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Morphology, phylogeny and pathogenicity of *Botryosphaeria* and *Neofusicoccum* species associated with drupe rot of olives in southern Italy

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Species of *Botryosphaeria* and *Neofusicoccum* are well known as pathogens of woody hosts. In this study the species that occur on rotting olive drupes in the main production areas of southern Italy were studied. Species were identified from the morphology of their conidial states in culture and from sequence data of the ITS rDNA operon and partial sequence of the translation elongation factor 1-α gene. *Botryosphaeria* and *Neofusicoccum* species were isolated from more than 60% of the affected drupes, suggesting that they are the main contributors to this disease. The most common species was *B. dothidea*, which was isolated from 34% of the drupes. However, *N. australe* and *N. vitifusiforme* were also common and were isolated from 16 and 12%, respectively. Two other species (*N. parvum* and *N. mediterraneum*) were uncommon and occurred on less than 1% of the drupes. All five species were pathogenic on the two cultivars of olive tested. The most aggressive species was *N. vitifusiforme*, followed by *N. australe* and *B. dothidea*. The two olive cultivars differed in their susceptibility to the pathogens. The results show that *B. dothidea*, *N. vitifusiforme* and *N. australe* are important pathogens of olives.

Keywords: disease severity, *Fusicoccum*, ITS, *Olea europaea*, phylogeny

Introduction

The European olive (*Olea europaea)* is an important crop in southern Italy. According to the Italian National Institute of Statistics (http://www.istat.it) olives were cultivated on 906 k ha throughout this region in 2006. Of the 2960 k tonnes of olives harvested, 2908 k tonnes were destined for oil production while the remainder were used as table olives. The greatest production was in the Calabria (1111 k tonnes) and Puglia (1090 k tonnes) regions with significant production in Sicilia (286 k tonnes) and Basilicata (42 k tonnes). Thus, the olive industry contributes significantly to the economy and employment of the southern regions of Italy.

Diseases of olive drupes can cause financial losses through direct loss of rotted drupes, reduced cosmetic value of table olives and reduced quality of the oil due to fungal infections. González *et al*. (2006) showed that oil from infected drupes had higher acidity, higher peroxide levels and lower stability than oil from healthy drupes.

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The disease of olive drupes known locally as 'Lebbra' is caused by *Colletotrichum acutatum*. This disease is easily recognized by the large quantities of orange conidia that are produced by the pathogen. A similar disease, but distinctive in the absence of orange conidial masses, has been known for many years and appears to be widespread throughout the Mediterranean region (Trapero & Blanco, 2004). This disease is commonly known as 'drupe rot' but it has also been referred to as 'Dalmation disease' (González *et al*., 2006).

Since it was first described in 1883, the causal agent of drupe rot has undergone several taxonomic changes. It was first described as *Phyllosticta dalmatica* and subsequently transferred to other genera such as *Phoma dalmatica*, *Macrophoma dalmatica*, *Sphaeropsis dalmatica* and *Camarosporium dalmaticum*. Phillips *et al*. (2005a) determined that all these names are synonyms of *Fusicoccum aesculi*, which is the anamorph of *Botryosphaeria dothidea*. They further determined that this fungus is the main cause of drupe rot of olives in central Greece.

A recent phylogenetic study of the Botryosphaeriaceae revealed that *Botryosphaeria* is composed of several distinct lineages that correspond to individual genera

(Crous *et al.*, 2006). Only *B. dothidea* and *B. corticis* were retained in *Botryosphaeria*, while other species with *Fusicoccum*-like anamorphs were transferred to the new genus *Neofusicoccum*.

The only *Neofusicoccum* species that has been reported on olives is *Neofusicoccum ribis* (= *Botryosphaeria ribis*) (Romero *et al.*, 2005). Nevertheless, *Neofusicoccum* spp. are known to be pathogens of a wide range of woody hosts causing diebacks and fruit rots (Pennycook & Samuels, 1985; Phillips *et al.*, 2002; Denman *et al.*, 2003; Niekerk *et al.*, 2004; Slippers *et al.*, 2005). There have been no detailed studies of the *Botryosphaeria* and *Neofusicoccum* species that occur on olives.

In a recent survey of drupe rot in southern Italy, a large collection of isolates corresponding morphologically to *Fusicoccum* and *Neofusicoccum* species were isolated. The aim of the present study was to determine the identity of the species and to determine their pathogenicity on two olive cultivars. Species were identified from their morphology and a study of combined ITS and EF1-α sequence data.

Materials and methods

Isolates and morphology

Between 70 and 100 olive drupes with symptoms of drupe rot were collected from each of 23 localities in the main olive producing regions of southern Italy (Table 1). The drupes were examined with a dissecting microscope and isolations were made by spreading the cirrus of conidia exuding from pycnidia on potato dextrose agar (PDA) (Difco) supplemented with 500 μg mL^{-1} streptomycin (PDAS). After incubating at 25°C for 18 h, single germinating conidia were transferred to fresh PDA. When no cirri could be seen, the drupes were washed with running tap water for 10 min, dipped in 70% ethanol for 2 min, and pieces of tissue taken from the edge of the lesion and transferred to PDAS. Putative *Botryosphaeria* and *Neofusicoccum* isolates, recognized by their rapidly growing colonies with grey mycelium, were transferred to fresh plates of PDA. Identity of the two genera was confirmed from conidial morphology and mode of conidiogenesis (Crous *et al.*, 2006). Isolates were stored on PDA slopes at 5°C. Representative cultures were deposited at the Centraalbureau voor Schimmelcultures (CBS), Utrecht, the Netherlands.

For studies on growth rates and colony morphology, isolates were grown on half strength-PDA and incubated at 25°C in darkness (for growth rates) or on the laboratory bench where they received diffused daylight (for colony morphology). To induce sporulation, cultures were grown on 2% w/v water agar bearing pieces of sterilized pine needles and kept on the laboratory bench. Conidia oozing from the pycnidia were transferred to a drop of water on a glass slide and when the water had almost completely dried out, a drop of 100% v/v lactic acid was added and a coverslip applied. The conidiogenous layer was dissected out from the pycnidia and mounted in 100% lactic acid. Microscope images were recorded with a Leica DFC320 digital camera from images recorded with the ×100 objective. Conidia were measured with the Leica IM500 measurement module. The mean, standard deviation and 95% confidence limits were calculated from at least 20 conidia of each isolate.

DNA isolation and amplification

DNA was isolated from fungal mycelium by the method of Möller *et al.* (1992). PCR reactions were carried out with *Taq* polymerase, nucleotides and buffers supplied by MBI Fermentas and PCR reaction mixtures were prepared according to Alves *et al.* (2004), with the addition of 5% v/v DMSO to improve the amplification of some difficult DNA templates. All primers were synthesized by MWG Biotech AG. The ITS region was amplified using the primers ITS1 and ITS4 (White *et al.*, 1990) as described by Alves *et al.* (2004). The primers EF1-728F and EF1-986R (Carbone & Kohn, 1999) were used to amplify part of the EF1-α gene as described by Phillips *et al.* (2005b).

The amplified PCR fragments were purified with the JETQUICK PCR Purification Spin Kit (GENOMED). Both strands of the PCR products were sequenced by STAB Vida Lda. The nucleotide sequences were read and edited with FinchTV 1·4·0 (Geospiza Inc.; http://www.geospiza.com/finchtv). All sequences were checked manually and nucleotide arrangements at ambiguous positions were clarified using both primer direction sequences. Sequences were deposited in GenBank (Table 1).

Phylogenetic analyses

Sequences of the strains isolated in this study were assembled with nucleotide sequences of additional isolates of *Botryosphaeria* and *Neofusicoccum* spp. retrieved from GenBank (Table 2). The sequences were aligned with ClustalX version 1·83 (Thompson *et al.*, 1997) using the following parameters: pairwise alignment gap opening = 10, gap extension = 0·1; multiple alignment gap-opening = 10, gap extension = 0·2; delay divergent sequences = 25%; transition weight = 0·5. Alignments were checked and manual adjustments were made where necessary. Phylogenetic analyses of sequence data were done using PAUP* v.4·0b10 (Swofford, 2003). The trees were rooted to *Diplodia seriata* and *D. mutila* and visualized with TreeView (Page, 1996).

The HKY85 nucleotide substitution model (Hasegawa *et al.*, 1985) was used for distance analysis. All characters were unordered and of equal weight. Bootstrap values were obtained from 1000 neighbour joining (NJ) bootstrap replicates.

Maximum parsimony (MP) analyses were performed using the heuristic search option with 1000 random taxa additions and tree bisection and reconnection (TBR) as the tree swapping algorithm. All characters were unordered and of equal weight and gaps were treated as a fifth character. Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved.

Table 1 Isolates of *Botryosphaeria* and *Neofusicoccum* species from olive drupes in southern Italy used in this study

Region	Province	Locality	No. infected drupes	Colony morphology group[a]	No. isolates in group	Isolatenumber	Identity	No of isolates of each species	GenBank Accession No. ITS	GenBank Accession No. EF
Puglia	Foggia	Borgo Cervaro, Foggia	61	38	27	CAP 288	*B. dothidea*	27	EF638755	EF638732
Puglia	Bari	Bari	52	11	26	CAP 226	*N. australe*	26	EF638774	EF638737
				12	19	CAP 227	*N. vitifusiforme*	19	EF638785	EF638744
Puglia	Bari	Monopoli	51	25	39	CAP 246	*B. dothidea*	39	EF638766	EF638730
Puglia	Taranto	Massafra	53	41	39	CAP 297	*B. dothidea*	39	EF638758	
Puglia	Brindisi	Brindisi	57	21	27	CAP 242	*B.dothidea*	53	EF638763	
				39	26	CAP 294	*B. dothidea*		EF638756	
Puglia	Brindisi	Lamaforca, Carovigno	56	36	26	CAP 286	*B. dothidea*	45	EF638753	EF638731
				37	19	CAP 287	*B. dothidea*		EF638754	
Puglia	Brindisi	Mesagne	56	19	26	CAP 240	*B. dothidea*	26	EF638761	
Puglia	Brindisi	Ostuni	78	17	25	CAP 237	*B. dothidea*	73	EF638759	
				22	17	CAP 243	*B. dothidea*		EF638764	
				23	31	CAP 244	*B. dothidea*		EF638765	
Puglia	Brindisi	Squinzano	61	24	33	CAP 245	*N. australe*	33	EF638776	EF638739
Puglia	Brindisi	Villanova	63	18	37	CAP 238	*B. dothidea*	37	EF638760	EF638729
Puglia	Lecce	Boschetto, Poggiardo	63	30	35	CAP 252	*N. australe*	35	EF638777	EF638740
Puglia	Lecce	Cursi	57	9	41	CAP 213	*N. vitifusiforme*	41	EF638784	
Puglia	Lecce	Latiano	65	20	29	CAP 241	*B. dothidea*	29	EF638762	
Puglia	Lecce	Lepre, Scorrano	59	31	11	CAP 253	*N. mediterraneum*	11	EF638787	EF638746
Puglia	Lecce	Maglie	77	8	36	CAP 212	*N. vitifusiforme*	36	EF638783	
Puglia	Lecce	Melissano	61	5	34	CAP 209	*N. vitifusiforme*	34	EF638780	
Puglia	Lecce	Miggiano	62	10	39	CAP 221	*N. australe*	39	EF638773	EF638736
Puglia	Lecce	Montesano Salentino	69	16	31	CAP 236	*N. australe*	67	EF638775	EF638738
				32	36	CAP 258	*N. australe*		EF638778	EF638741
Puglia	Lecce	Poggiardo	56	7	31	CAP 211	*N. vitifusiforme*	31	EF638782	
Puglia	Lecce	Surano	59	6	26	CAP 210	*N. vitifusiforme*	26	EF638781	EF638743
Puglia	Lecce	Taviano	65	1	32	CAP 162	*N. australe*	32	EF638770	EF638733
Puglia	Lecce	Tricase	62	3	25	CAP 178	*N. australe*	25	EF638772	EF638735
				4	17	CAP 201	*N. vitifusiforme*	17	EF638779	EF638742
Basilicata	Potenza	Potenza	57	15	14	CAP 234	*B. dothidea*	36	EF638749	
				40	22	CAP 296	*B. dothidea*		EF638757	
				26	4	CAP 247	*N. parvum*	4	EF638786	EF638745
Basilicata	Matera	Rotondella	59	2	31	CAP 173	*N. australe*	31	EF638771	EF638734
Calabria	Cosenza	Cosenza	57	27	28	CAP 248	*B. dothidea*	28	EF638767	
Calabria	Reggio Calabria	Gioia Tauro	54	33	38	CAP 281	*B. dothidea*	38	EF638750	
Calabria	Reggio Calabria	Reggio Calabria	52	34	26	CAP 282	*B. dothidea*	42	EF638751	
				35	16	CAP 283	*B. dothidea*		EF638752	
Sicilia	Catania	Linguaglossa	61	28	16	CAP 249	*B. dothidea*	38	EF638768	
				29	22	CAP 250	*B. dothidea*		EF638769	
Sicilia	Palermo	Palermo	64	13	16	CAP 232	*B. dothidea*	35	EF638747	EF638727
				14	19	CAP 233	*B. dothidea*		EF638748	EF638728
		Total	1747		1092			1092		

[a]Isolates were placed in one of 41 groups according to colony morphology.

Table 2 Additional isolates included in the phylogenetic study of species associated with drupe rot of olives

Isolate number[a]	Species	Collector	Host	Locality	GenBank Accession No. ITS	EF
CMW 9072	*Neofusicoccum australe*	J. Roux	*Acacia* sp.	Australia	AY339260	AY39268
CBS 119046	*N. australe*	E. Diogo	*Rubus* sp.	Portugal	DQ299244	EU017541
STE-U 4425	*N. australe*	F. Halleen	*Vitis vinifera*	South Africa	AY343388	AY343347
CBS110299	*Neofusicoccum luteum*	A.J.L. Phillips	*Vitis vinifera*	Portugal	AY259091	AY573217
CMW 9076	*N. luteum*	S.R. Pennycook	*Malus* x *domestica*	New Zealand	AY339257	AY339265
CBS 110887	*Neofusicoccum vitifusiforme*	J. Van Niekerk	*Vitis vinifera*	South Africa	AY343383	AY343343
CBS 110880	*N. vitifusiforme*	J. Van Niekerk	*Vitis vinifera*	South Africa	AY343382	AY343344
WAC 12398	*Dichomera eucalypti*	T. Burgess/K.-LGoei	*Eucalyptus diversicolor*	Australia	AY744371	DQ093214
CMW 15952	*D. eucalypti*	T. Burgess/K.-LGoei	*Eucalyptus diversicolor*	Australia	DQ093194	DQ093215
CMW 15953	*D. eucalypti*	T. Burgess/K.-LGoei	*Eucalyptus diversicolor*	Australia	DQ093195	DQ093216
CAA 002	*Neofusicoccum mediterraneum*	T.J. Michailides	*Pistacia vera* var. 'Kerman'	USA	EU017537	EU017538
CBS 112878	*Neofusicoccum viticlavatum*	F. Halleen	*Vitis vinifera*	South Africa	AY343381	AY343342
CBS 112977	*N. viticlavatum*	F. Halleen	*Vitis vinifera*	South Africa	AY343380	AY343341
CBS 110301	*Neofusicoccum parvum*	A.J.L. Phillips	*Vitis vinifera*	Portugal	AY259098	AY573221
CMW 9081	*N. parvum*	G.J. Samuels	*Populus nigra*	New Zealand	AY236943	AY236888
CBS 121·26	*Neofusicoccum ribis*	N.E. Stevens	*Ribes rubrum*	USA	AF241177	AY236879
CBS 115475	*N. ribis*	B. Slippers	*Ribes* sp.	USA	AY236935	AY236877
CBS 118531	*Neofusicoccum mangiferum*	G.I. Johnson	*Mangifera indica*	Australia	AY615185	DQ093221
CBS 118532	*N. mangiferum*	G.I. Johnson	*Mangifera indica*	Australia	AY615186	DQ093220
CBS 115791	*Neofusicoccum eucalyptorum*	H. Smith	*Eucalyptus grandis*	South Africa	AF283686	AY236891
CMW 10126	*N. eucalyptorum*	H. Smith	*Eucalyptus grandis*	South Africa	AF283687	AY236892
CBS 115766	*Neofusicoccum eucalypticola*	M.J. Wingfield	*Eucalyptus rossii*	Australia	AY615143	AY615135
CMW 6539	*N. eucalypticola*	M.J. Wingfield	*Eucalyptus rossii*	Australia	AY615141	AY615133
CBS 116741	*Botryosphaeria dothidea*	I. Rumbos	*Olea europaea*	Greece	AY640254	AY640257
CBS 110300	*B. dothidea*	A.J.L. Phillips	*Populus nigra*	Portugal	AY640253	AY640256
CBS 110302	*B. dothidea*	A.J.L. Phillips	*Vitis vinifera*	Portugal	AY259092	AY573218
CBS 115476	*B. dothidea*	B. Slippers	*Prunus* sp.	Switzerland	AY236949	AY236898
CBS 119048	*Botryosphaeria corticis*	P.V. Oudemans	*Vaccinium corymbosum*	USA	DQ299246	EU017540
CBS 119047	*B. corticis*	P.V. Oudemans	*Vaccinium corymbosum*	USA	DQ299245	EU017539
CBS 112553	*Diplodia mutila*	A.J.L. Phillips	*Vitis vinifera*	Portugal	AY259093	AY573219
CBS 112555	*Diplodia seriata*	A.J.L. Phillips	*Vitis vinifera*	Portugal	AY259094	AY573220

[a]Designation of isolates and culture collections: CAA = A. Alves, Universidade de Aveiro, Portugal; CAP = AJL Phillips, Universidade Nova de Lisboa, Portugal; CBS = Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CMW = MJ Wingfield, FABI, University of Pretoria, South Africa; STE-U = University of Stellenbosch, South Africa.

The robustness of the most parsimonious trees was evaluated by 1000 bootstrap replications (Hillis & Bull, 1993). Other measures used were consistency index (CI), retention index (RI), and homoplasy index (HI). A partition homogeneity test was done with PAUP to asses the validity of combining the ITS and EF1-α data.

Pathogenicity

Pathogenicity of the 41 selected isolates was tested on healthy drupes of the olive cultivars Coratina and Ogliarola. The isolates were grown on PDA at 25°C for 7 days prior to inoculation.

Olive drupes were collected from trees in the Molfetta area in the province of Bari, Puglia in January 2007. Five drupes for each combination of cultivar and isolate were washed with 1% v/v Tween 20 and placed on sterilized metal grids inside 20 cm diameter glass Petri dishes lined with filter papers, that were kept moist throughout the experiment. Drupes were damaged at six closely spaced points by pricking with a sterile needle (area wounded = 16 mm^2). A small piece of agar (4 × 4 mm) colonized with the test strain of each isolate was placed on the wounded zone. Controls were inoculated with pieces of uncolonized PDA. The Petri dishes were sealed with Parafilm and incubated at 22 ± 2°C in darkness for 20 days. There were five replicates for each combination of isolate and cultivar arranged as a fully randomized experiment. Aggressiveness of isolates was determined after 20 days from the percentage of the surface showing signs of rotting, which was assessed by referring to a series of standard photographs of drupes with various degrees of rotting. Significance of differences in susceptibility of the two cultivars was gauged by a *t*-test, while differences in aggressiveness of isolates was determined by analysis of variance.

Results

Isolations

Between October 2000 and December 2005, 1747 rotted drupes were collected from 23 sites in southern Italy.

Isolations on PDAS resulted in 1092 isolates of *Botryosphaeria* and *Neofusicoccum* spp. (Table 1). The other 655 rotted olives yielded species of *Fusarium*, *Colletotrichum*, *Alternaria* and *Phoma*, which were not considered further in this study. The *Botryosphaeria* and *Neofusicoccum* isolates from each locality were grouped according to their morphology. This resulted in 41 groups of isolates, and one isolate from each group was selected for detailed studies on their morphology, for pathogenicity testing and for sequence analysis (Table 1).

Phylogenetic analysis

ITS phylogeny

PCR products of ~580 bp were obtained for all isolates and ~520 bp were used in the phylogenetic analysis. After alignment the dataset, composed of 41 isolates from olives and 34 sequences retrieved from GenBank, consisted of 537 characters including gaps. Of these, 412 were constant and 12 were parsimony uninformative. Maximum parsimony analysis of the remaining 113 parsimony-informative characters resulted in four trees of 174 steps (TreeBase Accession No. SN 3487). The four trees differed only in the arrangement of isolates in the terminal clades, while the overall topology was the same.

Two major clades were resolved in the MP analysis. One clade corresponded to *Neofusicoccum* species while the other consisted of two species of *Botryosphaeria*, namely *B. dothidea* and *B. corticis*. Most of the isolates from olives (23) clustered in the *B. dothidea* clade. The remaining 18 olive isolates fell within four sub-clades in the *Neofusicoccum* clade. Nine isolates clustered close to *N. australe* and *N. luteum* and seven isolates clustered with *N. vitifusiforme* and *Dichomera eucalypti*. A single isolate clustered with *N. parvum* and another with some isolates from pistachio. However, species in the *Neofusicoccum* clade were not clearly resolved and bootstrap support for the branches was generally low. Therefore, representative isolates from the *Botryosphaeria* clade and the four groups within *Neofusicoccum* were selected for sequencing of the EF1-α gene (Table 1).

Combined ITS and EF phylogeny

A partition homogeneity test showed no significant difference ($P = 0{\cdot}27$) between the data from the different gene regions, indicating that they could be combined in a single dataset. The sequence alignment of 20 isolates from olives, 30 sequences from GenBank and two outgroup species consisted of 536 characters for the ITS region and 307 for the EF1-α gene, including alignment gaps. The combined dataset consisted of 843 characters, of which 522 were constant and 39 were parsimony-uninformative. Maximum parsimony analysis of the remaining 282 parsimony-informative characters resulted in seven most parsimonious trees (TL = 509 steps, CI = 0·845, RI = 0·964, HI = 0·155). The seven trees differed only in the arrangement of isolates within the terminal clades while their overall topology was the same. One of the trees is shown in Fig. 1. Neighbour-joining analysis resulted in a tree with essentially the same topology as the MP tree.

The combination of ITS and EF1-α revealed 10 well-supported clades in *Neofusicoccum* and two in *Botryosphaeria*. The isolates from olives clustered within four *Neofusicoccum* clades and with *B. dothidea*. The combined dataset confirmed that a single isolate belonged to *N. parvum* and another single isolate clustered with isolates of *N. mediterraneum* from pistachio. The remaining *Neofusicoccum* isolates clustered with either *N. australe* (nine isolates) or in a clade composed of isolates of *Dichomera eucalypti* and *N. vitifusiforme* (seven isolates). All the olive isolates that clustered with *N. australe* lay within a single sub-clade of this species, with low MP bootstrap support (64%) but high NJ bootstrap support (99%). Despite the high NJ bootstrap support, only a single base pair difference in ITS and 3 bp in EF 1-α separated the olive isolates from *N. australe*.

Morphology

Most of the isolates sporulated within 14 days of incubation on pine needles on water agar. Morphology of the isolates within the *B. dothidea* clade were typical of that species in their cylindrical conidiogenous cells (Fig. 2a,b) and fusiform, hyaline, aseptate conidia (Fig. 2c) that measured (11·7–)21·4–22·4(–33·3) × (3·9–)5·8–6(–8·5) μm. In some isolates, the conidium wall became darker and thicker, and some conidia developed one or two septa (Fig. 2d,e).

Conidia of isolates in the *N. australe* clade were hyaline, aseptate and sub-fusiform (Fig. 2f,g) measuring (16·2–)18·4–19·5(–20·7) × (5·8–)6·4–6·8(–7·6) μm, which is somewhat smaller than is typical for *N. australe* but similar to the dimensions reported for *N. luteum*. In culture, these isolates produced a yellow pigment that after 5–6 days became violaceous and then darkened, which is typical for both *N. australe* and *N. luteum*. Conidiogenous cells of the isolates in the *N. vitifusiforme*/*D. eucalypti* clade were long and cylindrical (Fig. 2h,i) and the conidia (Fig. 2j) were fusiform measuring (17–)18·5–22·5(–22·8) × (4·5–)5–6(–6·5) μm, which is within the range reported for that species. Morphologically the single isolate of *N. parvum* (Fig. 2k,l) corresponded well with the description by Pennycook & Samuels (1985). A single isolate of *N. mediterraneum* (CAP253) grouped with isolates from pistachio (Fig. 2m,n). The *Dichomera* synanamorph was not seen in any of the *Neofusicoccum* species studied here.

Pathogenicity

A preliminary analysis of the data indicated that no significant differences could be detected between isolates of each species. Therefore the data for all isolates of a given species were pooled (Table 3) so that differences in aggressiveness of the species could be tested. A Student's *t*-test on the pooled data for each cultivar indicated significant differences in the severity of disease ($t_{0{\cdot}05} = 5{\cdot}33$, $P < 0{\cdot}001$) with cv. Coratina being the most susceptible.

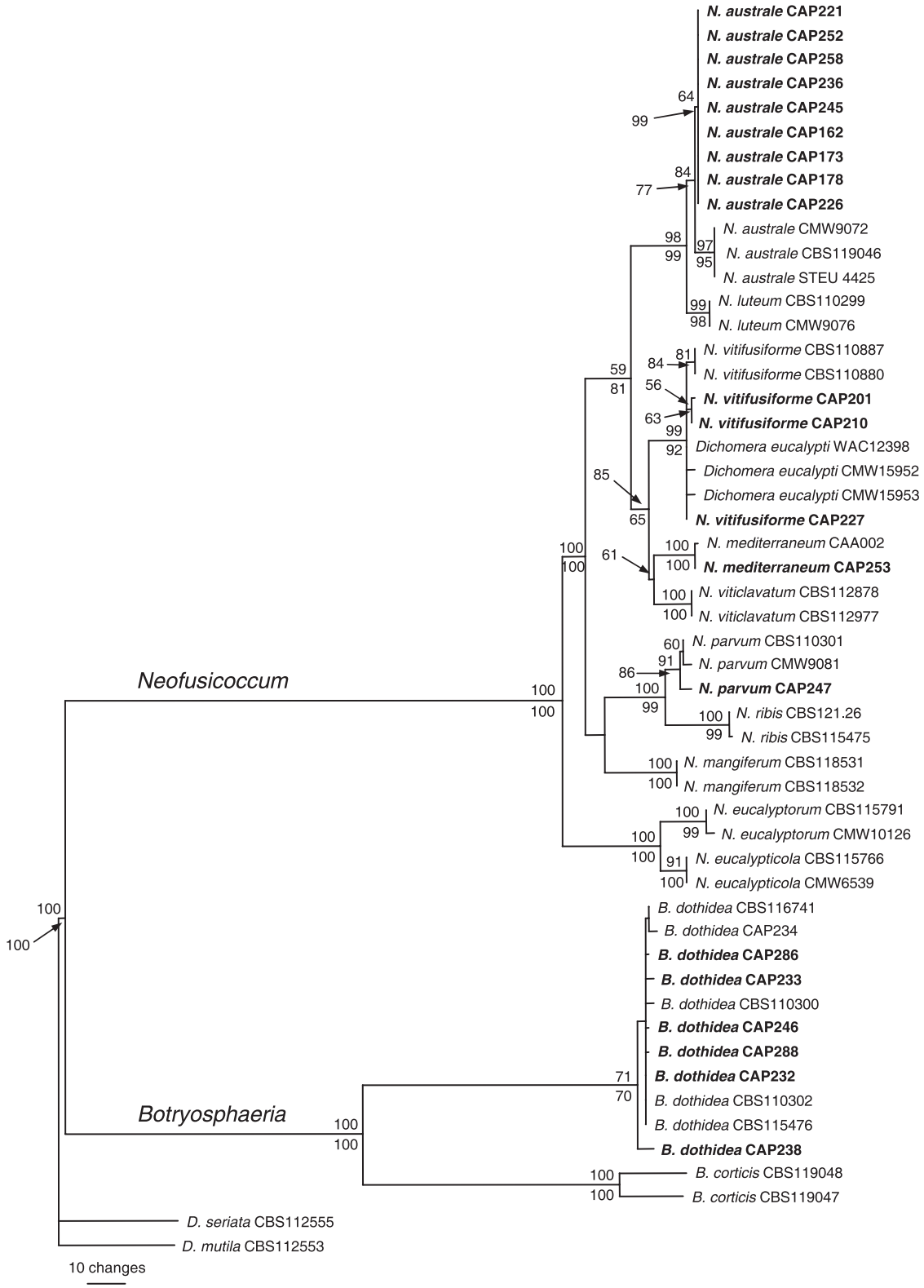

Figure 1 One of seven most parsimonious trees generated from combined ITS and EF1-α sequence data of isolates of *Botryosphaeria*, *Dichomera* and *Neofusicoccum* species. Isolate names in bold type are from olives. Maximum parsimony bootstrap values from 1000 replicates are given above the nodes, with neighbour joining bootstrap values below the nodes. The tree was rooted to *Diplodia seriata* and *D. mutila*.

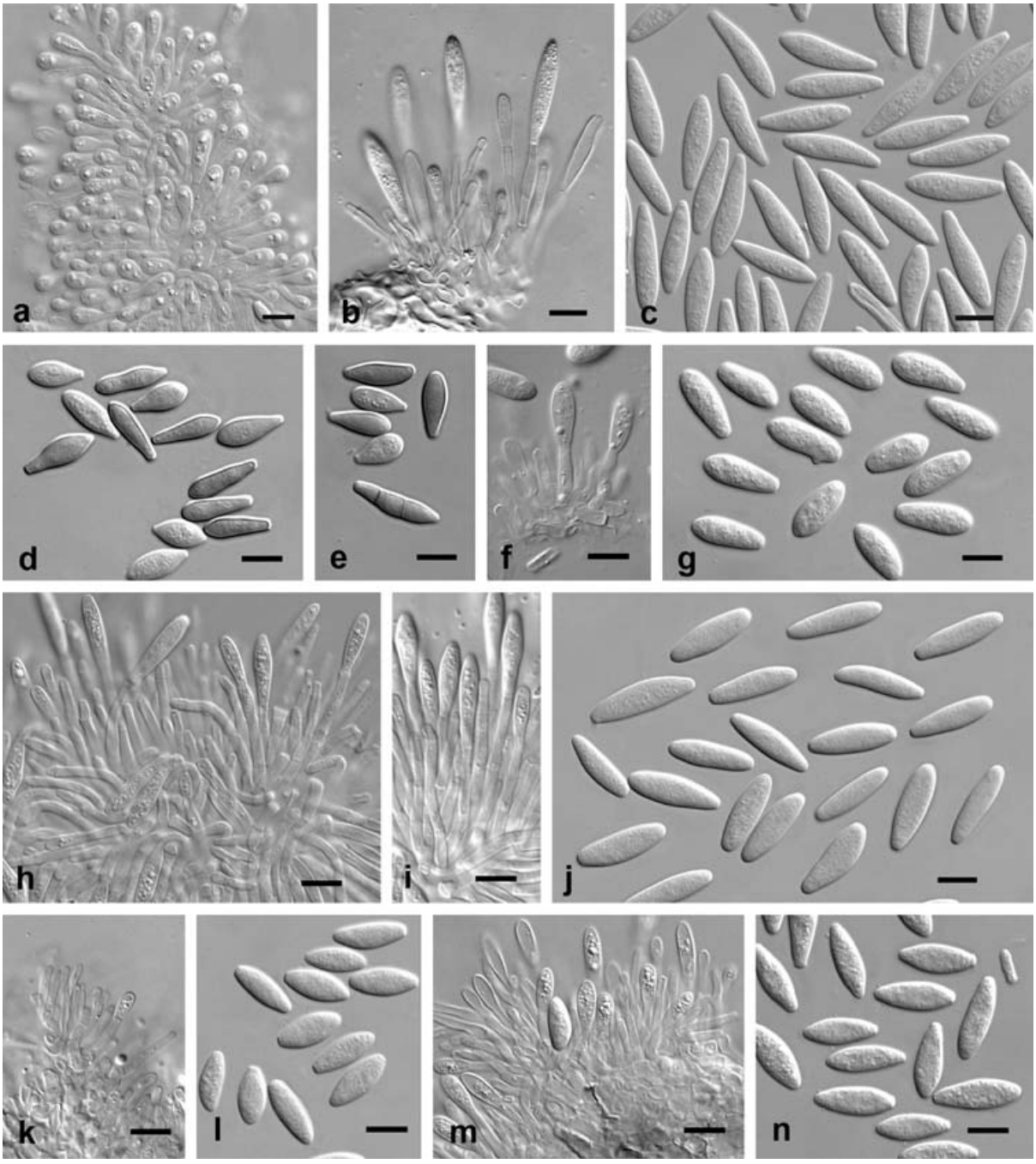

Figure 2 *Botryosphaeria* and *Neofusicoccum* species isolated from olive drupes. (a–e) *B. dothidea*. (a) conidiogenous layer; (b) conidiogenous cells and developing conidia; (c) hyaline conidia; (d–e) darkened, thicker walled, occasionally septate conidia. (f–g) *N. australe*. (f) conidiogenous cells; (g) conidia. (h–j) *N. vitifusiforme*. (h–i) conidiogenous cells; (j) conidia. (k–l) *N. parvum*. (k) conidiogenous cells; (l) conidia. (m–n) *Neofusicoccum mediterraneum*. (m) conidiogenous cells; (n) conidia. Bars = 10 μm.

Analysis of variance indicated significant differences in the aggressiveness of the five species on cv. Coratina ($F_{0{\cdot}05\,4,\,200}$ = 5·559, $P < 0{\cdot}001$) and on cv. Ogliarola ($F_{0{\cdot}05\,4,\,200}$ = 6·438, $P < 0{\cdot}001$). On both cultivars the most aggressive species was *N. vitifusiforme* followed by *N. australe* and *B. dothidea*. The data for *N. parvum* and *N. mediterraneum* are less reliable because only a single isolate of each was available and the degree of the variability within each species cannot be judged.

Discussion

This study represents the first attempt to characterize the species of *Botryosphaeria* and *Neofusicoccum* associated

Table 3 Percentage disease severity on olive drupes inoculated with different fungi as determined from the percentage of each drupe rotted

Species	Number of isolates	Cultivar	
		Coratina	Ogliarola
Botryosphaeria dothidea	23	36·6	15·9
Neofusicoccum australe	9	51·1	24·4
Neofusicoccum vitifusiforme	7	73·4	34·6
Neofusicoccum parvum	1	20·0	20·0
Neofusicoccum mediterraneum	1	80·0	100
Means ($n = 5$)		46·73	23·12
LSD 5% for species comparisons		±6·45	±5·56

with drupe rot of olives in an extensive collection of isolates, and integrating morphology, pathology and molecular data. Preliminary identifications were based on morphology of the anamorphs in culture. Although ITS sequence data allowed definitive identification of *B. dothidea* it was necessary to include sequence data from the translation elongation factor 1-α to identify unambiguously the *Neofusicoccum* species. In the present study, five species were identified on olives, of which only one (*B. dothidea*) has previously been associated with this host (Phillips *et al*., 2005a; González *et al*., 2006).

The five species isolated from olive drupes were identified from a combination of morphological (conidial) and molecular (ITS and EF1-α sequence) characters. The *B. dothidea* isolates clustered with an ex-epitype strain of this species in both the ITS tree and the tree constructed from combined ITS + EF1-α sequence data. The strains of *N. vitifusiforme* isolated from olives clustered with isolates of *N. vitifusiforme* from South African grapevines and isolates of *Dichomera eucalypti* from Australia. Only 2 bp in ITS separated the isolates in this clade, while EF1-α sequences for all these isolates were identical. Barber *et al.* (2005) showed that several '*Botryosphaeria*' species from *Eucalyptus* form a *Dichomera* synanamorph in culture, but they did not associate it with any *Neofusicoccum* species. When Crous *et al.* (2006) introduced *Neofusicoccum* they stated that the formation of a *Dichomera* synanamorph is the main feature that differentiates it from *Fusicoccum*. Since the isolates studied by Barber *et al.* (2005) clustered with ex-type cultures of *N. vitifusiforme* in this study, it is clear that *D. eucalypti* is the synanamorph of *N. vitifusiforme*. If this is accepted, the host range of *N. vitifusiforme* can be broadened to include *Eucalyptus* and *Olea*.

Of the 41 strains studied, nine clustered as a sub-clade within the *N. australe* clade. Although this sub-clade was supported by a high NJ bootstrap value (99%), MP bootstrap was low (64%) and only 1 bp difference in ITS and 3 bp differences in EF1-α separated these isolates from *N. australe*. All these isolates produced a yellow pigment typical of *N. luteum* and *N. australe*. Phylogenetically they were considered to be *N. australe*, but morphologically they were closer to *N. luteum*. Until more isolates from a wider geographic range have been studied, it is preferable to consider these isolates as *N. australe*.

Botryosphaeria and *Neofusicoccum* species were isolated from more than 60% of the rotted drupes sampled, thus indicating that they contribute significantly to drupe rots in the regions surveyed. The most common species was *B. dothidea*, which was isolated from 34% of the rotted drupes. This fungus is known to be associated with a wide range of hosts in most regions of the world (Slippers *et al*., 2004a, 2007). Furthermore, it has long been associated with olive drupe rot, albeit under a variety of different names (Phillips *et al*., 2005a). In a previous study (Phillips *et al*. 2005a) of the same disease in Greece (based on a relatively small sample of olives), only *B. dothidea* was associated with the disease. According to González *et al.* (2006), this fungus invades the drupes through wounds caused by the olive fruit fly (*Bactrocera oleae*) and may even be transmitted by it. However, the present study has shown that besides *B. dothidea*, two species in the closely related genus *Neofusicoccum* (*N. australe* and *N. vitifusiforme*) are commonly associated with olive drupe rot and cause the same symptoms.

Since it was first described (Niekerk *et al*., 2004) *N. vitifusiforme* has rarely been reported. Indeed it was thought to be a weak pathogen restricted to *Vitis vinifera* in South Africa (Niekerk *et al*., 2004). However, its occurrence on 12% of the rotted olive drupes sampled in southern Italy indicates that it is more widely distributed and has a wider host range than originally thought. *Neofusicoccum australe* was also relatively common and was isolated from 16% of the rotted drupes. Therefore, these two species contribute significantly to drupe rot of olives.

In addition to *N. vitifusiforme* and *N. australe*, two other *Neofusicoccum* species were occasionally isolated. One of these clustered with known isolates of *N. parvum*. This species is common on many woody hosts including *Vitis*, *Actinidia* and *Populus* species (Pennycook & Samuels, 1985) and thus it seems to be a common, widespread, plurivorous pathogen. On some hosts (e.g. *Vitis vinifera*) it is known to be an aggressive pathogen (Niekerk *et al*., 2004). Because it was relatively uncommon on olives (0·2% of the isolates), it is probably of little importance on this host. The other species (*N. mediterraneum*), which clustered with four isolates from pistachio, was isolated from only 11 of the 1747 rotted drupes sampled.

The two olive cultivars tested differed significantly in their susceptibility to infection by *B. dothidea* and the *Neofusicoccum* species. This indicates that a certain level of resistance exists in some cultivars, which indicates that breeding for resistance in olive varieties may be possible. González *et al.* (2006) demonstrated that the state of maturity of the olive fruits affects their susceptibility and this must be taken into account in any testing of pathogenicity within a breeding programme.

Botryosphaeria and *Neofusicoccum* were the main causes of drupe rot in all provinces except Foggia, Cosenza, Matera and Palermo, where they were isolated from similar proportions of rotted drupes as the other fungi associated with drupe rot. In most provinces, the most prevalent species was *B. dothidea*, but in Lecce *N. australe*

and *N. vitifusiforme* prevailed with less than 4% of the drupes affected by *B. dothidea*. In Matera, no *B. dothidea* was detected. These differences may be due to a number of factors including the predominant cultivars in each province, differences in climate and soil type and cultivation practices.

Judging from the frequency of isolation, it seems that *B. dothidea* is the most common cause of drupe rot of olives in southern Italy. This confirms the report by Phillips *et al*. (2005a) for the same disease in Greece and by González *et al*. (2006) in south-east Spain. However, *N. vitifusiforme* was the most aggressive species in the pathogenicity tests and *N. australe* also caused severe symptoms in artificial inoculations. Both of these two species were relatively frequent in the field and therefore they should also be taken into account in the development of disease control measures.

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