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

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.

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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 EF-1α 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 (EF-1α) 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 plant 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 wk, 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).

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 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 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

166 (Geospiza, Inc.; http://www.geospiza.com/finchtv) and then compared with reference
167 sequences retrieved from GenBank in BLAST searches (Altschul et al. 1990).
168 Nucleotide sequences of additional isolates included in this study were retrieved from
169 GenBank (Table 2).
170
171 Phylogenetic analysis
172 The ITS and EF1-α sequences of *Lasiodiplodia* isolates obtained in this study were
173 combined and the dataset, including sequences of 21 other species of *Lasiodiplodia*
174 downloaded from GenBank, was compiled with the outgroup *Diplodia mutila* (Fr.) Fr.
175 and *D. seriata* (Table 2). Sequences were aligned with ClustalX v. 1.83 (Thompson et
176 al. 1997), using the default parameters. Alignments were checked and manual
177 adjustments were made where necessary. Incomplete portions at either end of the
178 alignment and the ambiguously aligned portion spanning the first 60 bases of the EF1-α
179 locus were excluded from the analyses. A comparison of highly supported clades
180 (bootstrap support values ≥ 70%) among trees generated from ML analyses of
181 individual data sets was performed in order to detect conflict between individual
182 phylogenies (Alves et al. 2008).
183 Maximum likelihood (ML) analyses were done using RAxML (Stamatakis 2006) on
184 the webserver (Stamatakis et al. 2008) at http://phylobench.vital-it.ch/raxml-bb.php
185 with the gamma model of rate heterogeneity in effect and maximum likelihood search.
186 Bayesian analyses were done with Mr Bayes v.3.0b4 (Ronquist and Huelsenbeck 2003)
187 employing a Markov Chain Monte Carlo (MCMC) method. The general time-reversible
188 model of evolution (Rodriguez et al. 1990), including estimation of invariable sites and
189 assuming a discrete gamma distribution with six rate categories was used. Four MCMC
190 chains were run simultaneously, starting from random trees for 10^6 generations. Trees
191 were sampled every 100th generation for a total of 10^4 trees. The first 10^3 trees were
192 discarded as the burn-in phase of each analysis. Posterior probabilities (Rannala and
193 Yang 1996) were determined from a 50% majority-rule consensus tree generated with
194 the remaining 9,000 trees. This analysis was repeated three times starting from different
195 random trees to ensure trees from the same tree space were sampled during each
196 analysis. Trees were visualized with TreeView (Page 1996).
197
198 Pathogenicity tests

199 Pathogenicity of two *Lasiodiplodia mediterranea* strains (BL1 and BL101) was
200 verified by inoculating ten 2-year-old holm oak seedlings (strain BL1) and five excised
201 grapevine shoots and five lignified canes from cv. Cannonau (strain BL101). A mycelial
202 plug (3–4 mm^2) taken from the margin of an actively growing colony on PDA was
203 placed in a shallow wound (~3 mm) made with a scalpel at the middle of each shoot and
204 cane and at the stem base of each seedling. The inoculation point was covered with
205 cotton wool soaked in sterile water and wrapped with Parafilm®. The inoculated shoots
206 were placed in a beaker containing 200 mL of sterile distilled water and then enclosed in
207 a transparent plastic bag for twelve days, whereas the bottom and top end of each cane
208 was sealed with a synthetic grafting resin to prevent drying and contamination and then
209 enclosed in a transparent plastic bag for fifty days. Inoculated grapevine samples were
210 kept in the laboratory in daylight and at 18–26 °C. Inoculated seedlings were watered
211 every 3 days and kept in the laboratory for 2 months. Five grapevine shoots and canes
212 and ten holm oak seedlings inoculated with a PDA plug were used as control. At the end
213 of each experiment, re-isolation was attempted by transferring to PDA 10 surface-
214 sterilized pieces of inner bark and xylem tissue taken around the margin of each lesion
215 on grapevine samples and stem of holm oak seedlings.
216
217 **Results**
218 Botryosphaeriaceous species associated with symptomatic grapevines
219 Wedge-shaped necrotic sectors on cordon and trunk represented the most frequent
220 symptom detected (58.5% of the samples processed) on investigated grapevine plants.
221 Black stripes in the wood was the second most frequent symptom (23%) followed by
222 black spots in the wood (11.5%) and necrotic cane lesions (7%). From 113 grapevine
223 samples processed, 48 botryosphaeriaceous isolates representing 10 distinct species
224 namely *Botryosphaeria dothidea*, *Diplodia africana* Damm & Crous, *D. mutila*, *D.*
225 *olivarum* A.J.L. Phillips, Frisullo & Lazzizera, *D. seriata*, *Lasiodiplodia mediterranea*
226 sp. nov., *Neofusicoccum australe* (Slippers, Crous & M.J. Wingf.) Crous, Slippers &
227 A.J.L. Phillips, *N. cryptoaustrale* Pavlic, Maleme, Slippers & M.J. Wingf., *N. luteum*
228 (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips and *N. parvum* were isolated
229 and identified from the 6 grapevine cultivars and 18 sites sampled (Table 3). The
230 identity of isolates of each species was confirmed by analysis of ITS and EF1-α
231 sequences. For all species BLAST searches in GenBank showed 99–100% similarity

232 with reference sequences of representative strains including those of ex-type isolates.
233 New sequences were deposited in GenBank (Table 3).
234 *Neofusicoccum* was the genus most frequently isolated: 16.8% of samples from 8
235 sites, whereas *D. seriata* was the most frequently isolated species (10.6% of samples
236 from 9 sites), followed by *N. parvum* (9.7% of samples from 4 sites). These two species
237 together with *B. dothidea* and *L. mediterranea* (here described as a new species) were
238 the only species associated with both V-shaped necrosis and brown vascular stripes on
239 cordons. Isolations from V-shaped necrotic sectors overall yielded 8
240 botryosphaeriaceous species (Table 3). However, apart from *D. seriata* and *N. parvum*,
241 the other 6 species were isolated only in one site. In particular, *L. mediterranea* was
242 obtained from all samples collected in one vineyard located in the north of Sardinia.
243 From sectioned cordons showing black spots the only species isolated was *N. parvum*,
244 no other fungal pathogens such as *Phaeoacremonium* spp. and *Phaeomoniella*
245 *chlamydospora* (W. Gams, Crous, M.J. Wingf. & Mugnai) Crous & W. Gams, typically
246 associated with this grapevine symptom, were obtained. Four species, namely *D.*
247 *africana*, *D. mutila*, *N. australe* and *N. luteum* were obtained from cane samples
248 showing inner bark necrotic lesions.
249
250 DNA phylogeny of *Lasiodiplodia* isolates
251 Fragments of approximately 500 and 300 bases were determined for ITS and EF1-α
252 regions, respectively. New sequences were deposited in GenBank (Table 2) and the
253 alignment in TreeBase (15565). Individual gene phylogenies revealed no major
254 conflicts thus indicating that the two loci could be combined. The combined ITS and
255 EF1-α dataset consisted of 850 characters (including alignment gaps) for 53 ingroup and
256 2 outgroup *taxa*. ML and Bayesian analyses generated trees with essentially the same
257 topology (TreeBase 15565). The ML tree is shown in Figure 2 with ML bootstrap
258 support values and Bayesian posterior probability scores at the nodes. In the
259 phylogenetic analysis 22 clades corresponding to species were recognized. Of these, 20
260 included all *Lasiodiplodia* spp. previously known from culture and for which molecular
261 data are available (Fig. 2). The other two well supported clades included the
262 *Lasiodiplodia* isolates obtained in this study.
263 The first clade (ML bootstrap = 98%, posterior probability = 1.00) containing the
264 *Lasiodiplodia* isolates from broom bush in Tunisia and pistachio (*Pistacia vera* L.) in
265 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.

Taxonomy

Lasiodiplodia euphorbiicola A.R. Machado & O.L. Pereira, Fungal Diversity (In press)

MycoBank: MB 804872

Lasiodiplodia euphorbicola A.R. Machado & O.L. Pereira, Fungal Diversity (In press)

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 wk, 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 d 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 wk, 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 d 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 d (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.

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 phylogenic and morphological study on genera of the *Botryosphaeriaceae* (Liu et al. 2012, ***Phillips et al. 2013) and it is now relatively easy to identify taxa to genera and species (Hyde et al. 2014). 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 they play in the aetiology of Botryosphaeria dieback in Sardinia, or their possible synergistic interaction.

In this study 22 clades were resolved within *Lasiodiplodia* which is **** as Hyde et al. (2014). 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 supports the plurivorous nature of *L. exigua* and at the same time adds further evidence to the fact that the name *L. theobromae* has been applied to a 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 analysis, 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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Legends

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

Fig. 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.

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

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