Mycologia, 96(4), 2004, pp. 781–798.

DNA phylogeny, morphology and pathogenicity of *Botryosphaeria* species on grapevines

Jan M. van Niekerk

Department of Plant Pathology, University of Stellenbosch, P. Bag X1, Matieland 7602, South Africa

Pedro W. Crous[1]
J.Z. (Ewald) Groenewald

Centraalbureau voor Schimmelcultures, Fungal Biodiversity Centre, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands

Paul H. Fourie

Department of Plant Pathology, University of Stellenbosch, P. Bag X1, Matieland 7602, South Africa

Francois Halleen

ARC Infruitec-Nietvoorbij, P. Bag X5026, Stellenbosch 7599, South Africa

Abstract: Several species of *Botryosphaeria* are known to occur on grapevines, causing a wide range of disorders including bud mortality, dieback, brown wood streaking and bunch rot. In this study the 11 *Botryosphaeria* spp. associated with grapevines growing in various parts of the world, but primarily in South Africa, are distinguished based on morphology, DNA sequences (ITS-1, 5.8S, ITS-2 and EF1-α) and pathological data. *Botryosphaeria australis, B. lutea, B. obtusa, B. parva, B. rhodina* and a *Diplodia* sp. are confirmed from grapevines in South Africa, while *Diplodia porosum, Fusicoccum viticlavatum* and *F. vitifusiforme* are described as new. Although isolates of *B. dothidea* and *B. stevensii* are confirmed from grapevines in Portugal, neither of these species occurred in South Africa, nor were any isolates of *B. ribis* confirmed from grapevines. All grapevine isolates from Portugal, formerly presumed to be *B. ribis,* are identified as *B. parva* based on their EF1-α sequence data. From artificial inoculations on grapevine shoots, we conclude that *B. australis, B. parva, B. ribis* and *B. stevensii* are more virulent than the other species studied. The *Diplodia* sp. collected from grapevine canes is morphologically similar but phylogenetically distinct from *D. sarmentorum. Diplodia sarmentorum* is confirmed as anamorph of *Otthia spiraeae,* the type species of the genus *Otthia* (Botryosphaeriaceae). A culture identified as *O. spiraeae* clustered within *Botryosphaeria* and thus is regarded as probable synonym. These findings confirm earlier suggestions that the generic concept of *Botryosphaeria* should be expanded to include genera with septate ascospores and *Diplodia* anamorphs.

Key words: *Botryosphaeria,* Botryosphaeriaceae, *Diplodia,* EF1-α, *Fusicoccum,* ITS, *Otthia,* systematics

INTRODUCTION

Members of the genus *Botryosphaeria* Ces. & De Not. are known to be cosmopolitan, having broad host ranges and wide geographical distributions (Barr 1972, 1987). Symptoms caused by *Botryosphaeria* species on grapevines include bud mortality, dieback, brown streaking inside the wood, internal necrotic lesions and in some cases bunch rot (Castillo-Pando et al 2001; Lehoczky 1988; Phillips 1998, 2000), while leaf spots, cankers, dieback, and various other fruit, shoot and trunk diseases are common on other hosts (von Arx 1987, Denman et al 2000). Several species that are considered to be saprotrophic have been reported from grapevines, while others have been shown to be severe pathogens of this host. Species of *Botryosphaeria* readily infect wounds, and in the case of grapevines this is especially true for pruning wounds (Castillo-Pando et al 2001, Phillips 2002). Symptoms usually develop slowly, and severe symptoms become visible only in grapevines that are 8 or more yr old or that are subjected to stress (Boyer 1995, Larignon and Dubos 2001). Common species known from grapevines include *B. dothidea* (Moug. : Fr.) Ces. & De Not., *B. parva* Pennycook & Samuels, *B. obtusa* (Schwein.) Shoemaker, *B. stevensii* Shoemaker, *B. lutea* A.J.L. Phillips and *B. ribis* Grossenb. & Duggar (Pascoe 1998, Phillips 2002).

In spite of the range of symptoms associated with species of *Botryosphaeria,* field diagnosis of the causal organism is difficult because symptoms often resemble those of other diseases such as Phomopsis cane and leaf spot, caused by *Phomopsis viticola* (Sacc.) Sacc., or Eutypa dieback, caused by *Eutypa lata* (Pers.) Tul. & C. Tul. (Castillo-Pando et al 2001). Accurate identification of the causal species is difficult

Accepted for publication December 23, 2003.

[1] Corresponding author, extraordinary professor at the Department of Plant Pathology, University of Stellenbosch, South Africa. E-mail: crous@cbs.knaw.nl

TABLE I. Isolates subjected to DNA sequence analyses

Accession no.[1]	Species	Host	Country	Collector	GenBank No. ITS	GenBank No. EF-1α
STE-U 4599	*Botryosphaeria australis*	*Vitis vinifera*	R.S.A.	F. Halleen	AY 343408	
STE-U 4428	*B. australis*	*V. vinifera*	R.S.A.	F. Halleen	AY 343391	
STE-U 4596	*B. australis*	*V. vinifera*	R.S.A.	F. Halleen	AY 343405	
STE-U 4585	*B. australis*	*V. vinifera*	R.S.A.	F. Halleen	AY 343402	
STE-U 4590	*B. australis*	*Robinia pseudoacacia*	Portugal	A.J.L. Phillips	AY 343403	
STE-U 4582	*B. australis*	*V. vinifera*	R.S.A.	F. Halleen	AY 343401	
STE-U 4591	*B. australis*	*V. vinifera*	R.S.A.	F. Halleen	AY 343404	
STE-U 4434	*B. australis*	*V. vinifera*	R.S.A.	F. Halleen	AY 343396	
STE-U 4433	*B. australis*	*V. vinifera*	R.S.A.	F. Halleen	AY 343395	
STE-U 4430	*B. australis*	*V. vinifera*	R.S.A.	F. Halleen	AY 343393	
STE-U 4529	*B. australis*	*V. vinifera*	R.S.A.	F. Halleen	AY 343399	
STE-U 4416	*B. australis*	*V. vinifera*	R.S.A.	F. Halleen	AY 343386	
STE-U 4429	*B. australis*	*V. vinifera*	R.S.A.	F. Halleen	AY 343392	
STE-U 4528	*B. australis*	*V. vinifera*	R.S.A.	F. Halleen	AY 343398	
STE-U 4427	*B. australis*	*V. vinifera*	R.S.A.	F. Halleen	AY 343390	
STE-U 4435	*B. australis*	*V. vinifera*	R.S.A.	F. Halleen	AY 343397	
STE-U 4432	*B. australis*	*V. vinifera*	R.S.A.	F. Halleen	AY 343394	
STE-U 4415 CBS 112877	*B. australis*	*V. vinifera*	R.S.A.	F. Halleen	AY 343385	AY 343346
STE-U 4426	*B. australis*	*V. vinifera*	R.S.A.	F. Halleen	AY 343389	
STE-U 4425 CBS 112872	*B. australis*	*V. vinifera*	R.S.A.	F. Halleen	AY 343388	AY 343347
STE-U 5054	*B. australis*	*V. vinifera*	R.S.A.	F. Halleen	AY 343412	
STE-U 4535	*B. australis*	*V. vinifera*	R.S.A.	J.M. van Niekerk	AY 343400	
STE-U 5040	*B. australis*	*V. vinifera*	R.S.A.	F. Halleen	AY 343411	
STE-U 5033	*B. australis*	*V. vinifera*	R.S.A.	F. Halleen	AY 343409	
STE-U 5039	*B. australis*	*V. vinifera*	R.S.A.	F. Halleen	AY 343410	
STE-U 4597	*B. australis*	*V. vinifera*	R.S.A.	F. Halleen	AY 343406	
STE-U 4418	*B. australis*	*V. vinifera*	R.S.A.	F. Halleen	AY 343387	
STE-U 4598 CBS 110864	*B. australis*	*V. vinifera*	R.S.A.	F. Halleen	AY 343407	AY 343348
STE-U 5149 Cap 007	*B. dothidea*	*V. vinifera*	Portugal	A.J.L. Phillips	AY 343415	
STE-U 5045 CBS 110484	*B. dothidea*	*V. vinifera*	Argentina	G. Marta	AY 343414	AY 343350
STE-U 4595 Cap 086 CBS 110487[2]	*B. dothidea* (= *B. populi*)	*Populus nigra*	Portugal	A.J.L. Phillips	AY 343413	AY 343349
STE-U 4593 Cap 002[2]	*B. lutea*	*V. vinifera*	Portugal	A.J.L. Phillips	AY 343417	AY 343352

TABLE I. Continued

Accession no.[1]	Species	Host	Country	Collector	GenBank No. ITS	GenBank No. EF-1α
STE-U 4594 Cap 037	*B. lutea*	*V. vinifera*	Portugal	A.J.L. Phillips	AY 343418	
STE-U 4592 Cap 058 CBS 110862	*B. lutea*	*Sophora japonica*	Portugal	A.J.L. Phillips	AY 343416	AY 343351
STE-U 4542 CBS 112876	*B. obtusa*	*V. vinifera*	R.S.A.	F. Halleen	AY 343434	AY 343353
STE-U 4539	*B. obtusa*	*V. vinifera*	R.S.A.	F. Halleen	AY 343432	
STE-U 4541	*B. obtusa*	*V. vinifera*	R.S.A.	F. Halleen	AY 343433	
STE-U 4436	*B. obtusa*	*V. vinifera*	R.S.A.	F. Halleen	AY 343419	
STE-U 4531	*B. obtusa*	*V. vinifera*	R.S.A.	F. Halleen	AY 343426	
STE-U 5037	*B. obtusa*	*V. vinifera*	Portugal	A.J.L. Phillips	AY 343446	
STE-U 5043	*B. obtusa*	*V. vinifera*	R.S.A.	F. Halleen	AY 343448	
STE-U 5034 CBS 112978	*B. obtusa*	*V. vinifera*	R.S.A.	F. Halleen	AY 343445	AY 343354
STE-U 5031	*B. obtusa*	*V. vinifera*	R.S.A.	F. Halleen	AY 343443	
STE-U 5042	*B. obtusa*	*V. vinifera*	R.S.A.	F. Halleen	AY 343447	
STE-U 5032	*B. obtusa*	*V. vinifera*	R.S.A.	F. Halleen	AY 343444	
STE-U 4538	*B. obtusa*	*V. vinifera*	R.S.A.	F. Halleen	AY 343431	
STE-U 4537	*B. obtusa*	*V. vinifera*	R.S.A.	F. Halleen	AY 343430	
STE-U 4581	*B. obtusa*	*V. vinifera*	France	P. Larignon	AY 343439	
STE-U 4587	*B. obtusa*	*V. vinifera*	France	P. Larignon	AY 343441	
STE-U 5162	*B. obtusa*	*V. vinifera*	R.S.A.	J.M. van Niekerk	AY 343458	
STE-U 5143	*B. obtusa*	*V. vinifera*	R.S.A.	J.M. van Niekerk	AY 343455	
STE-U 4532	*B. obtusa*	*V. vinifera*	R.S.A.	F. Halleen	AY 343427	
STE-U 4527	*B. obtusa*	*V. vinifera*	R.S.A.	F. Halleen	AY 343425	
STE-U 5147	*B. obtusa*	*V. vinifera*	R.S.A.	F. Halleen	AY 343456	
STE-U 4440	*B. obtusa*	*V. vinifera*	R.S.A.	F. Halleen	AY 343420	
STE-U 4442	*B. obtusa*	*V. vinifera*	R.S.A.	F. Halleen	AY 343422	
STE-U 4443	*B. obtusa*	*V. vinifera*	R.S.A.	F. Halleen	AY 343423	
STE-U 4545	*B. obtusa*	*V. vinifera*	R.S.A.	F. Halleen	AY 343437	
STE-U 4543	*B. obtusa*	*V. vinifera*	R.S.A.	F. Halleen	AY 343435	
STE-U 5052	*B. obtusa*	*V. vinifera*	R.S.A.	J.M. van Niekerk	AY 343449	
STE-U 5053	*B. obtusa*	*V. vinifera*	R.S.A.	J.M. van Niekerk	AY 343450	
STE-U 5161	*B. obtusa*	*V. vinifera*	R.S.A.	J.M. van Niekerk	AY 343457	
STE-U 5163	*B. obtusa*	*V. vinifera*	R.S.A.	J.M. van Niekerk	AY 343459	
STE-U 4441	*B. obtusa*	*V. vinifera*	R.S.A.	F. Halleen	AY 343421	
STE-U 4544	*B. obtusa*	*V. vinifera*	R.S.A.	F. Halleen	AY 343436	
STE-U 4444	*B. obtusa*	*V. vinifera*	R.S.A.	F. Halleen	AY 343424	
STE-U 4536	*B. obtusa*	*V. vinifera*	R.S.A.	F. Halleen	AY 343429	
STE-U 4546	*B. obtusa*	*V. vinifera*	R.S.A.	F. Halleen	AY 343438	

TABLE I. Continued

Accession no.[1]	Species	Host	Country	Collector	GenBank No. ITS	GenBank No. EF-1α
STE-U 5139	*B. obtusa*	*V. vinifera*	R.S.A.	J.M. van Niekerk	AY 343452	
STE-U 5140	*B. obtusa*	*V. vinifera*	R.S.A.	J.M. van Niekerk	AY 343453	
STE-U 5141	*B. obtusa*	*V. vinifera*	R.S.A.	J.M. van Niekerk	AY 343454	
STE-U 5129	*B. obtusa*	*V. vinifera*	R.S.A.	J.M. van Niekerk	AY 343451	
STE-U 5164	*B. obtusa*	*V. vinifera*	R.S.A.	J.M. van Niekerk	AY 343460	
STE-U 4586	*B. obtusa*	*V. vinifera*	R.S.A.	F. Halleen	AY 343440	
STE-U 4588	*B. obtusa*	*V. vinifera*	R.S.A.	F. Halleen	AY 343442	
STE-U 4533	*B. obtusa*	*V. vinifera*	R.S.A.	F. Halleen	AY 343428	
STE-U 4437 CBS 112931	*B. parva*	*V. vinifera*	R.S.A.	F. Halleen	AY 343466	AY 343358
STE-U 4439 CBS 113032	*B. parva*	*V. vinifera*	R.S.A.	F. Halleen	AY 343468	AY 343360
STE-U 4438 CBS 112930	*B. parva*	*V. vinifera*	R.S.A.	F. Halleen	AY 343467	AY 343359
STE-U 5253 CBS 110888	*B. parva*	*V. vinifera*	Portugal	A.J.L. Phillips	AY 343477	AY 343367
STE-U 4341	*B. parva*	*V. vinifera*	R.S.A.	F. Halleen	AY 343464	
STE-U 4530 CBS 110852	*B. parva*	*V. vinifera*	R.S.A.	F. Halleen	AY 343469	AY 343361
STE-U 5049 CBS 112879	*B. parva*	*V. vinifera*	R.S.A.	F. Halleen	AY 343474	AY 343365
STE-U 4534	*B. parva*	*V. vinifera*	R.S.A.	F. Halleen	AY 343465	
STE-U 4417 CBS 112871	*B. parva*	*V. vinifera*	R.S.A.	F. Halleen	AY 343461	AY 343355
STE-U 4420 CBS 110867	*B. parva*	*V. vinifera*	R.S.A.	F. Halleen	AY 343462	AY 343356
STE-U 5130	*B. parva*	*V. vinifera*	R.S.A.	J.M. van Niekerk	AY 343475	
STE-U 5142 CBS 110871	*B. parva*	*V. vinifera*	R.S.A.	J.M. van Niekerk	AY 343476	AY 343366
STE-U 4424 CBS 112932	*B. parva*	*V. vinifera*	R.S.A.	F. Halleen	AY 343463	AY 343357
STE-U 5035	*B. parva*	*V. vinifera*	Portugal	A.J.L. Phillips	AY 343473	
STE-U 4584 CBS 110952	*B. parva*	*V. vinifera*	France	P. Larignon	AY 343471	AY 343363
CMW 9077	*B. parva*	*Actinidia deliciosa*	New Zealand	S.R. Pennycook	AY 236939	AY 236884
CMW 9078	*B. parva*	*A. deliciosa*	New Zealand	S.R. Pennycook	AY 236940	AY 236885
CMW 9079	*B. parva*	*A. deliciosa*	New Zealand	S.R. Pennycook	AY 236941	AY 236886
CMW 9080	*B. parva*	*Populus nigra*	New Zealand	G.J. Samuels	AY 236942	AY 236887
STE-U 4540 CBS 110860	*B. parva*	*V. vinifera*	R.S.A.	F. Halleen	AY 343470	AY 343362

TABLE I. Continued

Accession no.[1]	Species	Host	Country	Collector	GenBank No. ITS	GenBank No. EF-1α
STE-U 4589 CBS 113050	*B. parva*	*V. vinifera*	R.S.A.	F. Halleen	AY 343472	AY 343364
STE-U 5051 CBS 110495	*B. rhodina*	*V. vinifera*	Argentina	M. Gatica	AY 343483	AY 343369
STE-U 4583	*B. rhodina*	*V. vinifera*	R.S.A.	F. Halleen	AY 343482	
STE-U 4423	*B. rhodina*	*V. vinifera*	R.S.A.	F. Halleen	AY 343481	
STE-U 4421	*B. rhodina*	*V. vinifera*	R.S.A.	F. Halleen	AY 343479	
STE-U 4422	*B. rhodina*	*V. vinifera*	R.S.A.	F. Halleen	AY 343480	
STE-U 4419 CBS 112874	*B. rhodina*	*V. vinifera*	R.S.A.	F. Halleen	AY 343478	AY 343368
CMW 7772	*B. ribis*	*Ribes* sp.	U.S.A.	B. Slippers & G. Hudler	AY 236935	AY 236877
CMW7773	*B. ribis*	*Ribes* sp.	U.S.A.	B. Slippers & G. Hudler	AY 236936	AY236878
STE-U 5038 CBS 112875	*B. stevensii*	*V. vinifera*	Portugal	A.J.L. Phillips	AY 343484	AY 343370
STE-U 5046 CBS 110574	*Diplodia porosum*	*V. vinifera*	R.S.A.	J.M. van Niekerk	AY 343378	AY 343339
STE-U 5132 CBS 110496[2]	*D. porosum*	*V. vinifera*	R.S.A.	J.M. van Niekerk	AY 343379	AY 343340
CBS 120.41	*D. sarmentorum*	*Prunus communis*	Norway	H.W. Wollenweber	AY 343377	AY 343338
STE-U 5131	*Diplodia* sp.	*V. vinifera*	R.S.A.	J.M. van Niekerk	AY 343374	
STE-U 5048 CBS 112869	*Diplodia* sp.	*V. vinifera*	R.S.A.	J.M. van Niekerk	AY 343373	AY 343336
STE-U 5148 CBS 112870	*Diplodia* sp.	*V. vinifera*	R.S.A.	J.M. van Niekerk	AY 343376	AY 343337
STE-U 5146	*Diplodia* sp.	*V. vinifera*	R.S.A.	J.M. van Niekerk	AY 343375	
STE-U 5041 CBS 112977	*Fusicoccum viticlavatum*	*V. vinifera*	R.S.A.	F. Halleen	AY 343380	AY 343341
STE-U 5044 CBS 112878[2]	*F. viticlavatum*	*V. vinifera*	R.S.A.	F. Halleen	AY 343381	AY 343342
STE-U 5252 CBS 110887[2]	*F. vitifusiforme*	*V. vinifera*	R.S.A.	J.M. van Niekerk	AY 343383	AY 343343
STE-U 5050 CBS 110880	*F. vitifusiforme*	*V. vinifera*	R.S.A.	J.M. van Niekerk	AY 343382	AY 343344
IMI 063581b CBS 113091	*Otthia spiraeae*	*Ulmus* sp.	U.K.	A. Sivanesan	AY 343384	AY 343345

[1] Acronyms of culture collections: STE-U—Department of Plant Pathology, University of Stellenbosch, South Africa; Cap—A.J.L. Phillips, Universidade Nova de Lisboa, Portugal; CMW—M.J. Wingfield, FABI, University of Pretoria, South Africa; IMI—CABI Bioscience, Egham, U.K.; CBS—Centraalbureau voor Schimmelcultures, Utrecht, Netherlands.

[2] Ex-type cultures.

because *Botryosphaeria* teleomorphs are encountered rarely in nature (Shoemaker 1964, Jacobs and Rehner 1998) and teleomorphs rarely form in culture. The diversity among these teleomorphs is insufficient to allow clear differentiation at species level (Shoemaker 1964, Laundon 1973). Thus, the taxonomy and identification of *Botryosphaeria* species is based mainly on the anamorphic characters (Denman et al 2000, Phillips 2002), which frequently are combined with molecular data (Jacobs and Rehner 1998; Denman et al 2003; Phillips et al 2002; Slippers et al 2004a, b). The diversity of anamorph states of *Botryosphaeria* have added to the taxonomic confusion. Seven anamorph genera have been applied to asexual states of species of *Botryosphaeria*. Recent research suggests that anamorphs of *Botryosphaeria* belong to either *Fusicoccum* Corda (hyaline, thin-walled conidia), or *Diplodia* Fr. (pigmented, thick-walled conidia) (Pennycook and Samuels 1985, Crous and Palm 1999, Denman et al 2000, Zhou and Stanosz 2001, Phillips 2002).

A major problem facing the grapevine industry remains the correct identification of the *Botryosphaeria* species causing disease on vines from different cultivars, localities and countries. Species occurring on grapevines in different countries have been shown to differ in pathogenicity; this has led to confusion and conflicting reports about which species of *Botryosphaeria* are important pathogens of grapevines (Phillips 2002). These species differ in their epidemiology, the disease symptoms they cause, their relative importance and the control strategies that should be followed to combat the various diseases.

In South Africa, several species of *Botryosphaeria* have been reported as pathogens of grapevines, including *B. obtusa, B. dothidea* and *B. ribis* (Crous et al 2000), as well as *B. vitis* (Schulzer) Sacc. (Doidge 1950). *Botryosphaeria* is regarded as an important pathogen of grapevines in South Africa and is frequently isolated from grapevines with canker and dieback symptoms (Fourie and Halleen 2001). The identity of the various causal species, however, as well as their relative importance, remains unknown. The aims of this study were to use molecular methods and morphological characteristics to compare South African *Botryosphaeria* isolates with those associated with grapevine diseases elsewhere and to determine which species should be regarded as potentially important pathogens of this host.

MATERIALS AND METHODS

Isolates.—Isolations were made routinely for the past 5 yr from symptomatic material of diseased grapevines (TABLE I). Some isolates also were obtained from young asymptomatic nursery plants (shoots). Plant tissue was surface-sterilized by placing in 70% ethanol for 30 s, 1% NaOCl for 1 min and again in 70% ethanol for 30 s before drying under a laminar-flow hood. Small pieces of tissue were taken from the margin between necrotic and apparently healthy tissue and plated onto 2% potato dextrose agar (PDA; Biolab, Midrand, South Africa). Hyphae growing out from the tissue pieces were subcultured onto fresh PDA plates, incubated, and hyphal-tipped to obtain pure cultures. To enhance sporulation, isolates were plated out on water agar (WA; Biolab) plates, to which 3 cm pieces of double-autoclaved pine needles were added. The plates were incubated at 25 C under near-ultraviolet light in a 12 h light–dark regime for 2–3 wk. The 95% confidence intervals of conidial dimensions were derived from at least 30 observations at 1000× magnification. Growth rates, cultural characteristics and cardinal temperatures for growth were determined for isolates plated onto PDA in 90 mm diam Petri dishes and incubated in the dark 7 d at seven temperatures, 5–35 C in 5-degree intervals. Three plates were used for each isolate at each temperature, and the experiment was repeated once. Radial mycelial growth was measured perpendicularly for each plate and the mean calculated to determine the growth rates for each species. Colony colors were described from isolates incubated at 25 C under near-ultraviolet light for 7 d, according to Rayner (1970). Cultures are maintained in the culture collection of the Department of Plant Pathology, University of Stellenbosch (STE-U) and at the Centraalbureau voor Schimmelcultures in Utrecht, the Netherlands (CBS) (TABLE I).

Sequence comparisons.—A total of 122 *Botryosphaeria* isolates were used for ITS sequence analysis (TABLE I), their phylogeny determined (results not given) and a subset of 39 chosen for analysis of the translation elongation factor 1-α (EF1-α) gene. The isolation protocol of Lee and Taylor (1990) was used to extract genomic DNA from fungal mycelia grown on PDA. The primers ITS1 and ITS4 were used to amplify part of the nuclear rRNA operon using the PCR conditions recommended by the authors (White et al 1990). The primers EF1–728F and EF1–986R (Carbone and Kohn 1999) were used to amplify part of the EF1-α gene. PCR conditions were the same for this region, except for the $MgCl_2$ concentration, which was increased to 4.0 mM. PCR products were separated by electrophoresis at 80 V for 1 h in a 0.8% (w/v) agarose gel in 0.5× TAE running buffer (0.4 M Tris, 0.05 M NaAc, and 0.01 M EDTA, pH 7.85) and visualized under UV light using a GeneGenius Gel Documentation and Analysis System (Syngene, Cambridge, United Kingdom) after ethidium bromide staining.

The amplification products were purified with a NucleoSpin® Extract 2 in 1 kit (Macherey-Nagel, Germany). The purified products were sequenced in both directions using the PCR primers and the cycle sequencing reaction was carried out as recommended by the manufacturer with an ABI Prism Big Dye Terminator version 3.0 Cycle Sequencing Ready Reaction Kit (PE Biosystems, Foster City, California) containing AmpliTaq DNA Polymerase. The resulting fragments were analyzed on an ABI Prism 3100 DNA Sequencer (Perkin-Elmer, Norwalk, Connecticut).

TABLE II. Mean lesion length caused by in vitro inoculations with isolates of *Botryosphaeria* species on green shoots of the grapevine cultivar 'Periquita'

Treatment	Mean lesion length (mm)[a]
Botrysophaeria australis (STE-U 4416, 4598, 5040)	65.29 a
B. parva (STE-U 5142, 4589, 4420)	57.30 a
B. rhodina (STE-U 5051, 4422, 4421)	17.22 b
Fusicoccum vitifusiforme (STE-U 5050, 5252)	10.74 b
F. viticlavatum (STE-U 5044, 5041)	10.36 b
B. obtusa (STE-U 4444, 4440, 5139)	7.97 b
Diplodia sp. (STE-U 5148, 5048, 5131)	4.86 b
D. porosum (STE-U 5046, 5132)	4.30 b
Agar plug	3.38 b
LSD ($P = 0.05$)	16.95

[a] Means followed by the same letter are not significantly different.

TABLE III. Mean lesion length caused by isolates of *Botryosphaeria* species following in vitro inoculations on mature canes of grapevine cultivars 'Chardonnay' and 'Cabernet Sauvignon'

Treatment	Mean lesion length (mm)[a]
Botryosphaeria ribis (CMW 7773)	26.75 a
B. australis (STE-U 4598, 4425, 4415)	18.17 b
B. stevensii (STE-U 5038)	17.56 b
B. parva (STE-U 4589, 4420, 5253)	11.25 c
Fusicoccum vitifusiforme (STE-U 5252)	7.69 d
Diplodia sp. (STE-U 5048)	7.31 d
B. dothidea (STE-U 5045)	7.13 d
B. obtusa (STE-U 5034)	7.13 d
F. viticlavatum (STE-U 5044)	7.10 d
D. porosum (STE-U 5046)	6.38 de
B. lutea (STE-U 4593)	6.31 de
B. rhodina (STE-U 5051)	5.75 de
Agar plug	3.81 e
No treatment	3.81 e
LD ($P = 0.05$)	2.922

[a] Means followed by the same letter are not significantly different.

The ITS and EF1-α sequences were assembled and added to outgroup sequences, *Cercospora beticola* Sacc. (STE-U 5073) and *Cercospora penzigii* Sacc. (STE-U 4001), using Sequence Alignment Editor version 2.0a11 (Rambaut 2002) and manual adjustments for improvement were made by eye where necessary. The phylogenetic analyses of sequence data were done using PAUP (Phylogenetic Analysis Using Parsimony) version 4.0b10 (Swofford 2000). Alignment gaps were treated as a fifth character state, and all characters were unordered and of equal weight. Maximum-parsimony analysis was performed for all datasets using the heuristic search option with 100 random taxa additions and tree bisection and reconstruction (TBR) as the branch-swapping algorithm. Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. The robustness of the most-parsimonious trees was evaluated by 1000 bootstrap replications (Hillis and Bull 1993). Other measures including tree length, consistency index, retention index and rescaled consistency index (CI, RI and RC) also were calculated. The resulting trees were printed with TreeView version 1.6.6 (Page 1996). A partition homogeneity test was conducted in PAUP (Swofford 2000) to examine the possibility of a joint analysis of the ITS and EF1-α datasets.

Pathogenicity.—In vitro screening on green shoots. A total of 21 isolates, representing eight different species of *Botryosphaeria* (TABLES II, III), were selected for pathogenicity screening. Isolates were plated on PDA and incubated at 25 C for 1 wk. Inoculations were made on green shoots of the grapevine cultivar Periquita. Shoots were cut from vines ca 2 mo after budburst, and internodes 4–6 were used for inoculations. A total of 12 shoots were used for each species. Shoots were wounded on internode five (2 mm deep) with a 4 mm cork borer. A colonized agar plug, cut from a 1 wk old culture was placed in the wound and covered with Parafilm. Inoculated shoots were incubated in the dark under moist conditions in the laboratory for 10 d at 23 C. After the incubation period, the shoots were split longitudinally through the wound and the internal lesions measured. The layout of the trial was a randomized design, and the data were statistically analyzed using SAS (SAS 1999). An analysis of variance and Student's t-tests for least significant differences were done. After the experiment, all plant material was destroyed by autoclaving the plants twice for 30 min.

In vitro screening on mature canes. A set of 16 *Botryosphaeria* isolates were selected to use in this experiment (TABLE IV). Three isolates, however, were selected for *B. australis* and *B. parva* because these species most commonly were isolated from diseased vines. Inoculations were done using mature canes of two grapevine cultivars, Cabernet Sauvignon and Chardonnay. Four canes were inoculated per cultivar for each isolate. Canes used for inoculations were cut from vines ca 2 mo after harvest. Canes were wounded on internode five (2 mm deep) with a 4 mm cork borer and inoculated as described above. Inoculated canes were incubated in the dark under moist conditions in the laboratory for 3 wk under strict quarantine conditions. Afterward, the canes were assessed and the data analyzed as described above.

In vivo pathogenicity on mature vines. For this trial the same set of 21 isolates was used as in the in vitro green shoots trial (TABLE III). The trial layout and number of replicates per species were also the same. Inoculations were made in a vineyard on 15 yr old grapevine plants of the cultivar Periquita. Inoculations were made on mature canes in the same manner as with the green shoots trial, but the canes were left attached to the plant. Inoculations also were made in mature wood by drilling a hole 4 mm wide and 1.5 cm deep into the arms of the vines. A colonized agar

TABLE IV. Mean lesion lengths in mature canes and mature wood (rotting and streaking) of grapevine cultivar 'Periquita', caused by in vivo inoculations with isolates of *Botryosphaeria* species

Treatment	Lesion length (mm)[a]		
		Mature wood	
	Mature canes	Rotting	Streaking
Botryosphaeria australis (STE-U 4416, 4598, 5040)	12.89 ab	25.90 a	48.85 a
Fusicoccum viticlavatum (STE-U 5044, 5041)	7.32 c	12.13 bc	36.60 b
F. vitifusiforme (STE-U 5050, 5252)	7.73 c	11.35 bcd	32.72 b
B. obtusa (STE-U 4444, 4440, 5139)	8.08 c	13.54 b	29.44 bcd
B. parva (STE-U 5142, 4589, 4420)	15.01 a	10.53 bcd	24.71 cd
Diplodia sp. (STE-U 5148, 5048, 5131)	7.18 c	6.80 d	24.25 cd
B. rhodina (STE-U 5051, 4422, 4421)	11.73 b	12.64 bc	22.67 d
D. porosum (STE-U 5046, 5132)	13.22 ab	7.96 cd	22.01 d
Agar plug	4.18 d	6.72 d	6.72 e
LSD ($P = 0.05$)	2.99	4.99	8.96

[a] Means followed by the same letter are not significantly different.

plug, cut from a 1 wk old culture, was placed in the wound. The wound was sealed with petroleum jelly and covered with Parafilm. After 6 mo, the inoculated arms and mature canes were collected. The canes were assessed for disease severity and the data analyzed as described above. Re-isolations were made from the leading edges of lesions and the cultures identified by inducing sporulation in the same manner as for the morphological descriptions to satisfy Koch's postulates. All plant material was destroyed by autoclaving twice for 30 min.

RESULTS

Phylogenetic analysis.—Approximately 550 and 300 bases were determined for the ITS region and EF 1-α gene, respectively, of the isolates (TABLE I). The manually adjusted alignment contained 41 isolates with 529 characters for the ITS region, and 357 characters for EF 1-α, including alignment gaps (data not shown). New ITS and EF1-α sequences were deposited in GenBank (TABLE I) and the alignments in TreeBASE (SN 1533). The result of the partition homogeneity test ($P = 0.22$, where $P \geq 0.05$) was significantly incongruent, indicating that the ITS and EF1-α datasets could be combined.

The combined dataset contained 886 characters, of which 498 were parsimony informative, 11 were variable and parsimony uninformative and 377 were constant. Maximum-parsimony analysis of the combined sequence data resulted in a single most-parsimonious tree (FIG. 1). The first clade (100% bootstrap support) contained two *B. rhodina* isolates (STE-U 5051, 4419). The second clade (100% bootstrap support) contained a *B. stevensii* isolate (STE-U 5038) isolated from grapevines in Portugal, which grouped separately from two isolates of *B. obtusa* (STE-U 5034, 4542), which formed a well-supported clade (100% bootstrap support). Two isolates of *Diplodia porosum* (STE-U 5046, 5132) isolated from grapevine pruning debris, again grouped separately (100% bootstrap support). The next clade (STE-U 5148, 5048) contained 2 isolates of a *Diplodia* sp. (100% bootstrap support), which also was obtained from pruning debris and clustered close to (100% bootstrap support) *Otthia spiraeae* (Fuckel) Fuckel (IMI 63 581) and its anamorph, *Diplodia sarmentorum* (Fr.) Fr. (CBS 120.41) (100% bootstrap support). *Botryosphaeria dothidea* was represented by one grapevine isolate from Portugal (STE-U 4595) and another from Argentina (STE-U 5045). However, no isolates of *B. dothidea* were isolated from vines in South Africa.

Two new *Fusicoccum* species isolated from grapevines, namely *F. viticlavatum* (STE-U 5041, 5044) and *F. vitifusiforme* (STE-U 5252, 5050), each clustered with 100% bootstrap support. Isolates of *B. lutea,* including the ex-type strain (STE-U 4593), also clustered with 100% bootstrap support, adjacent to *B. australis* Slippers, Crous & M.J. Wingf. (72% bootstrap support) (Slippers et al 2004b).

Isolates of *B. ribis* and *B. parva* could be separated based on their EF1-α data. The *B. ribis* clade was supported by a bootstrap value of 100% and the *B. parva* clade with 62%. The South African isolates from grapevines all fell into the *B. parva* clade. No isolates of *B. ribis* were isolated from grapevines in South Africa.

TAXONOMY

Botryosphaeria vitis (Schulzer) Sacc., Syll. Fung. 1: 463. 1882, homonym of *B. vitis* Niessl, in Beitr.:48. 1871.

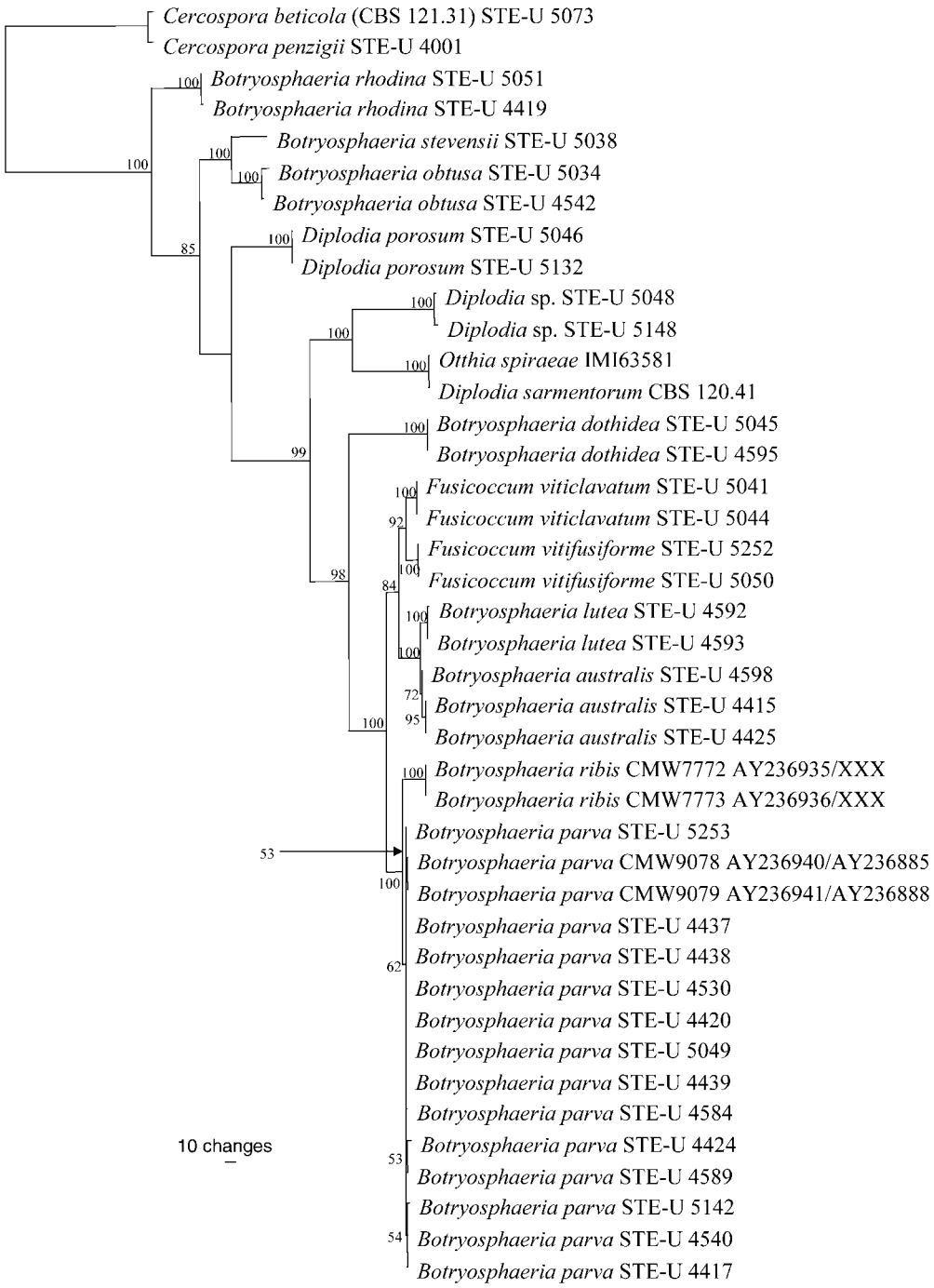


FIG. 1. Single most-parsimonious tree obtained from combined ITS and EF1-α sequence data. (PHT = 0.220; TL = 1070 steps, CI = 0.742, RI = 0.888, RC = 0.659). Bootstrap support values from 1000 replicates are shown at the nodes. The tree was rooted to *Cercospora beticola* and *Cercospora penzigii*. The bar represents 10 changes.

Basionym: *Gibbera vitis* Schulzer, in Verh. Zool.-Bot. Ges Wien 20:642. 1872.

Specimens examined. SOUTH AFRICA. WESTERN CAPE: Stellenbosch, Banhoek, on canes of *Vitis vinifera* L., *Buller,* PREM 46 581.

Notes. Botryosphaeria vitis Niessl has 1-septate, oblong ascospores, 14–16 × 4–5 μm, and subsequently was placed in *Lisea* Sacc. (= *Nectria fide* Rossman et al 1999) as *L. vitis* (Niessl) Sacc. (Michelia 1:43. 1879). The name proposed by Saccardo as *B. vitis* (Schulzer) Sacc. (1882) is thus illegitimate, and cannot be used. Nevertheless, Doidge (1950) reported *B. vitis* (Schulzer) Sacc. as occurring on grapevines in the Stellenbosch region of South Africa. Saccardo

(1882) cited this species as having ovate ascospores, 26–27 × 10–13 μm. On examination of the South African material (PREM 46581), ascospores were found to be 16–25 × 6–10 μm, thus significantly smaller than those originally reported for *B. vitis*. Furthermore, conidia were hyaline, fusoid–ellipsoidal, (17–)19–22(–23) × (5–)6–6.5(–7) μm (l : w ratio 3.3), thus closely resembling the *B. lutea-B. australis* Slippers, Crous & M.J. Wingf. complex, both of which are shown to occur on vines. Slippers et al (2004b) distinguished these two species by *B. australis* (l : w = 4.8) having conidia with a higher length : width ratio than *B. lutea* (l : w = 3.3), which suggests that the South African grapevine specimen is best accommodated in *B. lutea*.

Diplodia porosum Niekerk & Crous, sp. nov.

FIGS. 2–8

Pycnidia solitaria, globosa vel obpyriformia, ad 400 μm diam. Cellulae conidiogenae holoblasticae, hyalinae, subcylindricae vel ampulliformes, 6–10 × 5–7 μm. Conidia hyalina, guttulata, ovoidea vel late ellipsoidea, sursum hebete rotundata, ad basim raro truncata; pariete 2 μm crasso, multis poris 1 μm latis perforata, deinde brunnescentia, (38–)42–45(–47) × (20–)22–25(–30) μm in vitro (long: latitudo = 1.9).

Pycnidia solitary, unilocular, ostiolate, globose to obpyriform, up to 400 μm wide; pycnidial wall 4–8 cell layers thick, of dark brown textura angularis, becoming hyaline toward inner region. Conidiophores reduced to conidiogenous cells. Conidiogenous cells lining cavity, holoblastic, hyaline, subcylindrical to ampulliform, 6–10 × 5–7 μm, rarely proliferating percurrently. Conidia hyaline, guttulate, ovoid to broadly ellipsoid with a bluntly rounded apex, and flattened base; wall 2 μm thick, with pores 1 μm wide; becoming medium brown with age, (38–)42–45(–47) × (20–)22–25(–30) μm in vitro (l : w = 1.9). Colonies flat with undulating margins, dark green (27''i) on the surface and dull green (27''m) underneath, reaching a radius of 32 mm after 3 d at 25 C. Cardinal temperature requirements for growth: min. 10 C, max. 30 C, opt. 25 C.

Holotype. SOUTH AFRICA. WESTERN CAPE PROVINCE: Stellenbosch, on *Vitis vinifera,* 2002, *J.M. van Niekerk,* herb. CBS 7754, culture ex-type STE-U 5132, CBS 110 496.

Host. Vitis vinifera.

Known distribution. South Africa (Western Cape Province).

Notes. This species is unique within the genus *Diplodia* because of its large, thick-walled conidia with large pores (1 μm wide) and are clearly visible by light microscopy (FIGS. 7, 8). Conidia are initially hyaline but become pigmented while still in the pycnidial locule.

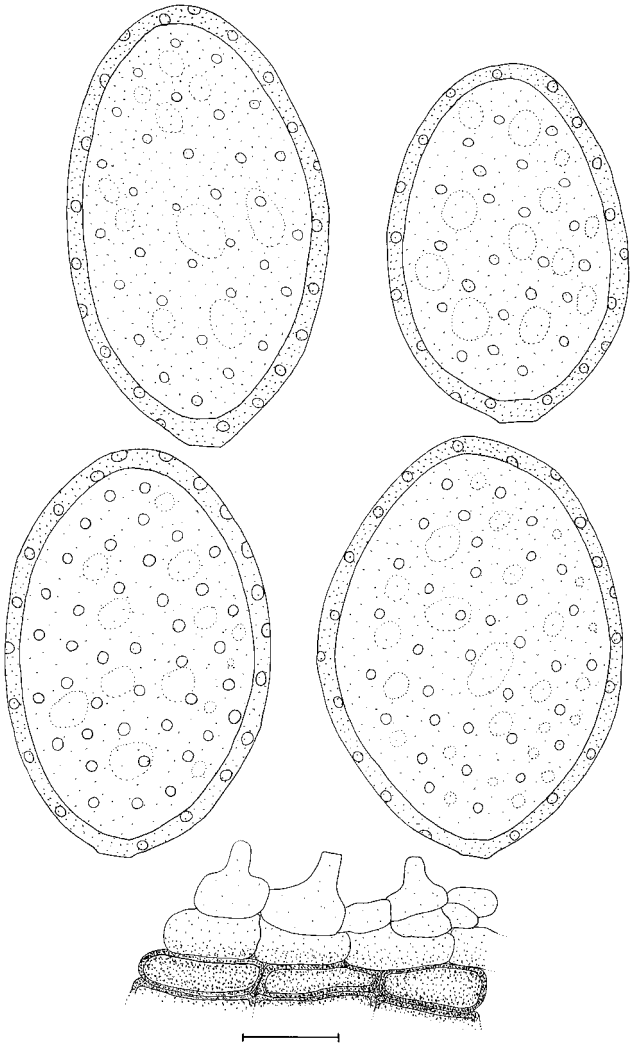


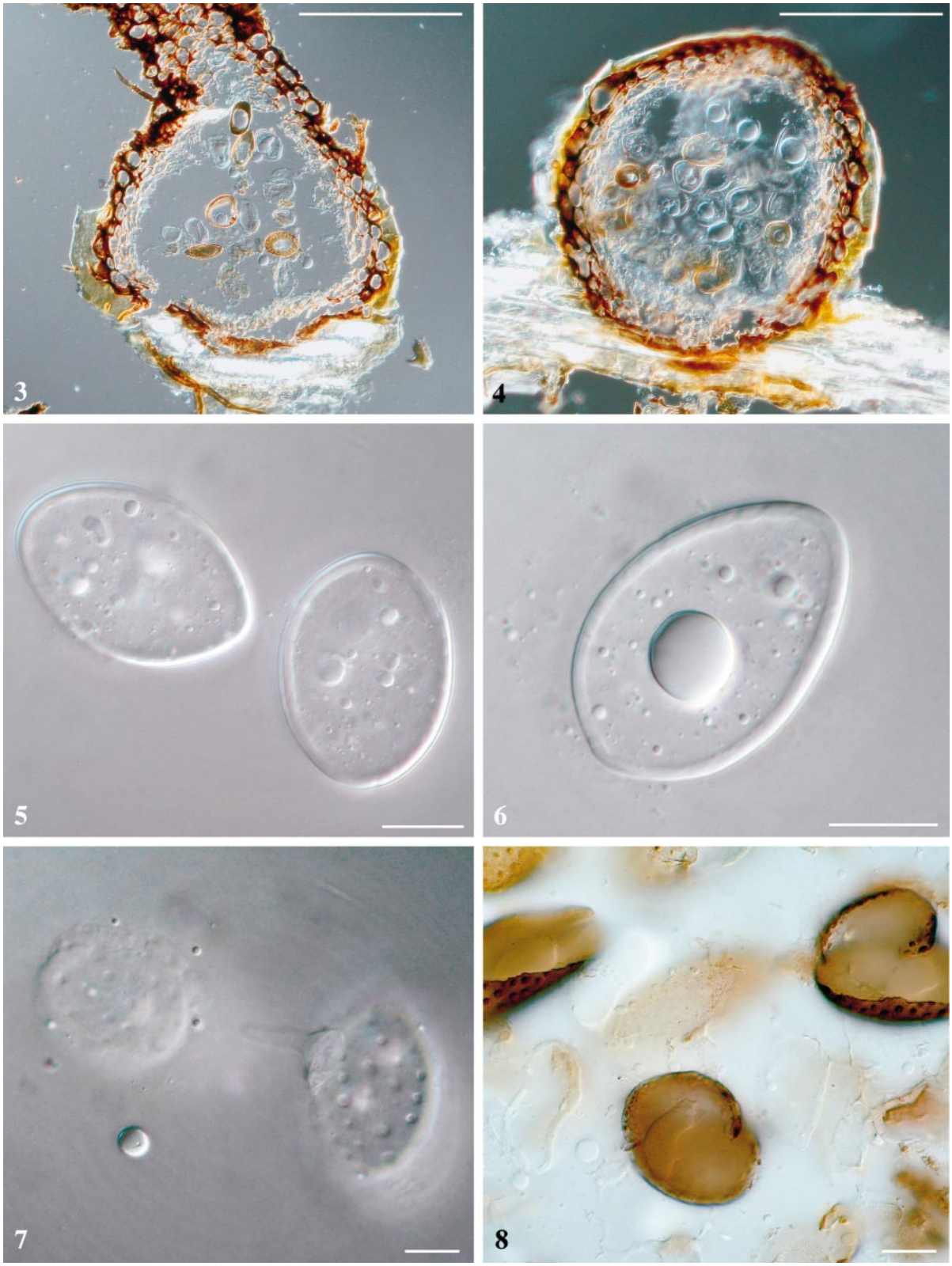
FIG. 2. Conidia and conidiogenous cells of *Diplodia porosum* (holotype). Note pores in conidium wall. Bar = 10 μm.

Diplodia sp. FIGS. 9, 10

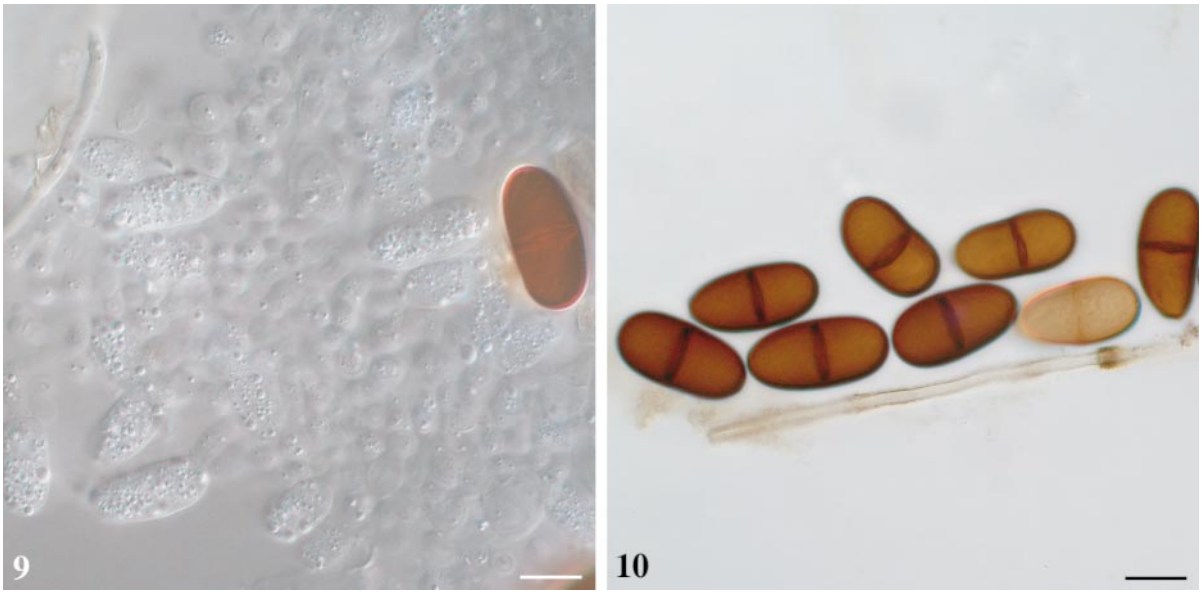
Host. Vitis vinifera.

Known distribution. South Africa (Western Cape Province).

Notes. Isolates of this *Diplodia* sp. had ovoid, brown, 1-septate conidia that are 18–22 ×10–12 μm, thus closely matching the description of *D. sarmentorum* (Wollenweber 1941, Booth 1958). In culture colonies are flat with undulating margins, dull green (27''m) on the surface, and greenish black (33''''k) underneath, reaching a radius of 33 mm after 7 d at 25 C. Cardinal temperatures for growth: min. 10 C, max. 30 C, opt. 25 C.



FIGS. 3–8. *Diplodia porosum* (holotype). 3, 4. Vertical section through pycnidia. 5, 6. Thick-walled conidia. 7. Pores visible on the conidial surface. 8. Broken, mature, pigmented conidia with pores. Bars = 200 μm in 3, 4; 10 μm in 5–8.



FIGS. 9, 10. Conidiogenous cells and conidia of a *Diplodia* sp. morphologically similar to *D. sarmentorum*. Bars = 10 μm.

Diplodia sarmentorum was reported as the teleomorph of *Otthia spiraeae,* a cosmopolitan fungus with a wide host range (Booth 1958). Although morphologically similar, the grapevine *Diplodia* isolates proved to be phylogenetically distinct from *D. sarmentorum* (FIG. 1). Because there are probably several cryptic species within *D. sarmentorum,* the grapevine isolates therefore will have to be compared to all 145 synonyms of *D. sarmentorum* (Wollenweber 1941), before their status can be resolved.

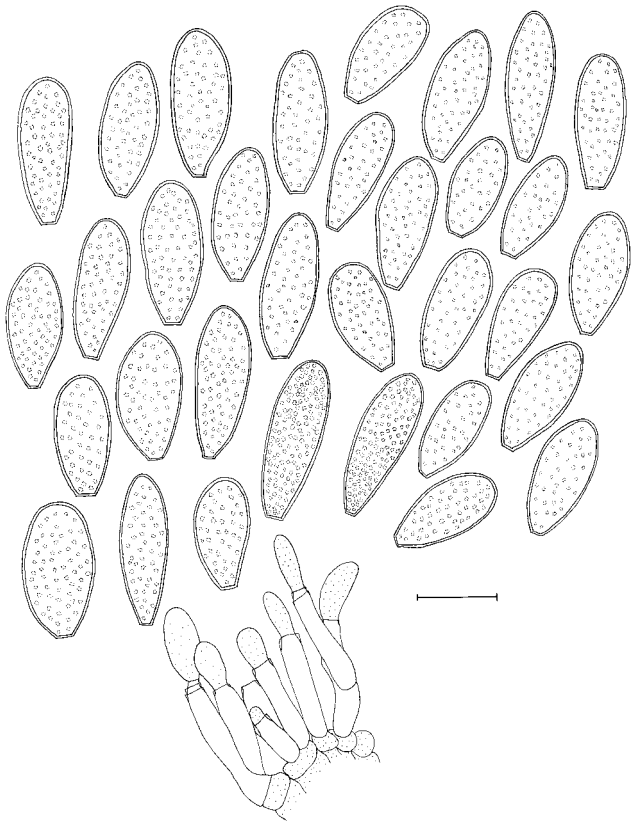


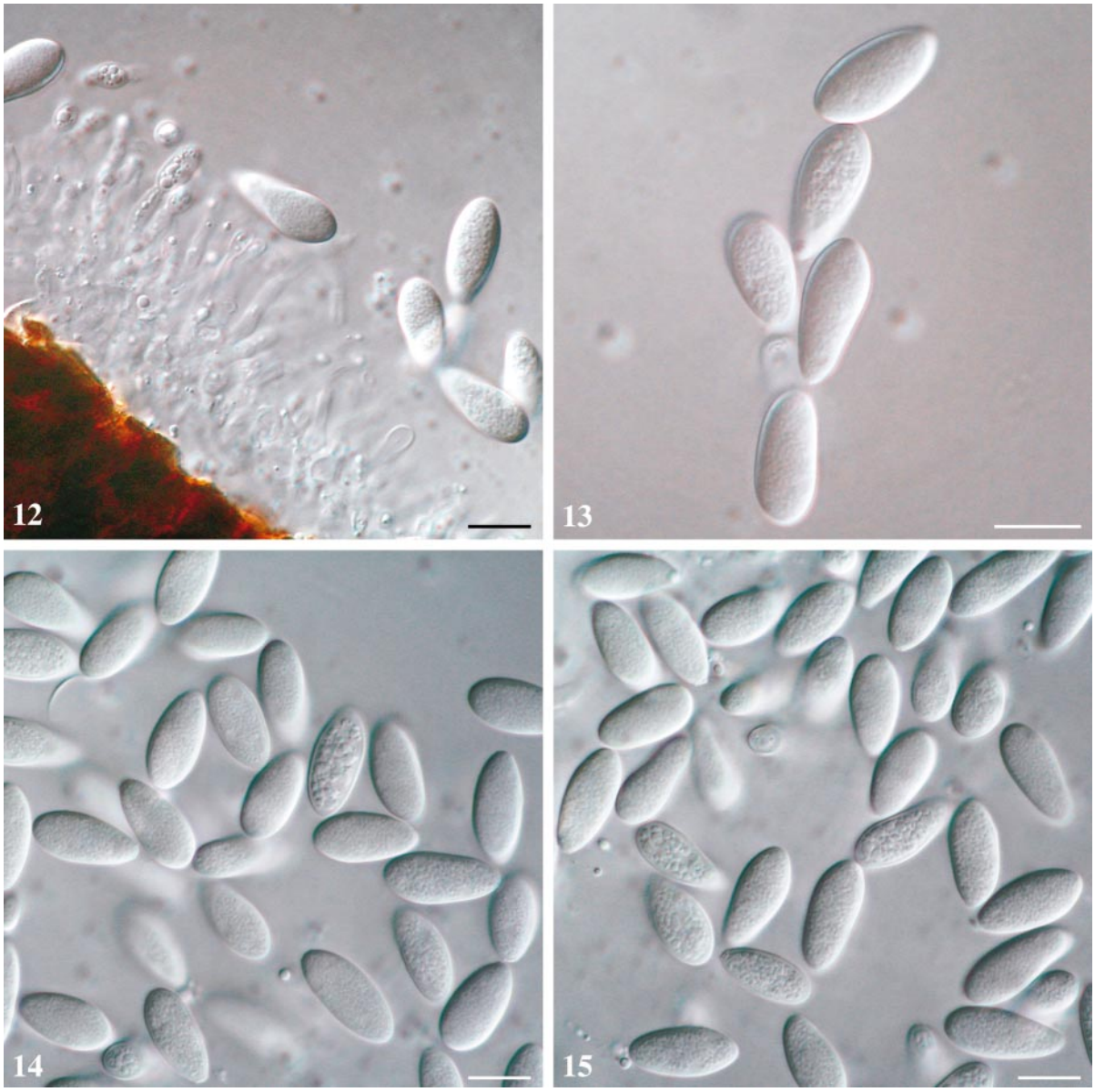
FIG. 11. Conidia and conidiogenous cells of *Fusicoccum viticlavatum* (holotype). Bar = 10 μm.

Fusicoccum viticlavatum Niekerk & Crous, sp. nov.
FIGS. 11–15

Fusicocco luteo simile, sed conidiis ellipsoideis vel clavatis, in vitro (15–)16–18(–20) × (6–)6.5–7.5(–8) μm (long.:lat. = 2.4) differens; coloniae pigmento luteo carentes.

Pycnidia embedded in host tissue, solitary, stromatic, globose, up to 450 μm wide; pycnidial wall 4–8 cell layers thick, of brown textura angularis, becoming hyaline toward inner region. Conidiophores 0–1-septate, hyaline, subcylindrical, 10–20 × 2.5–3.5 μm. Conidiogenous cells holoblastic, hyaline, subcylindrical, 7–15 × 2.5–3.5 μm, proliferating percurrently with 1–3 proliferations, or proliferating at same level (phialidic) with minute periclinal thickening. Conidia hyaline, guttulate, ellipsoid to clavate, widest in upper third, with an obtuse apex and flattened, subtruncate base, (15–)16–18(–20) × (6–)6.5–7.5(–8) μm in vitro (l:w = 2.4). Colonies umbonate with undulating margins, olivaceous (21''k) on the surface, and dull green (27''m) underneath, reaching a radius of 26 mm after 3 d at 25 C. Cardinal temperatures for growth: min. 10 C, max. 35 C, opt. 30 C.

Holotype. SOUTH AFRICA. WESTERN CAPE PROVINCE: Stellenbosch, on *V. vinifera,* 2002, *F. Halleen,* herb. CBS 7755, culture ex-type STE-U 5044, CBS 112 878.



FIGS. 12–15. *Fusicoccum viticlavatum* (holotype). 12. Conidiogenous cells giving rise to conidia. 13–15. Conidia. Bars = 10 μm.

Host. Vitis vinifera.

Known distribution. South Africa (Western Cape Province).

Notes. Fusicoccum viticlavatum is closely related to *F. australe* Slippers, Crous & M.J. Wingf. and *F. luteum* Pennycook & Samuels (FIG. 1) but readily can be distinguished from these taxa based on its characteristic conidial shape. Conidia are ellipsoid to clavate in *F. viticlavatum,* as opposed to the fusiform conidia in *F. luteum* and *F. australe.* Colonies of *F. viticlavatum* also do not produce yellow pigment in culture as observed in *F. luteum* and *F. australe* (Slippers et al 2004b).

Fusicoccum vitifusiforme Niekerk & Crous, sp. nov. FIGS. 16–24

Fusicocco luteo simile, sed conidiis brevioribus, in vitro, (18–)19–21(–22) × (4.5–)5.5–6.5(–8) μm (long.:lat. 3.3) differens; coloniae pigmento luteo carentes.

Pycnidia solitary, stromatic, globose to obpyriform, up to 450 μm diam; pycnidial wall 6–15 cell layers thick, of brown textura angularis, becoming hyaline toward inner region. Conidiophores 0–1-septate, hyaline, subcylindrical, 10–45 × 2.5–5 μm. Conidiogenous cells holoblastic, hyaline, subcylindrical, 10–30 × 2.5–3.5 μm, proliferating percurrently with numerous proliferations, or proliferating at the same level

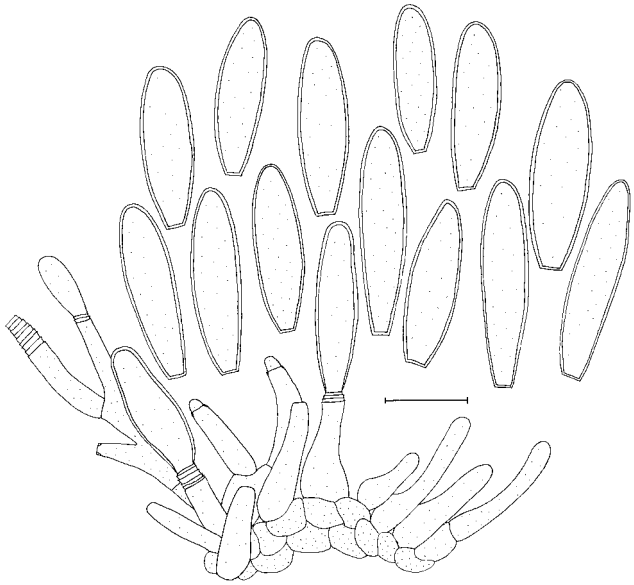


FIG. 16. Conidia and conidiogenous cells of *Fusicoccum vitifusiforme* (holotype). Bar = 10 μm.

(phialidic) with minute periclinal thickening. Conidia hyaline, granular, fusoid to ellipsoid, widest in the upper third with an obtuse apex and flattened, subtruncate base, (18–)19–21(–22) × (4.5–)5.5–6.5(–8) μm in vitro (l : w = 3.3). Colonies effuse with even, smooth margins, white on the surface, and greenish olivaceous (23'''i) underneath, reaching a radius of 31 mm after 3 d at 25 C. Cardinal temperatures for growth: min. 10 C, max. 35 C, opt. 30 C.

Holotype. SOUTH AFRICA. WESTERN CAPE PROVINCE: Stellenbosch, on *V. vinifera,* 2002, *J.M. van Niekerk,* herb. CBS 7756, culture ex-type STE-U 5252, CBS 110 887.

Host. Vitis vinifera.

Known distribution. South Africa (Western Cape Province).

Notes. Fusicoccum vitifusiforme is closely related to *F. australe* and *F. luteum,* and also has fusiform conidia, as in the case of the latter two species (Slippers et al 2004b). It is distinct, however, by not producing yellow pigment in culture and by having conidia that are shorter (up to 22 μm in length) than those of *F. australe* (18–30 μm) and *F. luteum* (15–30 μm).

Pathogenicity.—In vitro screening on green shoots. Mean lesion lengths caused by isolates of the *Botryosphaeria* spp. on green Periquita shoots are given in TABLE II. *Botryosphaeria australis* and *B. parva* caused significantly longer lesions (65.29 and 57.30 mm, respectively) than the other species tested (4.30–17.22 mm). All species tested caused markedly longer lesions compared to the agar plug-treated control (3.38 mm), although not all were significantly different.

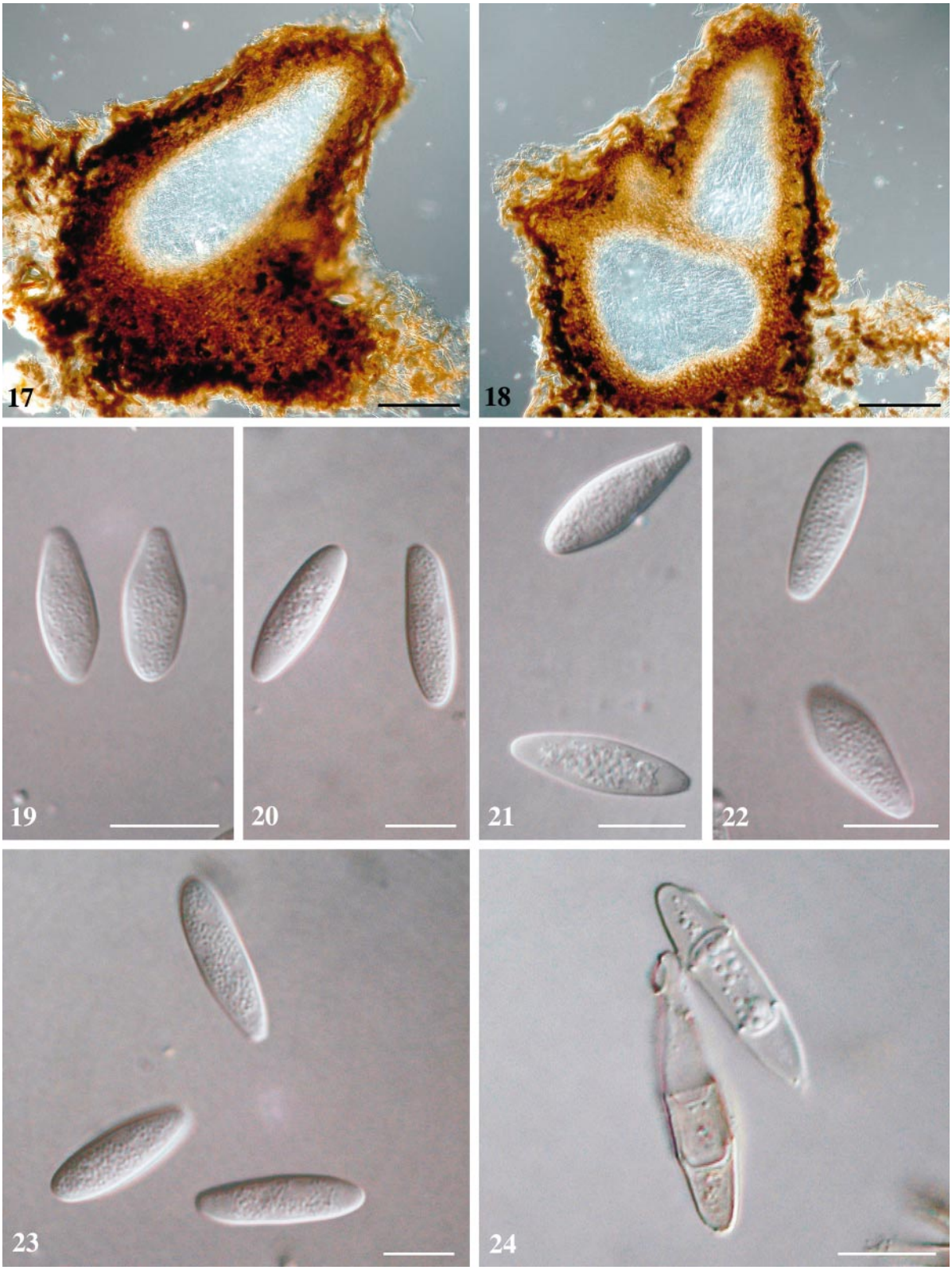
In vitro screening on mature canes. Analysis of variance showed no significant interaction between cultivar and treatment (P = 0.9431), and the lesion length measurements for both cultivars therefore were pooled (TABLE III). The most severe lesions were caused by *B. ribis* (26.75 mm long), followed by *B. australis* (18.17 mm), *B. stevensii* (17.56 mm), and *B. parva* (11.25 mm). *Fusicoccum vitifusiforme, Diplodia* sp., *B. dothidea, B. obtusa* and *F. viticlavatum* (7.69 mm to 7.10 mm) caused smaller but still significantly longer lesions than the controls (3.81 mm).

In vivo pathogenicity on mature vines. Mean lesion lengths caused by in vivo inoculations with *Botryosphaeria* spp. on mature canes and mature wood of the grapevine cultivar Periquita are given in TABLE IV. The species that caused the most severe lesions on mature canes were *B. parva* (15.01 mm long), *D. porosum.* (13.22 mm), *B. australis* (12.89 mm) and *B. rhodina* (11.73 mm). *Botryosphaeria obtusa,* the *Fusicoccum* spp. and *Diplodia* sp. caused significantly smaller lesions (8.08–7.18 mm) but still significantly larger than the agar plug-treated control (4.18 mm).

Two symptom types could be distinguished in the mature wood. The first type was black vascular streaking that originated from the inoculation site. The second symptom type was a brown wood-rotting lesion. Vascular streaking mostly extended beyond the rotting lesion. Because multiple isolates were not tested for each species, species and isolate interaction could not be determined in the analyses of variance of the rotting and streaking lesion lengths in the mature wood (TABLE IV). However, significant differences in the lesion lengths caused by different isolates from the same species were observed (data not shown). Mean rotting and streaking lengths are given in TABLE IV. *Botryosphaeria australis* caused the most severe rotting lesion (25.90 mm long), while the other species caused marginally to significantly longer lesions (6.80–13.54 mm) than the control (6.72 mm). *Botryosphaeria australis* also caused the most severe streaking (48.85 mm), with the other species causing vascular streaking of significantly larger proportions (22.06–36.60 mm) than the control (6.72 mm). Reisolations from the mature wood were successful, and all species were identified as being the same as originally used in inoculations, thereby satisfying Koch's postulates.

DISCUSSION

This study represents the first attempt to characterize species of *Botryosphaeria* on grapevines using an extensive collection of isolates and integrating morphology, pathology and molecular datasets. Eleven species were identified as occurring on grapevines, of



FIGS. 17–24. *Fusicoccum vitifusiforme* (holotype). 17, 18. Vertical section through pycnidia. 19–23. Mature conidia. 24. Conidia becoming 2-septate with age. Bars = 200 μm in 17, 18; 10 μm in 19–24.

which three are newly described. Because the present collection of strains had a strong bias toward South African vineyards, it is tempting to speculate that, if vineyards from other countries also were sampled more intensively, it would lead to the identification of yet more species from this host. Crous et al (2000) reported *B. ribis, B. obtusa* and *B. dothidea* as pathogens of grapevines in South Africa, of which only *B. obtusa* could be confirmed. New records from grapevines in South Africa include *B. rhodina, B. lutea, B. parva, B. australis, Diplodia* sp. (resembling *D. sarmentorum*)*, D. porosum, F. viticlavatum* and *F. vitifusiforme*. It was a surprise to discover that none of the collected isolates were representative of *B. dothidea* or *B. ribis,* which are the names commonly used for isolates causing Botryosphaeria dieback of grapevines (Pascoe 1998, Phillips 2002).

The in vitro and in vivo pathogenicity trials in this study tested the ability of the mycelium of *Botryosphaeria* spp. to infect wounded grapevine tissue at different stages of phenological development (green shoots, mature canes and mature wood). All species successfully were re-isolated from the respective lesions and thus should be considered as potential pathogens of grapevines. *Botryosphaeria australis* and *B. parva* were consistently among the species causing the most severe lesions on green shoots, mature canes and mature wood. However, all species showed variable degrees of lesion formation. For example, *B. rhodina* and *D. porosum* grouped among the most virulent species in the in vivo mature cane trial but in the in vitro mature cane trial they grouped with the least virulent species. This variability among species might be attributed to their reaction on host tissue at different stages of phenological development, physiological character of the host tissue, cultivar susceptibility and/or conditions and length of incubation. Variability was observed in the virulence of different isolates within a species. The *B. obtusa* isolate STE-U 5139 consistently caused lesions that were twice as large as the lesions caused by the other *B. obtusa* isolates (STE-U 4444 and STE-U 4440, results not shown). This phenomenon also was observed for *B. australis,* where isolate STE-U 5040 caused lesions twice as large as the other *B. australis* isolates (STE-U 4598 and STE-U 4416; results not shown). This might indicate that isolates within species can be divided into different virulence groups. This corresponds with findings of Larignon et al (2001) that isolates of *B. obtusa* could be divided into four virulence groups. The four species that were not found among the South African isolates, *B. dothidea, B. lutea, B. ribis* and *B. stevensii,* were tested in the in vitro mature cane trial only. *Botryosphaeria ribis* and *B. stevensii* should be considered as potentially important pathogens of grapevines, while data for *B. lutea* and *B. dothidea* suggest that they are less virulent species. The variability observed here is an indication that the protocols used for pathogenicity testing should be standardized and should employ inoculation techniques that simulate natural infection.

The multifaceted approach of using different datasets to identify cryptic species of *Botryosphaeria* is in contrast to the earlier, more simplistic view taken by von Arx and Müller (1954), where 183 taxa were reduced to a core 11 species. Although relatively easy to apply, this concept does not reflect the diverse species of *Botryosphaeria,* their relative pathogenicity, distribution and ecology. Furthermore, all published records since von Arx and Müller (1954) should be treated with caution. Regarding the *Fusicoccum* complex, progress has been made by the characterization and distinction of *B. dothidea, B. parva* and *B. ribis* (Slippers et al 2004a). The problem of dealing with these old names is one that will remain with us for some time to come. Approximately 2000 anamorph names are linked to the *Botryosphaeria* complex, with treatments of other genera continually adding more names. For instance, in their recent revision of *Phyllosticta* Pers. (van der Aa and Vanev 2002), an additional 18 species were recombined into *Fusicoccum*. Given the fact that none of these are known from culture and that recent studies suggest that culture and sequence data are required to clearly elucidate species of *Botryosphaeria,* it seems impossible to resolve the status of the old names in this group.

Although some species of *Botryosphaeria* appear to be host specific, such as *B. protearum* Denman & Crous on *Protea* spp. (Denman et al 2003), others, such as *B. obtusa, B. parva, B. rhodina,* appear to be common, having wider host ranges and distributions than initially accepted.

The delimitation of new species of *Fusicoccum* from the *B. ribis/parva* complex underlines the fact that these species will not be identifiable without molecular data. Their conidial shapes and dimensions show considerable overlap. The reference strains at CBS and sequence data available in GenBank will facilitate future identifications. It does raise perplexing questions for quarantine officers who need tools to make rapid decisions regarding the import and export of plant material.

The present phylogeny (FIG. 1) supports the decision of Denman et al (2000) and Zhou and Stanosz (2001) to place anamorphs of *Botryosphaeria* in either *Fusicoccum* (hyaline, thin-walled conidia) or *Diplodia* (pigmented, thick-walled conidia). Anamorph genera such as *Botryodiplodia* (Sacc.) Sacc. and *Sphaeropsis* Sacc. should be treated under *Diplodia* Fr. *Diplodia porosum* has thick-walled conidia that are ini-

tially hyaline, eventually turning brown at maturity within the pycnidial locule. A rather unusual character is the fact that the conidial wall is covered with large pores. The latter phenomenon has been noted in *Diplodia pinea* (Desm.) J. Kickx f., which has pitted and smooth conidial types, that seem to correlate with different cryptic species in this complex (de Wet et al 2003). The pores on conidia of *D. porosum* are unusual and distinct from the pits in the conidial wall of *D. pinea*. Furthermore, *D. porosum* clusters between *Diplodia* and *Fusicoccum* and might represent a distinct anamorph genus. Pores appear to be phylogenetically more informative than the striations observed on the inner conidial surface wall of *Botryodiplodia theobromae* Pat. These different anamorphs are, however, all part of *Botryosphaeria,* which appears to be monophyletic.

Otthia Nitschke ex Fuckel may be synonymous with *Botryosphaeria* (Booth 1958, Laundon 1973, Denman et al 2000). Booth (1958) obtained single ascospores of *Otthia spiraeae,* and via cultural studies linked this teleomorph to *D. sarmentorum,* a fungus regarded as cosmopolitan with a wide distribution (Wollenweber 1941). He also designated *O. spiraeae* as lectotype of the genus *Otthia*. Our sequence data (FIG. 1), confirm Booth's observations relating *D. sarmentorum* (CBS 120.43) to *O. spiraeae* (IMI 063581b). The grapevine isolates appear to be phylogenetically distinct, suggesting that they probably represent one of the 145 taxa regarded as synonyms of *D. sarmentorum* by Wollenweber (1941). An examination of the type specimen of *D. viticola* Desm. (PC), revealed that it is in fact a synonym of *Diplodia mutila* Fr. & Mont. (A.J.L. Phillips, personal communication) and thus unavailable for our isolates.

Laundon (1973) and Denman et al (2000) considered *Otthia* a probable synonym of *Botryosphaeria,* suggesting that ascospore septation is not useful at the generic level. The latter feature recently has been rejected in separating *Sphaerulina* Sacc. (3-septate ascospores), from *Mycosphaerella* Johanson (1-septate ascospores) (Crous et al 2003). The denominator common between species of *Mycosphaerella* and those of *Sphaerulina* that were shown to belong to *Mycosphaerella* was the morphology of their anamorphs. Similarly, this also appears to be the case for *Otthia* because a culture identified as *O. spiraeae* is shown here to belong to *Botryosphaeria*. The final synonymy of *Otthia* (1870) under *Botryosphaeria* (1863) as suggested by Denman et al (2000), however, awaits type studies and fresh collections from which single ascospore isolates can be obtained (FIG. 1). Given the plasticity of the current generic concept of *Botryosphaeria* and its anamorphs, we have chosen to describe *D. porosum* with its characteristic pored conidial wall in *Diplodia,* thus maintaining two anamorph genera for *Botryosphaeria,* namely *Diplodia* and *Fusicoccum*.

ACKNOWLEDGMENTS

The authors acknowledge the South African National Research Foundation (NRF) and Winetech for financial support. Bernard Slippers (FABI, University of Pretoria) is thanked for providing access to unpublished data, while Frikkie Calitz and Mardé Booyse (ARC-Agrimetrics Institute, Stellenbosch) are thanked for the statistical layout and analyses of the pathogenicity trials.

LITERATURE CITED

Barr ME. 1972. Preliminary studies on the Dothideales in temperate North America. Contr Univ Michigan Herbarium 9:523–638.

———. 1987. Prodromus to class Loculoascomycetes. Published by the author, Amherst, Massachusetts. 168 p.

Booth C. 1958. Studies of Pyrenomycetes. III. *Otthia spiraeae* (Fuckel) Fuckel, syn. *Diplodia sarmentorum* (Fr.) Fr. Trans Brit Mycol Soc 41:335−340.

Boyer, JS. 1995. Biochemical and biophysical aspects of water deficits and the predisposition to disease. Ann Rev Phytopathol 33:251–274.

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

Castillo-Pando M, Somers A, Green CD, Priest M, Sriskanthades M. 2001. Fungi associated with dieback of Semillon grapevines in the Hunter Valley of New South Wales. Aust Plant Path 30:59–63.

Crous PW, Palm ME. 1999. Reassessment of the anamorph genera *Botryodiplodia, Dothiorella* and *Fusicoccum*. Sydowia 51:167–175.

———, Phillips AJL, Baxter AP. 2000. Phytopathogenic fungi from South Africa. Stellenbosch, South Africa: Department of Plant Pathology Press, University of Stellenbosch Printers. 358 p.

———, Groenewald JZ, Wingfield MJ, Aptroot A. 2003. The value of ascospore septation in separating *Mycosphaerella* from *Sphaerulina* in the Dothideales: a Saccardoan myth? Sydowia 55:136–152.

Denman S, Crous PW, Taylor JE, Kang J-C, Pascoe I, Wingfield MJ. 2000. An overview of the taxonomic history of *Botryosphaeria,* and a re-evaluation of its anamorphs based on morphology and ITS rDNA phylogeny. Stud Myc 43:129–140.

———, ———, Groenewald JG, Slippers B, Wingfield BD, Wingfield MJ. 2003. Circumscription of *Botryosphaeria* species associated with Proteaceae based on morphology and DNA sequence data. Mycologia 95:294–307.

de Wet J, Burgess T, Slippers B, Preisig O, Wingfield BD, Wingfield MJ. 2003. Multiple gene genealogies and microsatellite markers reflect relationships between morphotypes of *Sphaeropsis sapinea* and distinguish a new species of *Diplodia*. Mycol Res 107:557–566.

Doidge EM. 1950. The South African fungi and lichens to the end of 1945. Bothalia 5:1–1094.

Fourie P, Halleen F. 2001. Diagnosis of fungal diseases and their involvement in dieback diseases of young vines. Winelands 2001(12):19–23.

Hillis DM, Bull JJ. 1993. An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. Syst Biol 42:182–192.

Jacobs KA, Rehner SA. 1998. Comparison of cultural and morphological characters and ITS sequences in anamorphs of *Botryosphaeria* and related taxa. Mycologia 90:601–610.

Larignon P, Dubos B. 2001. The villainy of Black Dead Arm. Wines & Vines 82:86–89.

———, Fulchic R, Cere L, Dubos B. 2001. Observation on black dead arm in French vineyards. Phytopath Mediter 40:S336–S342.

Laundon GF. 1973. *Botryosphaeria obtusa, B. stevensii* and *Otthia spiraeae* in New Zealand. Trans Brit Mycol Soc 61:369–374.

Lee SB, Taylor JW. 1990. Isolation of DNA from fungal mycelia and single spores. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, eds. PCR protocols: a guide to methods and applications. San Diego, California: Academic Press. p 282–287.

Lehoczky L. 1988. Black dead arm. In: Pearson, RC, Goheen AC, eds. Compendium of grape diseases. St. Paul, Minnesota: APS Press. 35 p.

Page RDM. 1996. TREEVIEW: An application to display phylogenetic trees on personal computers. Comp Applic Bios 12:357–358.

Pascoe I. 1998. Trunk diseases of grapevines—perspectives from a tour of California. The Aust Grapegr & Winem 417:68–71.

Pennycook SR, Samuels GJ. 1985. *Botryosphaeria* and *Fusicoccum* species associated with ripe fruit rot of *Actinidia deliciosa* (Kiwifruit) in New Zealand. Mycotaxon 24: 445–458.

Phillips AJL. 1998. *Botryosphaeria dothidea* and other fungi associated with excoriose and dieback of grapevines in Portugal. J Phytopath 146:327–332.

———. 2000. Excoriose, cane blight and related diseases of grapevines: a taxonomic review of the pathogens. Phytopath Mediter 39:341–356.

———. 2002. *Botryosphaeria* species associated with diseases of grapevines in Portugal. Phytopath Mediter 41:3–18.

Rambaut A. 2002. Sequence Alignment Editor. Version 2.0. Department of Zoology, University of Oxford, Oxford.

Rayner RW. 1970. A mycological colour chart. Kew, Surrey, UK: CMI and British Mycological Society. 17 sheets, 34 p.

Rossman AY, Samuels GJ, Rogerson CT, Lowen R. 1999. Genera of Bionectriaceae, Hypocreaceae and Nectriaceae (Hypocreales, Ascomycetes). Stud Myc 42:1–248.

Saccardo PA. 1882. Sylloge Fungorum. Pyrenomyceten omnium hucosque cognitorum. Padova, Italy. Vol. 1.

SAS. 1999. SAS/STAT User's Guide, Version 8.2, 1st printing. Vol. 2. SAS Institute Inc, SAS Campus Drive, Cary, North Carolina.

Shoemaker RA. 1964. Conidial states of some *Botryosphaeria* species on *Vitis* and *Quercus*. Can J Bot 42:1297–1301.

Sivanesan A. 1984. The bitunicate Ascomycetes and their anamorphs. Vaduz, Liechtenstein: J Cramer. 700 p.

Slippers B, Crous PW, Denman S, Coutinho TA, Wingfield BD, Wingfield MJ. 2004a. Combined multiple gene genealogies and phenotypic characters differentiate several species previously identified as *Botryosphaeria dothidea*. Mycologia 96:83-101.

———, Vermeulen G, Crous PW, Coutinho TA, Wingfield BD, Wingfield MJ. 2004b. Multiple gene sequence delimit *Botryosphaeria australis* sp. nov. as a sister species to *B. lutea*. Mycologia (In press).

Swofford DL. 2000. PAUP 4.0*: Phylogenetic Analysis Using Parsimony. Sinauer Associates, Sunderland, Massachusetts.

van der Aa HA, Vanev S. 2002. A revision of the species described in *Phyllosticta*. Utrecht, Netherlands: Centraalbureau voor Schimmelcultures. 510 p.

von Arx JA, Müller E. 1954. Die Gattung der amerosporen Pyrenomyceten. Beitr Kryptog fl Schweiz 11(1):1–434.

———. 1987. Plant pathogenic fungi. Beihef zur Nova Hedw 87:1–288.

White TJ, Bruns T, Lee S, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, eds. PCR protocols: a guide to methods and applications. San Diego, California: Academic Press. p 315–322.

Wollenweber HW. 1941. *Diplodia sarmentorum* Fries und ihre Verbreitung. Zentralbl Bakteriol Parasitendk, Abt 2, 103:347–357.

Zhou S, Stanosz GR. 2001. Relationships among *Botryosphaeria* species and associated anamorphic fungi inferred from the analyses of ITS and 5.8S rDNA sequences. Mycologia 93:516–527.