

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

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30 **Abstract**

31 **The** diversity of *Botryosphaeriaceae* species associated with “Botryosphaeria
32 dieback” of grapevine was investigated in 18 vineyards in Sardinia, Italy. *Lasiodiplodia*
33 isolates obtained from different woody hosts including holm oak, sweet orange and
34 broom bush in Italy, Algeria and Tunisia were **also** characterized. Morphological and
35 cultural characteristics as well as **ITS and EF-1 α** sequence data were used to identify the
36 fungal isolates. **Forty-eight** botryosphaeriaceous isolates were obtained from 113
37 symptomatic grapevine samples, from which **ten** species were identified. *Diplodia*
38 *seriata* was the dominant species (25% of isolates), followed by *Neofusicoccum parvum*
39 (21.7%). Two species, *Diplodia olivarum* and *D. africana* **are** reported for the first time
40 on grapevine. In addition, two new species namely *Lasiodiplodia mediterranea* sp. nov.
41 from grapevine, holm oak and sweet orange and *Lasiodiplodia exigua* sp. nov. from
42 broom bush **are** described. In artificial inoculation experiments conducted on excised
43 green grapevine shoots and lignified canes as well as holm oak seedlings, *L.*
44 *mediterranea* was **shown** to be an aggressive pathogen.

45

46 **Keywords** *Citrus × sinensis*, *Diplodia*, *Lasiodiplodia*, *Neofusicoccum*, *Quercus ilex*,
47 *Retama raetam*

48

49 **Introduction**

50 During the last decades an increase in grapevine trunk diseases, due to attack by
51 several fungal pathogens belonging mainly to the **family** *Botryosphaeriaceae*, has been
52 reported in both traditional and emerging grape-producing countries worldwide
53 (Larignon et al. 2001; Phillips 2002; van Niekerk et al. 2004; Úrbez-Torres et al. 2006;
54 Luque et al. 2009; Pitt et al. 2010; Mohammadi et al. 2013; Mondello et al. 2013; Yan et
55 al. 2013). Common external symptoms caused by infection of *Botryosphaeriaceae* on
56 grapevine include leaf spots, leaf wilting, fruit rots, bud necrosis and perennial cankers
57 which are often associated with a poor vine growth, cordon dieback and sudden death of
58 whole plant. Internal wood symptoms consist mainly of wedge-shaped necrotic sectors
59 and brown stripes below the bark. The name “Botryosphaeria dieback” has recently
60 been proposed to include all these decline-associated symptoms caused by species of
61 *Botryosphaeriaceae* (Úrbez-Torres 2011). Similar to other grape-growing regions also
62 in Sardinia (Italy), Botryosphaeria dieback represents a worrying problem for grape and
63 wine production (Linaldeddu et al. 2010). In Sardinia, grapevine trunk diseases were

64 originally linked to infections by *Eutypa lata* (Pers.) Tul. & C. Tul. and other fungi
65 commonly associated with “esca” disease (Serra et al. 2010), but recent investigations
66 have shown that in fact many species of *Botryosphaeriaceae* are directly involved in the
67 aetiology of wood symptoms on trunks and cordons (Deidda et al. 2012). However, the
68 information currently available about the occurrence, distribution and identity of the
69 species of *Botryosphaeriaceae* associated with the different grapevine wood symptoms
70 in Sardinia is still limited.

71 To date, at least 23 different taxa of *Botryosphaeriaceae* have been reported as weak
72 or aggressive pathogens on grapevine worldwide, many of which have been described
73 as new species during the last decade. Four species namely *Botryosphaeria dothidea*
74 (Moug.) Ces. & De Not., *Diplodia seriata* De Not., *Neofusicoccum parvum* (Pennycook
75 & Samuels) Crous, Slippers & A.J.L. Phillips and *Lasiodiplodia theobromae* (Pat.)
76 Griffon & Maubl. are usually recognised as the predominant species associated with
77 grapevine cankers and dieback worldwide (Úrbez-Torres 2011). Recent studies, based
78 on sequence data of the Internal Transcribed Spacers (ITS) of the ribosomal RNA
79 cluster and part of the translation Elongation Factor 1-alpha (EF-1 α) gene, have led to
80 the identification of cryptic species within the *L. theobromae* species complex (Alves et
81 al. 2008; Abdollahzadeh et al. 2010; Begoude et al. 2010; Liu et al. 2012; Úrbez-Torres
82 et al. 2012). Currently, six species of *Lasiodiplodia*, including *L. crassispora* T.I.
83 Burgess & Barber, *L. missouriana* Úrbez-Torres, Peduto & Gubler, *L. parva* A.J.L.
84 Phillips, A. Alves & Crous, *L. pseudotheobromae* A.J.L. Phillips, A. Alves & Crous, *L.*
85 *theobromae* and *L. viticola* Úrbez-Torres, Peduto & Gubler have been isolated from
86 grapevine (Úrbez-Torres 2011; Úrbez-Torres et al. 2012; Correia et al. 2013; Yan et al.
87 2013). It is likely that over the years the name *L. theobromae* has been applied to more
88 than one species. In order to stabilize the name and allow its unambiguous application, a
89 neotype specimen and an ex-neotype culture have recently been designated for *L.*
90 *theobromae* (Phillips et al. 2013). *Lasiodiplodia theobromae* is a plurivorous pathogen
91 with a worldwide distribution especially in tropical and subtropical regions where it
92 occurs mainly on woody plants including fruit and forest trees (Mohali et al. 2005;
93 Alves et al. 2008; Liu et al. 2012). Despite this, in Mediterranean countries few studies
94 have focused on geographic distribution, host range or genetic variability of this
95 pathogen and other species of *Lasiodiplodia*. *Lasiodiplodia theobromae* was previously
96 reported associated with dieback of grapevine in Sicily (Italy) and Spain (Aroca et al.
97 2008; Burruano et al. 2008). In recent years, during surveys carried out in Algeria,

98 Tunisia and Sardinia aimed at clarifying the causes of decline affecting different woody
99 plants such as sweet orange (*Citrus × sinensis*), broom bush (*Retama raetam* (Forssk.)
100 Webb & Berthel.) and holm oak (*Quercus ilex* L.), a large collection of *L. theobromae*-
101 like strains were isolated from trees showing cankers and a progressive dieback of
102 branches. The main aims of the work described here were: 1) to study the species
103 diversity and distribution of *Botryosphaeriaceae* associated with grapevine
104 *Botryosphaeria* dieback in Sardinia; 2) to characterize a collection of *Lasiodiplodia*
105 isolates obtained from different hosts and geographic origins in terms of morphological
106 and phylogenetic relationships to all *Lasiodiplodia* species known from culture.

107

108 **Materials and Methods**

109 **Sampling, fungal isolation and identification**

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

118 Grapevine plant were brought to the laboratory to be inspected and symptomatic
119 samples were initially cleaned of loose bark and then the outer bark surface tissue was
120 cut away by a scalpel. Longitudinal and transversal cuts from symptomatic canes,
121 cordons, and trunks were made to observe any internal symptom (Fig. 1). Isolations
122 were made from chips of xylem tissues, approx. 5 mm², cut by a sterile scalpel from the
123 margin of necrotic lesions. All chips were cultured on potato dextrose agar (PDA,
124 Oxoid Ltd.) in Petri dishes. After incubation at 25 °C for 1 wk, fungal colonies were
125 sub-cultured onto half-strength PDA or on water agar supplemented with autoclaved
126 poplar or holm oak twigs to enhance sporulation. All colonies were kept on the
127 laboratory bench at about 20–25 °C where they received diffused daylight. Putative
128 botryosphaeriaceous isolates were identified by reference to the keys and descriptions
129 data provided in Phillips et al. (2013). Monoconidial cultures were obtained by
130 spreading conidia on the surface of PDA and incubating overnight at 25 °C. Individual
131 germinating conidia were transferred to fresh plates of PDA. Representative isolates of

132 each species were stored on PDA slants under oil in the culture collection of the Sez. di
133 Patologia vegetale ed Entomologia, Dipartimento di Agraria, at the University of
134 Sassari. In addition, three strains of the two new *Lasiodiplodia* species were also
135 deposited at the Centraalbureau voor Schimmelcultures (CBS), Utrecht, the Netherlands
136 and nomenclatural data in MycoBank (Crous et al. 2004). Specimens were lodged with
137 the herbarium of Estação Agronómica Nacional, Oeiras, Portugal (LISE).

138

139 Morphology and cultural characteristics

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

145 For microscopy, the contents of conidiomata were dissected out and mounted in
146 100% lactic acid. Measurements of conidiogenous cells, conidia and paraphyses were
147 made with the Leica IM 500 measurement module from images recorded on a Leica
148 DFC 320 digital camera. From measurements of 50 conidia the mean, standard
149 deviation and 95% confidence intervals were calculated. Spore dimensions are
150 presented as mean values with extreme values in parentheses. Dimensions of other
151 structures are given as mean of at least 20 measurements.

152

153 DNA extraction, PCR amplification and sequencing

154 Following morphological identification, a subset of isolates of each species of
155 *Botryosphaeriaceae* obtained in this study was selected for DNA sequence analysis.
156 Instagene Matrix (BioRad Laboratories, Hercules, CA) was used to extract genomic
157 DNA from 5-day-old cultures grown on PDA and incubated at 25°C. The ITS region
158 was amplified and sequenced with primers ITS1 and ITS4 (White et al. 1990), while the
159 primers EF446f and EF1035r (Inderbitzin et al. 2005; 2010) were used to amplify and
160 sequence part of the EF-1 α gene. Polymerase chain reaction (PCR) mixtures and
161 amplification conditions were conducted as described by Linaldeddu et al. (2013). The
162 PCR products were purified using the EUROGOLD gel extraction kit (EuroClone
163 S.p.A.) following manufacturer's instructions. ITS and EF1- α regions were sequenced
164 in both directions by the BMR Genomics DNA sequencing service ([www.bmr-](http://www.bmr-genomics.it)
165 [genomics.it](http://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 $\geq 70\%$) 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 mm²) 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 & Lazzizzera, *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 *Phaeoconiella*
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

266 *Lasiodiplodia exigua* sp. nov. The second clade (ML bootstrap = 90%, posterior
267 probability = 0.86) including the *Lasiodiplodia* isolates obtained from grapevine and
268 holm oak in Italy and sweet orange in Algeria was considered to represent a further
269 distinct species, which is described here as *Lasiodiplodia mediterranea* sp. nov. The
270 isolate of *Lasiodiplodia jatrophiicola* A.R. Machado & O.L. Pereira (CMM 3610), a
271 species recently described by Machado et al. (2014), clustered in the *Lasiodiplodia*
272 *iraniensis* Abdollahz., Zare & A.J.L. Phillips clade.

273

274 Taxonomy

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

277 MycoBank: MB 804872

278

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

281

282 *Notes:* Orthography of the epithet is herein corrected.

283

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

285 MycoBank: MB 808355 (Fig. 3)

286 **Etymology:** in reference to the small conidia.

287

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

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

301 *Cardinal temperatures for growth*: minimum <10 °C, maximum <40 °C and
302 optimum 25-30 °C, covering the medium surface (90 mm) before 7 d in the dark.

303

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

305 *Known geographic distribution*: Tunisia and Arizona (USA).

306

307 *Specimens examined*: TUNISIA, Nabeul, isolated from a branch canker of *Retama*
308 *raetam*, 27 June 2012, Benedetto T. Linaldeddu, HOLOTYPE LISE 96302, a dried
309 culture sporulating on *Quercus ilex* twigs, culture ex-holotype CBS 137785 = BL104.
310 Other isolates examined are listed in Table 2.

311

312 *Notes*: Phylogenetically *L. exigua* is closely related to *Lasiodiplodia mahajangana*
313 Begoude, Jol. Roux & Slippers, but can easily be distinguished on average conidial
314 dimensions and l/w ratio. Moreover, average size of the septate paraphyses of *L. exigua*
315 are $80.1 \times 2.9 \mu\text{m}$, whereas aseptate paraphyses of *L. mahajangana* are $43 \times 3 \mu\text{m}$.

316

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

318 MycoBank: MB 808356 (Fig. 4).

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

321

322 **Sexual state**: Not seen. **Asexual state**: *Conidiomata* pycnidial formed on poplar twigs
323 in culture within 2–3 wk, uniloculate, dark brown to black, immersed in the host
324 becoming erumpent when mature. *Paraphyses* hyaline, cylindrical, septate, sometimes
325 branched, ends rounded, measuring $87 \pm 19.9 \times 2.7 \pm 0.6 \mu\text{m}$ (mean \pm S.D., n = 20).
326 *Conidiogenous cells* $13.6 \pm 2.2 \times 3.7 \pm 1 \mu\text{m}$ (mean \pm S.D., n = 20), hyaline, smooth,
327 cylindrical, sometimes slightly swollen at the base, holoblastic forming conidia at their
328 tips, proliferating internally giving rise to periclinal thickenings. *Conidia* subcylindrical
329 to elliptical, apex and base rounded, typically widest at the middle, thick-walled,
330 initially hyaline and aseptate and remaining so for a long time, becoming one or two-
331 septate and dark brown with age, with longitudinal striations $(26.3\text{--}30.6\text{--}37) \times (13.5\text{--}$

332)16.1(–18) μm , 95% confidence limits = 30–31.1 \times 15.9–16.3 μm (mean \pm S.D. = 30.6
333 \pm 2.8 \times 16.1 \pm 0.9 μm , l/w ratio = 1.9 \pm 0.2).

334 *Cultural characteristics:* Colonies on PDA grew rapidly, reaching 90 mm in
335 diameter before 7 d at 25 °C, the mycelium was moderately aerial, surface white at first
336 and later turned pale to dark grey from the centre to the margin and greyish to dark in
337 reverse. Isolates growing at 35 °C produced a diffusible pink pigment within 3 d (Fig.
338 4).

339 *Cardinal temperatures for growth:* minimum <10 °C, maximum <40 °C and
340 optimum 25-30 °C.

341

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

343 *Known distribution:* Italy and Algeria.

344

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

351

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

355

356 Pathogenicity tests

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

360 Twelve days after inoculation, all grapevine shoots inoculated with the pathogen
361 displayed dark-brown to black discoloration on bark and vascular tissues, measuring
362 10.3 \pm 3 cm (mean \pm S.D.). Fifty days after inoculation, the lignified canes displayed
363 dark-brown to black discoloration on bark and vascular tissues, measuring 8.1 \pm 1.8 cm
364 (mean \pm S.D.). In cross section all canes showed a wedge-shaped necrotic sector. On
365 holm oak seedlings *L. mediterranea* caused extensive necrotic lesions, which often

366 girdled the stem, causing leaf chlorosis and in most cases wilting of the distal portion of
367 the canopy. Wood necrosis on stems measured 8.3 ± 2.7 cm (mean \pm S.D.). The wilted
368 seedlings reacted by producing new shoots below the point of inoculation. Artificially
369 obtained symptoms were congruent with field observations. The pathogen was
370 successfully re-isolated from the margin of all symptomatic tissues, thus fulfilling
371 Koch's postulates. Control grapevine shoots and canes and holm oak seedlings
372 inoculated with sterile PDA plugs remained symptomless.

373

374 Discussion

375 There has been much recent phylogenetic and morphological study on genera of the
376 *Botryosphaeriaceae* (Liu et al. 2012, ***Phillips et al. 2013) and it is now relatively
377 easy to identify taxa to genera and species (Hyde et al. 2014). Thus the studies of
378 *Botryosphaeriaceae* on various hosts has multiplied. The present study represents the
379 first survey aimed at studying the occurrence and diversity of species of
380 *Botryosphaeriaceae* associated with grapevine in Sardinia. The results obtained have
381 given new insights into the complex aetiology associated with *Botryosphaeria* dieback.
382 Morphological studies and DNA sequence analyses allowed us to identify 10 different
383 botryosphaeriaceous species from V-shaped necrotic sectors, brown vascular stripes,
384 black spots and cane necrotic lesions, including *Botryosphaeria dothidea*, *Diplodia*
385 *africana*, *D. mutila*, *D. olivarum*, *D. seriata*, *Lasiodiplodia mediterranea* sp. nov.
386 *Neofusicoccum australe*, *N. cryptoaustrale*, *N. luteum* and *N. parvum*. All species found
387 in this study, except *D. africana*, *D. olivarum* and *L. mediterranea*, which are reported
388 for the first time on grapevine, have been detected in other grape-growing areas
389 worldwide, and are associated with a broad range of grapevine disease symptoms
390 including leaf spots, fruit rot, shoot dieback, bud necrosis, vascular discoloration of the
391 wood and perennial cankers (Úrbez-Torres 2011).

392 *Diplodia seriata* was the dominant species, sampled from nine sites and five
393 grapevine cultivars, followed by *Neofusicoccum parvum* and *Botryosphaeria dothidea*.
394 The high frequency of isolation of *Diplodia seriata* obtained in this study is in
395 accordance with results of previous studies conducted in France (Larignon et al. 2001),
396 Australia (Taylor et al. 2005; Pitt et al. 2010), Spain (Luque et al. 2009) and Chile
397 (Morales et al. 2012) where this pathogen was found as the dominant species isolated
398 from symptomatic grapevine samples. Although *D. seriata* has been reported from a
399 wide range of grapevine cultivars worldwide, there are conflicting reports regarding its

400 pathogenicity on this host: in particular, it has been considered to be a primary pathogen
401 by Larignon et al. (2001) in France, Auger et al. (2004) in Chile and van Niekerk et al.
402 (2004) in South Africa, as a secondary pathogen by Phillips (1998) in Portugal and
403 Úrbez-Torres and Gubler (2009) in California and as not pathogenic in Australia by
404 Taylor et al. (2005). These conflicting data may be a result of differences in inoculation
405 methods and experimental conditions, susceptibility among the various grapevine
406 cultivars, age and type of host tissue but may also be due to differences in strain
407 virulence. In this regard, Larignon et al. (2001) found significant differences in the
408 mean lesion lengths caused on 1-year-old canes by ten strains of *D. seriata* used in a
409 pathogenicity assay.

410 Apart from *Diplodia seriata*, *Neofusicoccum parvum* and *Botryosphaeria dothidea*
411 the other seven species of *Botryosphaeriaceae* obtained in this study were, in most
412 cases, isolated from a single site each thus suggesting that various site-specific
413 conditions (microclimate conditions, source of propagation material and the occurrence
414 of alternative hosts surrounding the vineyard) may influence the presence of these
415 species within vineyards. This aspect is supported by the fact that all of the species
416 isolated in this study are polyphagous and some of them are known to be able to infect
417 several forest trees such as cork oak, holm oak and juniper in Sardinia (Linaldeddu et al.
418 2006; 2007; 2011; 2014). In addition, given the low frequency of isolation of these
419 seven species, at the moment it is not possible to establish the exact role they play in the
420 aetiology of *Botryosphaeria dieback* in Sardinia, or their possible synergistic
421 interaction.

422 In this study 22 clades were resolved within *Lasiodiplodia* which is **** as Hyde et
423 al. (2014). Species names are available for 20 of these clades, for the other two clades,
424 which represent two new species obtained from different woody hosts in Italy, Algeria
425 and Tunisia the names *L. mediterranea* and *L. exigua* are introduced here.

426 *Lasiodiplodia mediterranea* was found associated with grapevine V-shaped necrotic
427 sectors. To date, six species of *Lasiodiplodia* have been associated with grapevine wood
428 diseases (Úrbez-Torres et al. 2012; Correia et al. 2013; Yan et al. 2013). All six species
429 were chiefly linked to cankers and wood symptoms according to results obtained in this
430 study for *L. mediterranea*. Phylogenetically, *L. mediterranea* is closely related to *L.*
431 *pseudotheobromae* but the two species differed in three bp in ITS and nine bp in EF1- α .
432 Morphologically *L. mediterranea* resembles *L. macrospora* A.R. Machado & O.L.
433 Pereira, a species recently described in Brazil on *Jatropha curcas* L. (Machado et al.

434 2014). However, *L. mediterranea* can be distinguished from other species on the basis
435 of its larger conidia (Table 4), and the size of its septate and branched paraphyses.
436 Besides grapevine, *L. mediterranea* has also been isolated from a cankered branch of
437 holm oak in Sardinia and cankered branches of sweet orange in Algeria indicating the
438 polyphagous nature of this new *Lasiodiplodia* species.

439 The second clade, which includes isolates from broom bush in Tunisia and one
440 isolate from pistachio in the USA previously identified as *L. theobromae* (strain
441 PD161), represents a previously unrecognized *Lasiodiplodia* species, which we
442 described here as *L. exigua* sp. nov. Although this species is phylogenetically closely
443 related to *L. mahajangana*, it is easily separated by its larger conidia (av. = 21.8×12.3
444 μm) as compared with *L. mahajangana* (av. = $17.5 \times 11.5 \mu\text{m}$). Because it was
445 impossible in this study to obtain broom bush seedlings the pathogenicity of *L. exigua*
446 was not assessed and thus Koch's postulates have not been satisfied. The data presented
447 here supports the plurivorous nature of *L. exigua* and at the same time adds further
448 evidence to the fact that the name *L. theobromae* has been applied to a number of
449 cryptic species. Given that a neotype specimen and ex-culture with related molecular
450 data were established for *L. theobromae* (Phillips et al. 2013), a more detailed analysis
451 of the current 990 sequences accessible in GenBank under the name *L. theobromae* will
452 be possible in the future.

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

463

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469

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614

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 μ m.

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 μ m.