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Morphology and phylogeny of *Botryosphaeria dothidea* causing fruit rot of olives

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Abstract

The taxonomic position of the causal agent of fruit rot of olives was determined from fresh collections of the fungus from central Greece. In culture it formed two types of conidia, namely fusiform, hyaline, aseptate conidia typical of the genus *Fusicoccum*, and dark-walled, ovoid, ellipsoid or fusiform, 1–2 septate conidia that are not typically observed in *Fusicoccum*. A phylogenetic analysis based on ITS and EF1-α sequences placed the fungus within the same clade as *Fusicoccum aesculi*, which is the anamorph of *Botryosphaeria dothidea*, and the type of the genus *Fusicoccum*.

Key words: *Botryosphaeria dothidea*, *Fusicoccum aesculi*, *Olea europaea*, systematics, taxonomy

Introduction

The European olive (*Olea europaea* L.) is a long-lived, drought resistant, evergreen tree native to the Mediterranean basin. It is hardy and suffers from few major disease problems. The most serious are bird's eye spot, caused by *Spilocaea oleaginea* (Castagne) S. Hughes [1], and verticillium wilt caused by *Verticillium dahliae* Kleb. [2, 3]. However, a fruit rot of olives has been known for many years [4–6] and appears to be widespread in the Mediterranean region [7].

Since it was first described in 1883, the causal agent of olive fruit rot has undergone several taxonomic changes. It was first described by Thümen [8] as *Phyllosticta dalmatica* Thüm. on olive fruits from Dalmatia, Croatia. Saccardo [9] considered this to be a species of *Phoma* Sacc. and made the new combination *Phoma dalmatica* (Thüm.) Sacc. When Berlese and Voglino [10] raised *Phoma* subgenus *Macrophoma* to generic status, they included *P. dalmatica* as *Macrophoma dalmatica* (Thüm.) Berl. & Vogl. However, Gigante [11] regarded it to be a species of *Sphaeropsis* Sacc. and made the new combination *Sphaeropsis dalmatica* (Thüm.) Gigante. Zachos and Tzavella-Klonari [7] studied the pathogen in detail and reported two types of conidia. In one type the conidia were fusiform, hyaline or yellow-brown, aseptate or 1–2(–8) septate, and measured 20–34 × 6.5–9 μm. In the second type they were hyaline, yellow-brown or brown, ovoid, ellipsoid, limoniform, or pyriform, with 1–5 transverse and 1–3 longitudinal septa, 10.6–23.5 × 6.5–9.5 μm. Since cultures derived from single conidia of either type produced both types of conidia, Zachos and Tzavella-Klonari [7] considered it to be a single species. On account of the longitudinal and transverse septation and the dark wall of some conidia, they reclassified *M. dalmatica* as *Camarosporium dalmaticum* (Thüm.) Zachos & Tzav.-Klon.

Based on Thümen's [8] description, van der Aa and Vanev [12] assigned *Phyllosticta dalmatica* to *Fusicoccum* Corda as *Fusicoccum dalmaticum* (Thüm.) Vanev.

We recently isolated a fungus from olive fruits that appeared to be a *Fusicoccum* sp., but in culture it formed brown, septate conidia in addition to the hyaline, fusiform ones typical of the genus. Therefore, the purpose of the work presented here was to determine the correct taxonomic position and name for the fungus that causes fruit rot of olives.

Materials and methods

Collection and isolation

Olive fruits showing typical rot symptoms were collected from Thessalia in central Greece. Conidiomata were dissected out and crushed in a drop of sterile water. The resulting suspension of conidia was spread over the surface of a plate of Difco potato dextrose agar (PDA) and incubated overnight at 22 °C. Single germinating conidia were transferred to fresh plates of PDA.

Morphological characterization

Cultures were grown on PDA or oatmeal agar (OA) [13] at 22 °C with 12 h of light per day from mixed UV and daylight fluorescent tubes. Conidia oozing from mature conidiomata were mixed with a small drop of water on a glass slide, the water was allowed to evaporate and the conidia were mounted in lactic acid. The conidiogenous layer was dissected from conidiomata and mounted in lactic acid. Digital images were recorded with a Leica DFC320 camera. Conidial measurements were made with the Leica IM500 measurement module. The mean, standard deviation (SD) and 95% confidence intervals were calculated from measurements of at least 50 conidia of each isolate. Dimensions of conidia are presented as the lower and upper 95% confidence limits with minimum and maximum dimensions in parentheses.

DNA extraction and amplification

The procedures described by Alves et al. [14] were used to extract genomic DNA from fungal mycelium and to amplify part of the nuclear rRNA operon using the primers ITS1 and ITS4 [15]. The primers EF1-728F and EF1-986R [16] were used to amplify part of the translation elongation factor 1-alpha (EF1-α) gene. PCR reactions were carried out with *Taq* polymerase, nucleotides and buffers supplied by MBI Fermentas (Vilnius, Lithuania) and PCR reaction mixtures were prepared according to Alves et al. [14]. The amplification conditions were as follows: initial denaturation of 5 min at 95 °C, followed by 30 cycles of 30 s at 94 °C, 45 s at 55 °C, and 1½ min at 72 °C, and a final extension period of 10 min at 72 °C. In some cases where amplification of the EF1-α region was not accomplished, a second PCR was performed using as template 1 μl of the first PCR amplification.

DNA sequencing and analysis

The amplified PCR fragments were purified with the JETQUICK PCR Purification Spin Kit (GENOMED, Löhne, Germany). Both strands of the PCR products were sequenced with the ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit with AmpliTAQ DNA Polymerase (PE Applied Biosystems, Foster City, California, USA) in a Bio-Rad iCycler Thermal Cycler. Cycle sequencing procedure was described elsewhere [14].

The sequences were obtained with the ABI PRISM® 310 Genetic Analyzer (PE Applied Biosystems, Foster City, California, USA). The sequences of the ITS (partial 18S, ITS1, 5.8S gene, ITS2, and partial 28S sequences) and partial EF1-α regions were read and edited with Chromas 1.45 (http://www.technelysium.com.au/chromas.html). All sequences were checked manually and nucleotide arrangements at ambiguous positions were clarified using both primer direction sequences. Sequences were deposited in the GenBank public database. Nucleotide sequences for both DNA regions of additional *Botryosphaeria* species were taken from GenBank (Table 1).

The ITS and EF1-α sequences were aligned with MegAlign (DNASTAR Inc, Madison, Wisconsin) and manual adjustments were made where necessary. Phylogenetic analyses of sequence data were done using PAUP version 4.0b10 [17]. All characters were unordered and of equal weight and alignment gaps were treated as a fifth character state. The ITS and EF1-α sequences were

Table 1. Isolates included in this study

Accession no.[a]	Species	Host	Collector	Locality	Genbank	
					ITS	EF1-α
CBS 112877	*Botryosphaeria australis*	*Vitis vinifera*	F. Halleen	South Africa	[b]*AY 343385*	*AY 343346*
CBS 112872	*B. australis*	*V. vinifera*	F. Halleen	South Africa	*AY 343388*	*AY* 343347
[c]**CBS 112549**	*B. corticola*	*Quercus suber*	A. Alves	Portugal, Aveiro	*AY 259100*	*AY 573227*
CMW 8000	*B. dothidea*	*Prunus sp.*	B. Slippers	Switzerland, Crocifisso	*AY 236949*	*AY 236898*
CBS 110302	*B. dothidea*	*V. vinifera*	A.J.L. Phillips	Portugal, Montemor-o-Novo	*AY 259092*	*AY* 573218
CBS 110300	*B. dothidea*	*Populus nigra*	A.J.L. Phillips	Portugal, Braga	*AY 640253*	*AY* 640256
CBS 116777	*B. dothidea*	*Olea europaea*	I. Rumbos	Greece, Thessalia	*AY* 786320	*AY* 786317
CBS 116741	*B. dothidea*	*Olea europaea*	I. Rumbos	Greece, Thessalia	*AY 640254*	*AY 640257*
CBS 116742	*B. dothidea*	*Olea europaea*	I. Rumbos	Greece, Thessalia	*AY* 786321	*AY* 786318
CBS 116743	*B. dothidea*	*Olea europaea*	I. Rumbos	Greece, Thessalia	*AY* 786322	AY 786319
CMW 10125	*B. eucalyptorum*	*Eucalyptus grandis*	H. Smith	South Africa, Mpumalanga	*AF 283686*	*AY 236891*
CMW 10126	*B. eucalyptorum*	*E. grandis*	H. Smith	South Africa, Mpumalanga	*AF 283687*	*AY 236892*
CBS 110299	*B. lutea*	*V. vinifera*	A.J.L. Phillips	Portugal, Oeiras	*AY 259091*	*AY* 573217
CMW 9076	*B. lutea*	*Malus* × *domestica*	S.R. Pennycook	New Zealand	*AY 236946*	*AY 236893*
CBS 112555	*B. obtusa*	*V. vinifera*	A.J.L. Phillips	Portugal, Montemor-o-Novo	*AY 259094*	*AY* 573220
CMW 9080	*B. parva*	*P. nigra*	G.J. Samuels	New Zealand	*AY 236942*	*AY 236887*
CBS 110301	*B. parva*	*V. vinifera*	A.J.L. Phillips	Portugal, Palmela	*AY 259098*	*AY* 573221
CMW 9074	*B. rhodina*	*Pinus sp.*	T. Burgess	Mexico	*AY 236952*	*AY* 236901
CMW 7772	*B. ribis*	*Ribes sp.*	B. Slippers, G. Hudler	USA, New York	*AY 236935*	*AY 236877*
CMW 7773	*B. ribis*	*Ribes sp.*	B. Slippers, G. Hudler	USA, New York	*AY 236936*	*AY 236878*
CBS 112553	*B. stevensii*	*V. vinifera*	A.J.L. Phillips	Portugal, Montemor-o-Novo	*AY 259093*	*AY* 573219
CBS 112878	*Fusicoccum viticlavatum*	*V. vinifera*	F. Halleen	South Africa	*AY 343380*	*AY* 343341
CBS 112977	*F. viticlavatum*	*V. vinifera*	F. Halleen	South Africa	*AY 343381*	*AY* 343342
CBS 110887	*F. vitifusiforme*	*V. vinifera*	J.M. van Niekerk	South Africa	*AY 343383*	*AY* 343343
CBS 110880	*F. vitifusiforme*	*V. vinifera*	J.M. van Niekerk	South Africa	*AY 343382*	*AY* 343344

[a] Acronyms of culture collections: CBS – Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CMW – M.J. Wingfield, FABI, University of Pretoria, South Africa.
[b] Sequence numbers in italics were retrieved from the GenBank public database. All others were obtained in this study.
[c] Isolate accession numbers in bold signify cultures ex-type, or from samples that have been linked morphologically to the type material.

combined and aligned and a partition homogeneity test was conducted in PAUP [17] to examine the possibility of a joint analysis of the two data sets. Maximum parsimony analyses were performed using the heuristic search option with 1000 random taxa addition and tree bisection and reconnection (TBR) as the branch-swapping algorithm. Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. Robustness of the branches was evaluated by 1000 bootstrap replications [18].

Results

Morphological characterization

Pycnidia on olive fruits were brown to black and immersed in the host, breaking through the epidermis to become partially erumpent. Conidia were hyaline, aseptate, thin-walled, fusiform to subclavate, apex subacute, base truncate with a minute marginal frill, (19.5–)22.3–23.6(–27.7) × (4.6–)5.2–5.5(–6.9) μm, mean ± S.D. of 33 coni-

dia = 22.9 ± 2.0 × 5.3 ± 0.5 μm, L/W ratio of 4.35 ± 0.5.

Colonies developing on OA and PDA were initially colourless, becoming dark olive from the centre with the dark colouration ultimately spreading to the entire colony. Conidiomata started to form after 10 days on OA at 22 °C. Conidiomata (Figure (1)1) were unilocular and pycnidial, or multilocular and eustromatic and were initially covered with olivaceous, appendage-like hyphae. The wall was dark brown to black, composed of thick-walled, dark brown *textura angularis* in the outer parts with hyaline, thin-walled cells towards the inner layers. Ostioles were circular, central, and well-defined. Conidiophores, when present, were hyaline, cylindrical, smooth and sparingly branched. Conidiogenous cells (Figure 1(2 and 3)) were hyaline, smooth, thin-walled, cylindrical, initially holoblastic, becoming enteroblastic, producing one or more conidia apically, proliferating internally to form periclinal thickenings and typical phialides [19] or proliferating percurrently resulting in 2–3 close- or wide-spaced annellations. Conidia were either hyaline, thin-walled and aseptate (Figure 1(4)), or brown, thick-walled and one- or two-septate (Figure 1 (5–9)). The hyaline conidia were smooth, aseptate, fusiform with a subacute apex and a rounded or truncate base that often bore a minute basal frill. Conidia measured (23.8–)26.4–27.4(–31.1) × (4.5–) 5.3–5.5(–6.2) μm with mean and SD of 50 conidia = 26.9 ± 1.7 × 5.4 ± 0.4 μm, length width ratios were in the range of (4.1–)4.9–5.2(–6.2) and the mean and SD = 5.0 ± 0.5. The brown-walled conidia had a smooth, moderately thick, pale brown wall and were 0–2(–3)-septate, subglobose,

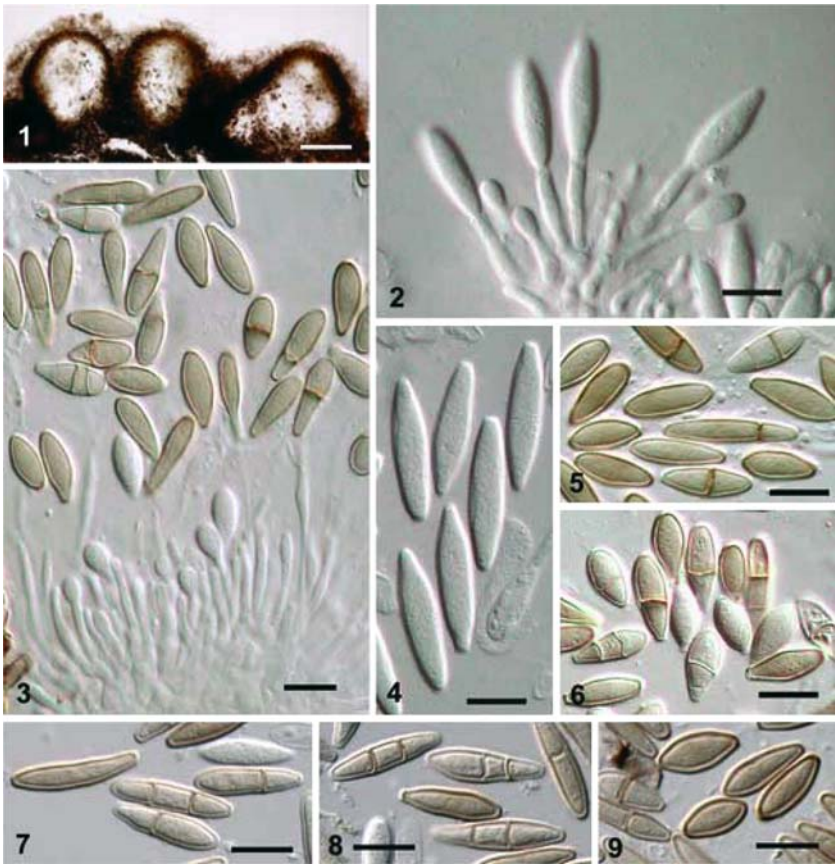

Figure 1. Fusicoccum anamorph of *Botryosphaeria dothidea* causing fruit rot of olives. (1) Sectioned pycnidia from oatmeal agar cultures; (2) Condiogenous cells and developing fusiform, hyaline conidia; (3) Conidiogenous cells and dark-walled, septate conidia; (4) Hyaline, aseptate conidia; (5–9). Dark-walled, 0–2 septate conidia. Scale bars: 1 = 100 μm, 2–9 = 10 μm.

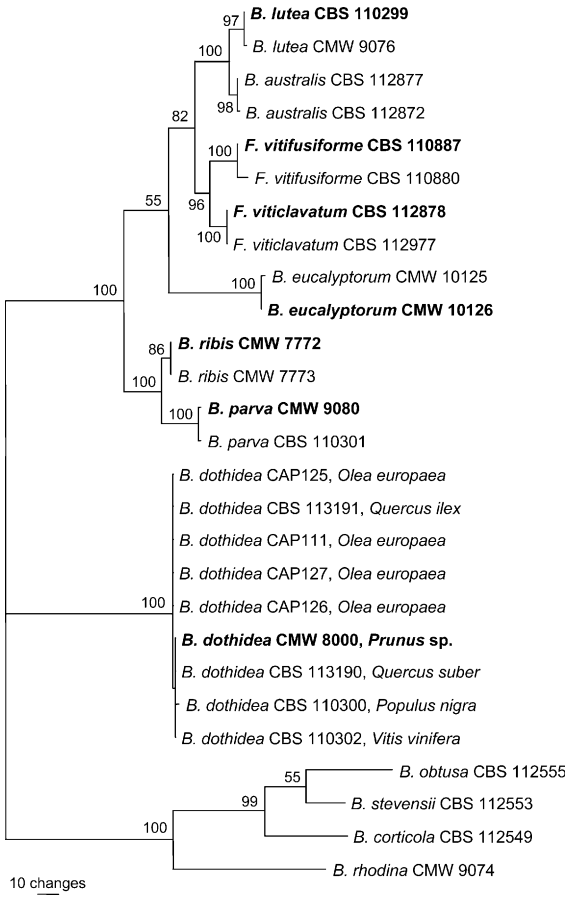

Figure 2. (10) One of four most parsimonious trees obtained from combined ITS and EF1-α sequence data. Cultures linked to the type material are indicated in bold. Host names are provided for the *B. dothidea* isolates. Bootstrap values from 1000 replications are shown at the nodes. The tree was rooted to *B. obtusa*, *B. stevensii*, *B. corticola* and *B. rhodina*. The bar represents 10 changes.

clavate, fusiform-ellipsoid or clavate, with a truncate base. Conidia measured (9.7–)15.1–15.9 (–22.9) × (3.1–)5.0–5.1(–6.7) μm with mean and SD of 150 conidia = 15.5 ± 2.7 × 5.1 ± 0.6 μm, length width ratios were in the range of (1.6–) 3.0–3.3(–5.2) and the mean and SD = 3.1 ± 0.8.

Phylogenetic analysis

Four isolates from olives were chosen for sequencing the ITS and EF1-α. ITS and EF1-α sequences of a further 19 isolates (Table 1), representing five isolates of *B. dothidea* and seven other *Botryosphaeria* species with *Fusicoccum* anamorphs, were retrieved from GenBank and included in the analysis. Since the focus of this study was to determine the position of the isolates from olives in relation to other species with *Fusicoccum* anamorphs, the tree was rooted to four species with *Diplodia* anamorphs. New ITS and EF1-α sequences were deposited in GenBank. A partition homogeneity test ($P = 0.34$) indicated that the ITS and EF1-α trees reflect the same underlying phylogeny and could be combined in a single analysis. After alignment, the combined dataset consisted of 850 characters including gaps. Of these, 519 characters were constant while 52 variable characters were parsimony – uninformative. Using the remaining 279 characters, four equally parsimonious trees were retained after heuristic searches in PAUP, one of which is shown in Figure 2(10). The four trees differed only in the positions of some isolates within the terminal clades with no differences in overall phylogeny. The eight species with *Fusicoccum* anamorphs were clearly distinguished and the species clades were supported by bootstrap values in excess of 98% except for the *B. ribis* clade, which received 86% support. The four isolates from olive fruits clustered within the clade containing isolates identified as *B. dothidea*. One of those (CMW8000) was isolated from the ex-epitype of *B. dothidea* [20], and in that respect it can be regarded as authentic for the name. The *B. dothidea* clade formed a lineage separate from all other species with *Fusicoccum* anamorphs.

Discussion

The fungus studied here and the one illustrated by Zachos and Tzavella-Klonari [7] are clearly not species of *Phyllosticta* since the conidia lack the mucilagenous, apical appendages or caps that are characteristic of that genus [12, 21]. Furthermore, the transfer to *Phoma* by Saccardo [9] is not suitable, according to the current concept of that genus, since the conidiogenous cells of the isolates from olives are longer and more conspicuous than those found in any species of *Phoma*. Although the fungus was known for many years as *Macrophoma dalmatica* the genus *Macrophoma* has been the source of considerable confusion. The name has been applied

indiscriminately to *Phoma*-like fungi with relatively large conidia and to species with dark conidia. When Berlese and Voglini [10] raised *Phoma* subgenus *Macrophoma* to the status of genus, they included species with hyaline, thin-walled conidia as well as species with brown, thick-walled conidia. However, they did not designate a species as type of the genus. Petrak and Sydow [22] took the first listed species, *M. macrosperma* (Karst.) Berl. & Vogl., as lectotype, but showed that *Sphaeria pinea* Desm. provided an earlier epithet and consequently they proposed *M. pinea* (Desm.) Petrak & Sydow as type. Subsequently, Petrak [23] showed that *M. pinea* is a later homonym of *M. pinea* Pass. (≡ *Dothiorella pinea* (Pass.) Petr. & Syd.) and so he replaced the name of the lectotype species with *M. sapinea* (Fr.) Petrak. Thus, the type species of *Macrophoma* (*M. sapinea*) has brown, thick-walled conidia. For this reason, Sutton [19] regarded *Macrophoma* as a synonym of *Sphaeropis*. Clearly, *Macrophoma* is not a suitable genus for the fungus causing fruit rot of olives.

Gigante [11] transferred *P. dalmatica* to *Sphaeropsis* presumably because of the brown-walled conidia. However, the distinction between *Sphaeropsis* and *Diplodia* has never been clear. Percurrent proliferation of conidiogenous cells has been regarded as more typical of *Sphaeropsis* than of *Diplodia* as defined by Sutton [19]. Since percurrent proliferation of conidiogenous cells is common in *Diplodia mutila* [14], the type species of *Diplodia*, there is no valid reason to separate these two genera and *Sphaeropsis* is considered to be a later synonym of *Diplodia*.

Morphologically the fungus studied in this paper correlated well with the one reported by Zachos and Tzavella-Klonari [7]. While Zachos and Tzavella-Klonari [7] regarded *Camarosporium* to be a suitable genus, the hyaline, thin-walled, aseptate conidia they also report are not typical of that genus and suggest *Fusicoccum*. Mode of conidiogenesis and morphology of the conidiogenous cells tend to confirm this. Dimensions and morphology of the hyaline conidia correlate well with *F. aesculi*, the anamorph of *B. dothidea* as defined by Slippers et al. [20]. Phylogenetically, the olive isolates were indistinct from other isolates of *B. dothidea*, including the ex-epitype strain. Therefore, we conclude that the fungus causing fruit rot of olives is *B. dothidea* and its anamorph is *Fusicoccum aesculi*.

The dark-walled conidia of the isolates from olive are unusual. Nevertheless, when Crous and Palm [24] emended the concept of *Fusicoccum* they included conidia that can become olivaceous with age. Furthermore, conidia of some *Fusicoccum* become brown and develop septa when aged or just before germination. For example, conidia of *B. parva* and *B. ribis* develop two septa before germination and in *B. parva* the central cell often becomes darker than the two terminal cells [20].

The inclusion of dark conidia in *Fusicoccum* makes the distinction between *Fusicoccum* and *Diplodia* less clear. As discussed by Alves et al. [14] the separation of these two anamorph genera on the basis of conidium colouration is tenuous. Indeed, Denman et al. [25] suggested that the two genera should be united under the older name of *Fusicoccum*. Although this would be desirable in order to link one teleomorph genus with a single anamorph genus, no formal synonymies are made in this paper.

Taxonomy

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Anamorph: *Fusicoccum aesculi* Corda in Sturm, Deutschland flora 2: 111. 1829.

= *Fusicoccum dalmaticum* (Thüm.) Vanev, in van der Aa and Vanev, A revision of the species described in *Phyllosticta* p. 179. 2002.

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