, ARS, USDA. Available at: https://nt.ars-grin.gov/fungaldatabases/.https://nt.ars-grin.gov/fungaldatabases/ Accessed July 4, 2019.

Giambra S., Piazza G., Alves A., Mondello V., Berbegal M., … S. Burruano, 2016. Botryosphaeriaceae species associated with diseased loquat trees in Italy and description of *Diplodia rosacearum* sp. nov. *Mycosphere* 7: 978–989.

Linaldeddu B., Scanu B., Maddau L., Franceschini A., 2011. *Diplodia africana* causing dieback disease on *Juniperus phoenicea*: a new host and first report in the Northern Hemisphere. *Phytopathologia Mediterranea* 50: 473–477.

Njuguna W., 2011. *Stem canker and dieback disease on Grevillea robusta Cunn ex R. Br. Distribution, causes, and implication in Agroforestry systems in Kenya.* PhD Thesis, Swedish University of Agricultural Sciences, Uppsala, Sweden., 57 pp.

Njuguna W., Barklund P., Ihrmark K., J. Stenlid, 2011. A canker and dieback disease threatening the cultivation of *Grevillea robusta* on small-scale farms in Kenya. *African Journal of Agricultural Research* 6: 748–756.

O'Donnell K., Cigelnik E., Nirenberg H.I., 1998. Molecular systematics and phylogeography of the *Gibberella fujikuroi* species complex. *Mycologia* 90: 465–493.

Phillips A.J.L., Alves A., Abdollahzadeh J., Slippers B., Wingfield M.J., … P.W. Crous, 2013. The *Botryosphaeriaceae*: Genera and species known from culture. *Study in Mycology* 76: 51–167.

Raimondo F.M., Venturella G., Schicchi R., Filippone A., 1995. Orti botanici e giardini storici: La componente legnosa del verde pubblico di Palermo: Analisi distributiva e sintesi cartografica. *Giornale Botanico Italiano* 129: 199.

Tamura K., Stecher G., Peterson D., Filipski A., Kumar S., 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution* 30: 2725–2729.

Thompson J.D., Gibson T.J., Plewniak F., Jeanmougin F., Higgins D.G., 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* 25: 4876–4882.

Toljander Y.K., Nyeko P., Stenström E., Ihrmark K., Barklund P., 2007. First Report of Canker and Dieback Disease of *Grevillea robusta* in East Africa Caused by *Botryosphaeria* spp. *Plant Disease* 91: 773.

Úrbez-Torres J.R., 2011. The status of Botryosphaeriaceae species infecting grapevines. *Phytopathologia Mediterranea* 50: 5–45.

Venturella G., Gambino A., Gendusa F., Surano N., Raimondo F.M., 1990. Indagini qualitative e distributive sulla dendroflora della città di Palermo. *Giornale Botanico Italiano* 124: 115.

White T.J., Bruns T., Lee S., Taylor J., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR Protocols, a Guide to Methods and Applications* (MA Innis, DH Gelfand, JJ Sninsky, J White, ed.), Academic Press, San Diego, Ca, USA, 315–322.