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RESEARCH PAPER

Phylogeny of *Neofusicoccum* species associated with grapevine trunk diseases in Algeria, with description of *Neofusicoccum algeriense* sp. nov.

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Summary. During a study of Botryosphaeriaceae species associated with grapevine trunk diseases in North Algeria, a collection of 67 *Neofusicoccum*-like strains were isolated from three cultivars (Cinsaut, Alphonse Lavallée and Cardinal) from two different locations. Based on morphology and DNA sequence data (ITS and TEF-1α), four species were identified. Of these, *Neofusicoccum parvum*, *N. mediterraneum* and *N. australe* are known, while *N. algeriense* is described here as new. These species are reported for the first time from Algeria. In this study, relationships between vascular lesions and *Neofusicoccum* species isolated were highlighted. The *Neofusicoccum* spp. were most often isolated from wedge-shaped and yellow soft wood rots and more rarely from central brown necrosis and black streaking. *Neofusicoccum parvum* was the most frequently isolated (48 isolates) followed by *N. algeriense* (four), while *N. mediterraneum* and *N. australe* were each found only once.

Key words: Botryosphaeriaceae, *Neofusicoccum*, phylogeny, taxonomy, *Vitis vinifera*.

Introduction

Viticulture in North Africa has a long history that began in antiquity, as in many other Mediterranean countries. Most of the vineyards were created by European colonizers for their need for wines, but the independence of Islamic countries has caused massive uprooting, which led to an end of the golden age of wine (Hildebert, 1949; Levadoux *et al*., 1971). During the last 20 years, the reconstitution of vineyards has been an essential element of the new Algerian agriculture policy. The National Fund for Regulation and Development of Agriculture (FNRDA) has emerged, strongly promoting viticulture. The trend is to increase and enhance production by financing plans for new vineyards and by improving grapevine productivity and quality. This important project is currently constrained by biotic and abiotic stresses that affect the vines. A major concern for growers is dieback that is frequently observed, and represents a real threat for the productivity and longevity of their vineyards. The intensity of this dieback has increased significantly, leading in some cases to the elimination of entire vineyards (Berraf and Péros, 2005). Recent research has indicated the involvement of grapevine trunk diseases (GTDs) that appear to be the main causes of dieback and mortality observed during the last decade (Berraf and Péros, 2005; Berraf-Tebbal *et al*., 2011).

Several Botryosphaeriaceae species are known to occur worldwide, causing dieback and canker of annual and perennial hosts, including grapevines. They include a range of diverse fungi that are either pathogens, endophytes or saprobes, mainly on woody hosts (Phillips *et al*., 2013). Based on extensive phylogenetic studies, 18 genera are known in culture in the Botryosphaeriaceae (Phillips *et al*., 2013), with at least 21 species recognized to be sig-

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nificant grapevine trunk disease pathogens, including species of *Neofusicoccum* (Úrbez-Torres, 2011). *Neofusicoccum* was introduced by Crous *et al.* (2006) to accommodate species with *Fusicoccum*-like anamorphs and *Dichomera*-like synamorphs. Currently, 20 species are recognized in *Neofusicoccum*, and they have been separated on the basis of their morphological characters and DNA-based techniques (Abdollahzadeh *et al.*, 2013; Crous *et al.*, 2013; Phillips *et al.*, 2013).

Despite the importance attributed to the species of this family, there have been no studies on the Botryosphaeriaceae in Algeria. The aims of the present study were to identify the *Neofusicoccum*-like isolates collected from vines with dieback symptoms in Northern Algeria. Species were identified based on their morphology and comparison of DNA sequence data for ITS rDNA and translation elongation factor 1-α. In addition, the localization of each species in the different types of wood lesions was studied.

Materials and methods

Fungal isolation

Grapevine branches and trunks displaying symptoms of dieback were collected from vineyards of Cinsaut (wine grapes), Alphonse-Lavallée and Cardinal (table grapes) cultivars. Cross sections were made at intervals of 10–20 cm along the trunks and arms of each vine to reveal the type and localization of internal necrosis. Isolations were made from each type of necrotic tissues. From each lesion, ten pieces of wood (10 × 5 × 5 mm) were cut from the margin of the soft white rot, the sectorial necrotic zone and the central brown zone as well as black spots, as described by Larignon and Dubos (1997) and Berraf and Péros (2005). The pieces of wood were surface-disinfested with calcium hypochlorite (3% available chlorine) for 10 min, rinsed twice in sterile water and then placed on potato dextrose agar (PDA, Difco Laboratories). Plates were incubated at room temperature and observed every 2–3 d. Colonies emerging from wood pieces that were recognized by their fast growth with grey mycelium as Botryosphaeriaceae species (Phillips *et al.*, 2008; Abdollahzadeh *et al.*, 2013) were subcultured to fresh PDA plates and incubated at 25°C. Preliminary identifications to genus and tentative species level were based on the keys provided in Phillips *et al.* (2013).

Morphological characterization

Cultures were transferred to 2% water agar (WA) with double-autoclaved pine needles or poplar twigs on the agar surface to enhance sporulation. Plates were incubated at 25°C under mixed near-UV and cool-white fluorescent light in a 12 h light 12 h dark regime for 2–6 weeks. A single pycnidium from each culture was dissected and the conidiogenous layer and conidia were mounted in 100% lactic acid. Digital images were recorded with a Leica DFC 320 camera on a Leica DMR HC light microscope, and measurements were made with the Leica IM500 measurement module. The mean, standard deviation and 95% confidence intervals were calculated from measurements of 50 conidia from each isolate. Dimensions are presented as the range of measurements with extremes in brackets followed by 95% confidence limits and mean ± standard deviation. Dimensions of other structures are presented as the range of at least 20 measurements. Cultural characteristics, growth rates and cardinal temperatures for growth were determined on 2% malt extract agar (MEA, Difco Laboratories) plates incubated at different temperatures (5–35°C at 5°C intervals) in the dark. Colony colours were determined with the reference to the colour chart of Rayner (1970) for isolates incubated on PDA at 25°C in the dark.

DNA isolation, PCR amplification and sequencing

Genomic DNA of all isolates identified morphologically as Botryosphaeriaceae was extracted from fresh mycelium grown on PDA plates in darkness at 25°C for 4–7 days, according to Abdollahzadeh *et al.* (2009). PCR reactions were carried out with Taq DNA 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% DMSO to improve the amplification of some difficult DNA templates. All primers used were synthesised by STAB Vida Lda. The ITS region plus the D1/D2 domain of the LSU was amplified with the primer pair ITS1 (White *et al.*, 1990) /NL4 (O'Donnell, 1993). The translation elongation factor 1-α (EF-1α) was amplified with primers EF1-688F/EF1-1251R (Alves *et al.*, 2008).

Nucleotide sequences of the ITS and EF-1α regions were determined using the primers ITS1/ITS4 (White *et al.*, 1990) and EF1-688F/EF1-1251R (Alves

et al., 2008). Both strands of the PCR products were sequenced by STAB Vida Lda. Newly generated sequences were deposited in GenBank (Table 1). Sequences for both DNA regions were retrieved in BLAST searches (Basic Local Alignment Search Tool) (Altschul *et al.*, 1990) from GenBank (Table 1). The sequences of *Botryosphaeria dothidea* (CBS 110302) and *B. corticis* (CBS 119047) were used as outgroups. Sequences were edited with BioEdit Alignment Editor V.7.0.9.0 (Hall, 1999) and aligned with Clustal X version 1.83 (Thompson *et al.*, 1997). Alignments were checked and manual adjustments were made when necessary.

Phylogenetic analyses were carried out using PAUP v4.0b10 (Swofford, 2003) for maximum-parsimony (MP) and Neighbour-joining (NJ) analyses and Mr Bayes 3.0b4 (Ronquist and Huelsenbeck, 2003) for the Bayesian analysis. Maximum parsimony analysis was performed with the heuristic search option with 1000 random taxon additions and tree bisection and reconnection (TBR) as the branch-swapping algorithm. All characters were unordered and of equal weight and gaps were treated as fifth character. 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 were consistency index (CI), retention index (RI) and homoplasy index (HI). Bayesian analyses employing a Markov chain Monte Carlo (MCMC) method were performed. The general time-reversible model of evolution (Rodriguez *et al.*, 1990), including estimation of invariable sites and assuming a discrete gamma distribution with six rate categories (GTR + Γ + C), was used. Four MCMC chains were run simultaneously, starting from random trees, for 10^6 generations. Trees were sampled every 100th generation for a total of 10^4 trees. The first 10^3 trees were discarded as the burn-in phase of each analysis. Posterior probabilities (Rannala and Yang, 1996) were determined from a majority rule consensus tree generated from the remaining 9000 trees. The analysis was repeated three times starting from different random trees to ensure trees from the same tree space were being sampled during each analysis. The trees were visualized with TreeView (Page, 1996). New sequences were deposited in GenBank (Table 1) and the alignment and trees in TreeBASE (S15530).

Results

Morphology

Colonies grew rapidly covering the agar surface in 9 cm diam. Petri dishes within 7 d. Mycelium was initially white, turning grey and fluffy. All isolates studied produced pycnidia either on pine needles or poplar twigs on WA within 2–4 weeks. No teleomorph structures were observed for these isolates. Hyaline, aseptate, thin-walled conidia were produced in single or grouped, black, globose pycnidia. Isolates were grouped according to their colony characteristics and micromorphology, and representative isolates chosen from each group were selected for DNA sequencing.

DNA sequencing and phylogenetic analyses

A total of 67 isolates of *Neofusicoccum* species were obtained from branches and trunks of diseased vines. To confirm the identity of the isolates, ITS and EF-1α were sequenced for 17 representative isolates selected based on their colony characteristics and micromorphology. The two datasets were combined and aligned in a single matrix with sequences of 44 isolates retrieved from GenBank, representing a selection of all known *Neofusicoccum* species. The combined alignment consisted of 672 characters (including alignment gaps). Of these, 152 were parsimony-informative, 24 were variable and parsimony-uninformative and 496 were constant. After a heuristic search, 209 parsimonious trees with the same overall topology were retained (Length = 290; CI = 0.772; RI = 0.930, HI = 0.228). One of the trees is shown in Figure 1. The phylogenetic analyses of Maximum-parsimony (MP), Neighbour joining (NJ) and Bayesian methods (BM) produced trees with essentially identical topologies (NJ and BM trees not presented). In these trees, the 17 strains isolated from Algerian grapevines grouped in four subclades. The first clade included 11 isolates, which clustered with *Neofusicoccum parvum*, forming a single monophyletic group with a high bootstrap support. The second group of four isolates resided in a well-defined clade with strong bootstrap support (MP/NJ/BM = 94/95/1.00). These isolates formed a distinct subclade, sister to *N. kwambonambiense* and *N. occulatum*. A single isolate clustered with *N. mediterraneum*. Only one base pair difference in ITS separated this isolate from the ex-type isolate, while EF-1α sequences were

Table 1. Details and GenBank accession numbers of fungal isolates and included in the phylogenetic analysis.

Species	Isolate number[a, b]	Origin	Host	Collector	GenBank accession numbers[c]	
					ITS	EF-1α
Botryosphaeria dothidea	CBS 110302	Portugal	*Vitis vinifera*	A.J.L. Phillips	AY259092	AY573218
B. corticis	CBS 119047	USA	*Vaccinium corymbosum*	P.V. Oudemans	DQ233245	EU01539
Neofusicoccum algeriense	**CBS 137504**	Algeria	*Vitis vinifera*	A. Berraf-Tebbal	KJ657702	KJ657715
	ALG9	Algeria	*Vitis vinifera*	A. Berraf-Tebbal	KJ657704	KJ657721
	ALG11	Algeria	*Vitis vinifera*	A. Berraf-Tebbal	KJ657705	KJ657722
	ALG12	Algeria	*Vitis vinifera*	A. Berraf-Tebbal	KJ657703	KJ657716
N. andinum	**CBS 117453**	Venezuela	*Eucalyptus* sp.	S. Mohali	AY693976	AY693977
	CBS 117452	Venezuela	*Eucalyptus* sp.	S. Mohali	DQ306263	DQ306264
N. arbuti	**CBS 116131**	USA	*Arbutus menziesii*	M. Elliott	AY819720	KF531792
	CBS 117090	USA	*Arbutus menziesii*	M. Elliott	AY819724	KF531791
N. australe	**CMW 6837**	Australia	*Acacia* sp.	M.J. Wingfield	AY339262	AY339270
	CMW 6853	Australia	*Sequoiadendron* sp.	M.J. Wingfield	AY339263	AY339263
	ALG69	Algeria	*Vitis vinifera*	A. Berraf-Tebbal	KJ657714	KJ657731
N. batangarum	**CBS 124924**	Africa	*Terminalia catappa*	D. Begoude ,J. Roux	FJ900607	FJ900653
	CBS 124923	Africa	*Terminalia catappa*	D. Begoude, J. Roux	FJ900608	FJ900654
N. brasiliense	CMM 1269	Brazil	*Mangifera indica*	M.W. Marques	JX513629	JX513609
	CMM 1285	Brazil	*M. indica*	M.W. Marques	JX513628	JX513608
N. cordaticola	**CBS 123634**	South Africa	*Syzygium cordatum*	D. Pavlic	EU821898	EU821868
	CBS 123635	South Africa	*S. cordatum*	D. Pavlic	EU821903	EU821873
N. cryptoaustrale	CMW 23786	South Africa	*Eucalyptus sp.*	H.M. Maleme	FJ752744	FJ752714
	CMW 23787	South Africa	*Eucalyptus sp.*	H.M. Maleme	FJ752743	FJ752711
N. eucalypticola	**CBS 115679**	Australia	*Eucalyptus rossii*	M.J. Wingfield	AY615141	AY615133
	CBS 115766	Australia	*E. rossii*	M.J. Wingfield	AY615143	AY615135
N. eucalyptorum	**CBS 115791**	South Africa	*Eucalyptus grandis*	H. Smith	AF283686	AY236891
	CMW 10126	South Africa	*E. grandis*	H. Smith	AF283687	AY236892

(Continued)

Table 1. (Continued).

Species	Isolate number[a, b]	Origin	Host	Collector	GenBank accession numbers[c]	
					ITS	EF-1α
N. kwambonambiense	CBS 123643	South Africa	*S. cordatum*	D. Pavlic	EU821894	EU821954
	CBS 123639	South Africa	*S. cordatum*	D. Pavlic	EU821900	EU821870
N. luteum	**CBS 110299**	Portugal	*Vitis vinifera*	A.J.L. Phillips	AY259091	AY573217
	CBS 110497	Portugal	*Vitis vinifera*	A.J.L. Phillips	EU673311	EU673277
N. macroclavatum	**CBS 118223**	Australia	*E. globulus*	T.I. Burguess	DQ093196	DQ093217
	WAC 12445	Australia	*E. globulus*	T.I. Burguess	DQ093197	DQ093218
N. mangiferae	CBS 118531	Australia	*Mangifera indica*	G.I. Johnson	AY615185	DQ093221
	CBS 118532	Australia	*M. indica*	G.I. Johnson	AY615186	DQ093220
N. mediterraneum	**CBS 121718**	Greece	*Eucalyptus* sp.	P.W. Crous, M.J. Wingfield, A.J.L.Phillips	GU251176	GU251836
	CBS 121558	Italy	*Olea europaea*	C. Lazzizera	GU799463	GU799462
	ALG77	Algeria	*Vitis vinifera*	A. Berraf-Tebbal	KJ657706	KJ657723
N. nonquaesitum	**CBS 126655**	California	*Umbellularia*	F. P. Trouillas	GU251163	GU251295
	PD 301	Chile	*Vaccinium corymbosum*	E.X. Briceño, J.G. Espinoza, B.A.	GU251164	GU251296
N. occulatum	**CBS 128008**	Australia	*Eucalyptus grandis* hybrid	T.I. Burgess	EU301030	EU339509
	MUCC 286	Australia	*Eucalyptus pellita*	T.I. Burgess	EU736947	EU339511
N. parvum	CBS 110301	Portugal	*Vitis vinifera*	A.J.L. Phillips	AY259098	AY573221
	ATCC 58191	New Zealand	*Actinidia deliciosa*	S.R. Pennycook, G.J. Samuels	AY236943	AY236888
	ALG73	Algeria	*Vitis vinifera*	A. Berraf-Tebbal	KJ657710	KJ657727
	ALG75	Algeria	*Vitis vinifera*	A. Berraf-Tebbal	KJ657708	KJ657725
	ALG70	Algeria	*Vitis vinifera*	A. Berraf-Tebbal	KJ657713	KJ657730
	ALG72	Algeria	*Vitis vinifera*	A. Berraf-Tebbal	KJ657711	KJ657728
	ALG76	Algeria	*Vitis vinifera*	A. Berraf-Tebbal	KJ657707	KJ657724
	ALG74	Algeria	*Vitis vinifera*	A. Berraf-Tebbal	KJ657709	KJ657726
	ALG71	Algeria	*Vitis vinifera*	A. Berraf-Tebbal	KJ657712	KJ657729

(Continued)

Table 1. (Continued).

Species	Isolate number[a, b]	Origin	Host	Collector	GenBank accession numbers[c]	
					ITS	EF-1α
N. ribis	**CBS 115475**	USA	*Ribes* sp.	B. Slippers, G. Hudler	AY236935	AY236877
	CBS 121.26	USA	*R. rubrum*	B. Slippers	AF241177	AY236879
N. ursorum	**CBS 122811**	South Africa	*Eucalyptus arboretum*	H.M. Maleme	FJ752746	FJ752709
	CMW 23790	South Africa	*Eucalyptus arboretum*	H.M. Maleme	FJ752745	FJ752708
N. umdonicola	**CBS 123645**	South Africa	*Syzygium cordatum*	D. Pavlic	EU821904	EU821874
	CBS 123646	South Africa	*Syzygium cordatum*	D. Pavlic	EU821905	EU821875
N. viticlavatum	**CBS 112878**	South Africa	*Vitis vinifera*	F. Halleen	AY343381	AY343342
	CBS 112977	South Africa	*Vitis vinifera*	F. Halleen	AY343380	AY343341
N. vitifusiforme	**CBS 110887**	South Africa	*Vitis vinifera*	J. Van Niekerk	AY343383	AY343343
	CBS 110880	South Africa	*Vitis vinifera*	J. Van Niekerk	AY343382	AY343344

[a] Ex-type isolates are given in bold typeface.
[b] Acronyms of culture collections: **ATCC**: American Type Culture Collection, Virginia, USA; **ALG**: Personal culture collection A. Berraf-Tebbal, **CBS**: CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands; **CMW**: Tree Pathology Co-operative Program, Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa; **PD**: Culture collection, University of California, Davis, USA; **WAC**: Department of Agriculture, Western Australia Plant Pathogen Collection, South Perth, Western Australia. **MUCC**: Culture Collection, Laboratory of Plant Pathology, Mie University, Tsu, Mie prefecture, Japan. **CMM:** culture collection of Phytopathogenic Fungi "Prof. Maria Menezes",Universidade Federal Rural de Pernambuco, Recife, Brazil.
[c] KJ-numbers refer to newly generated sequences.

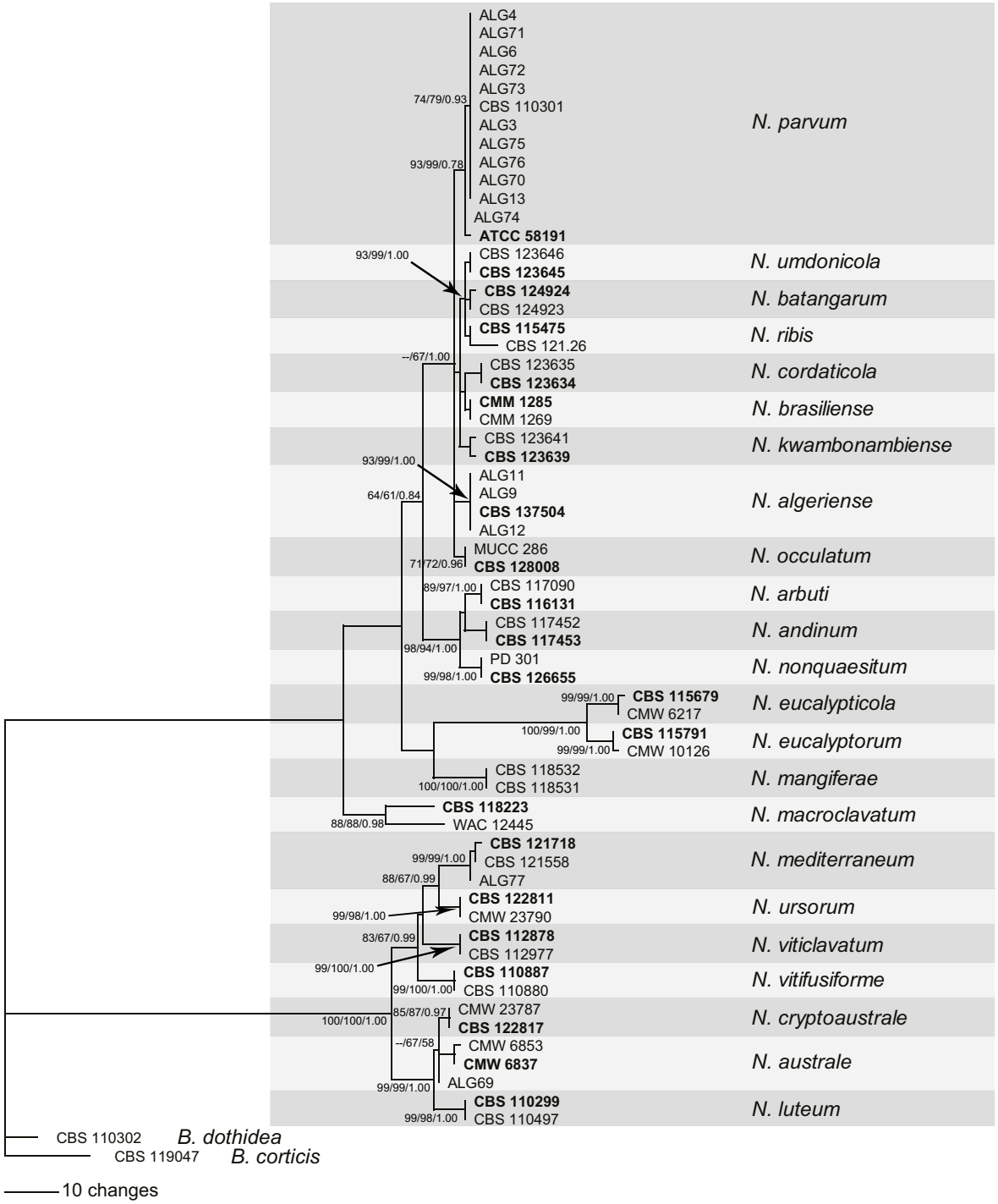

Figure 1. One of 209 equally parsimonious trees resulting from the alignment of 672 characters of combined *ITS* and *EF-1α* partial sequences. Length = 296; consistency index (CI) = 0. 774; retention index (RI) = 0.930; homoplasy index (HI) = 0.228. Ex-type isolates are given in bold typeface. Bootstrap values from 1000 replications are shown for Maximum Parsimony (MP), Neighbour-Joining (NJ) and Bayesian methods (BM) at the tree nodes (MP/NJ/BM). Branches marked with a minus (–) are not present in NJ tree. *Botryosphaeria dothidea* (CBS 110302; GenBank ITS: AY259092; TEF: AY573218) and *Botryosphaeria corticis* (CBS 119047; GenBank ITS: DQ233245; TEF: EU01539) were included as outgroups.

identical. Another isolate clustered in a subclade together with *N. australe* and *N. cryptoaustrale*. This isolate differed from these two species by a single base pair in ITS, one base pair in EF-1α from *N. australe* and three base pairs in EF-1α from *N. cryptoaustrale*.

Taxonomy

Based on ITS and EF-1α sequence data and anamorph morphology, three of the four species are known (*N. parvum*, *N. mediterraneum*, *N. australe*). One set of isolates was phylogenetically distinct from all other known species and is described here as new.

Neofusicoccum algeriense A. Berraf-Tebbal & A.J.L. Phillips, sp. nov.
MycoBank **MB 808496**

Etymology. Name after Algeria where the fungus was discovered.

Teleomorph not seen. *Conidiomata* pycnidial, produced on poplar twigs or pine needles on WA within 2–4 wk, solitary or aggregated, dark brown to black, uniloculate, immersed in the host becoming erumpent when mature. *Conidiophores* cylindrical, hyaline, smooth, thin-walled, septate. *Conidiogenous cells* cylindrical, smooth, holoblastic, phialidic with periclinal thickening, 11.5–14(–16.5) × 3–2.5(–5) μm. *Conidia* hyaline, fusiform, base subtruncate to bluntly rounded, non-septate, rarely forming a septum before germination, (14.5–)17–18(–21) × (4.5–)5.5–5.7(–6.5) μm (av. ± SD of 50 conidia = 17.6±1.3 × 5.6±0.4 μm). *Spermatia* hyaline, cylindric to allantoid, aseptate, 3.2–7.5 × 2.8–4 μm.

Culture characteristics. Colonies white with abundant aerial mycelium reaching 90 mm in diameter within 7 d on PDA at 25ºC. Aerial mycelium becoming smoke grey (21‴f) to olivaceous grey (21‴i) at the surface and dull green (27″m) to brown vinaceous (5‴m) at the reverse after 14 d in the dark at

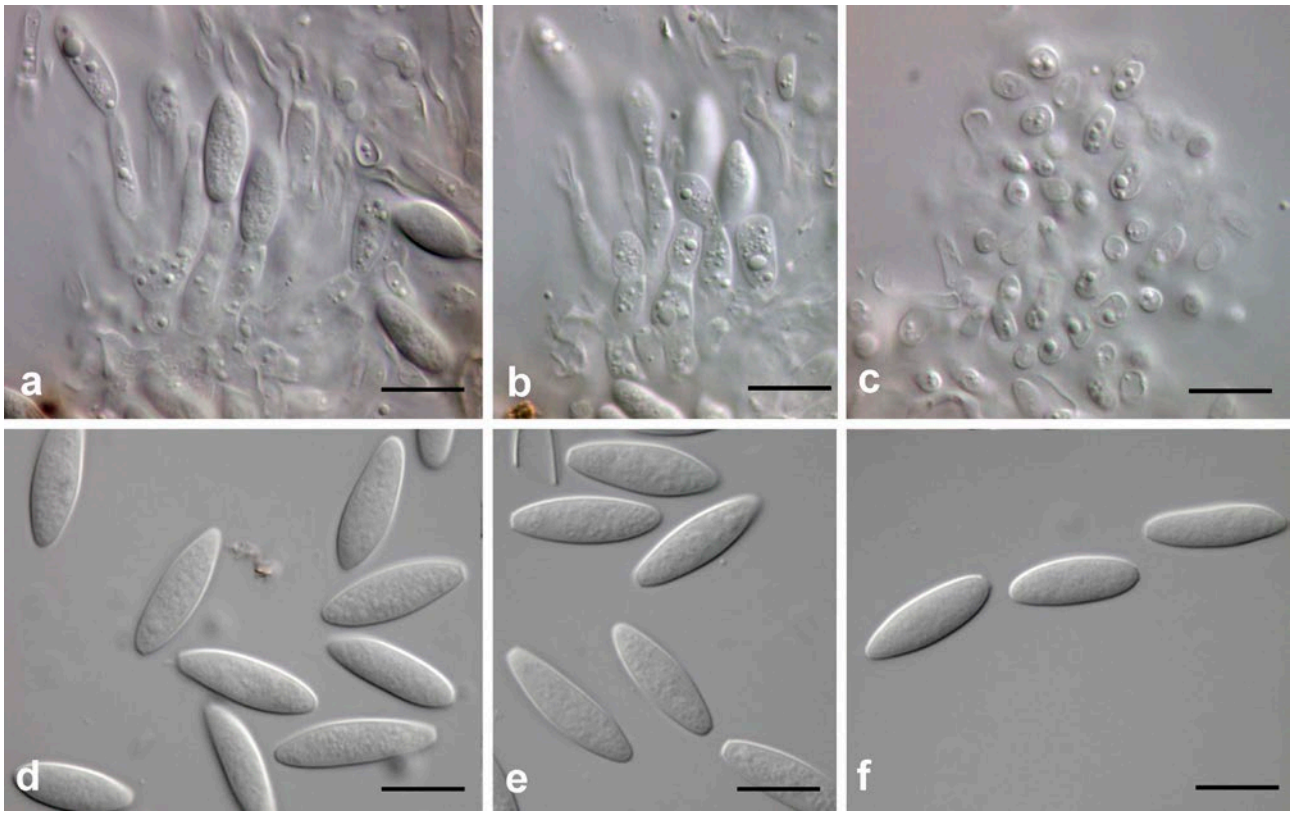

Figure 2. *Neofusicoccum algeriense*. a, b. Conidia developing on conidiogenous cells. c. Spermatia. d–f. Hyaline mature conidia. Bars: 1000 μm.

Table 2. *Neofusicoccum* species isolated from different types of diseased tissues in grapevines with dieback symptoms

Species	Wedge-shaped necrosis	Brown central necrosis	Black streaking	Yellow soft wood rot
Neofusicoccum parvum	44	2	2	13
N. algeriense	3	0	0	1
N. mediterraneum	0	0	1	0
N. australe	1	0	0	0
Total	48	2	3	14

25ºC. Cardinal temperatures for growth; minimum ≤ 5ºC, maximum ≥ 35ºC, optimum 25ºC.

Habitat. trunks and branches of *Vitis vinifera*.

Known distribution. Northern Algeria.

Holotype. Algeria. Ain-Benian, on trunks and branches of *Vitis vinifera*, May 2013, A. Berraf-Tebbal (LISE 9630, deposited in Herbarium of the National Agronomic Station, Oeiras, Portugal), a dry culture of ALG1 on poplar twigs, ex-type culture ALG1 = CBS 137504).

Notes. Two unique, fixed alleles in ITS and one in EF1-α separate *N. algeriense* from all other *Neofusicoccum* species. Conidia of *N. algeriense* are larger (17.6 × 5.6 μm) than those of *N. occulatum* (15.5 × 5.8 μm), but smaller than those of *N. kwambonambiens*e (22.3 × 6.3 μm).

Frequency and localization of the *Neofusicoccum* species

The frequency of the different species in the sample of 67 isolates could be determined by relating the identities of the representative isolates, based on ITS and EF-1α sequence data, to the morphological groupings. *Neofusicoccum parvum* was the most frequently isolated species, followed by *N. algeriense*. Only one isolate corresponded to each of *N. mediterraneum* and *N. australe*.

Internal symptom types in host stems were classified as wedge-shaped necrosis, brown central necrosis, black streaking, and yellow soft wood rot. Among the four types of wood alteration considered, *Neofusicoccum* species were most frequently isolated from wedge-shaped necrosis (48 isolates) followed by yellow soft wood rot (14), then black streaking (three) and brown central necrosis (two) (Table 2). The other species were rarely encountered.

Discussion

Several species of *Neofusicoccum* have been found associated with grapevine trunk diseases worldwide. However, this paper is the first study of the identity and frequency of *Neofusicoccum* species associated with Algerian grapevines, and forms part of a study of the Botryosphaeriaceae from Algeria. This is the first attempt to assess the diversity of *Neofusicoccum* species on grapevines with dieback symptoms in North Algeria. Integration of morphology, cultural characters and DNA sequence data revealed the presence of four species, namely *N. parvum*, *N. mediterraneum*, *N. australe* and *N. algeriense*, which is described as new.

In this study, *N. parvum* was the most frequently encountered species, with an incidence of 91%. Isolates of this species were sampled from three cultivars from two localities. This species is common on many woody hosts including *Vitis*, *Actinidia*, *Populus* and *Prunus.* On grapevine, *N. parvum* is known to be an aggressive pathogen (Van Niekerk *et al.*, 2004; Slippers *et al.*, 2007; Úrbez-Torres *et al.*, 2011; Gramaje *et al.*, 2012). Abdollahzadeh *et al.* (2013) identified *N. parvum* on three other hosts including willow, cherry and raspberry. In a recent study of the diversity and distribution of *N. parvum*, Sakalidis et *al.* (2013) recorded *N. parvum* in six continents and 29 countries, with wide host ranges including 90 host species. They also suggested that *N. parvum* has probably been dispersed through the transport of infected plant material into new *Vitis vinifera* growing areas.

One isolate was obtained of each of *N. mediterraneum* and *N. australe*. They represented only 1.5%

of the total number of isolates obtained in this study. *Neofusicoccum australe* has also been rarely isolated from *Vitis vinifera* in Australia. Although this species has often been implicated in grapevine trunk diseases (e.g. Van Niekerk *et al*., 2004; Úrbez-Torres *et al*., 2011; White *et al*., 2011), only a single isolate was obtained from two different vineyards in Australia (Wunderlich *et al*., 2011), Thus, the importance of *N. australe* in grapevine wood disease is not clear, although it does appear to play a minor role. Nevertheless, Van Niekerk *et al.* (2004) and White *et al.* (2011) confirmed that *N. australe* is pathogenic on grapevines. This species has widespread distribution across ten countries and has been reported from 46 host species in 18 plant families (Sakalidis *et al*., 2011). Recently, *N. cryptoaustrale* was described from *Eucalyptus* sp. (Crous *et al*., 2013). Only 1 bp difference in ITS and 3 bp differences in EF-1α separated the strain of *N. australe* in the present study from *N. cryptoaustrale*. Further analyses using additional gene regions will be required to clarify the status of species and strains within this group. Morphologically, conidia of the strain examined in the present study were much shorter (20.5 × 5.5 μm) than typical of *N. australe* (24.5 × 5.0 μm), but longer than *N. cryptoaustrale* (19 × 5.5 μm).

Neofusicoccum mediterraneum was first described by Crous *et al.* (2007) from *Eucalyptus* in Greece. Lazzizera *et al*. (2008) isolated this species from olive drupes in southern Italy. Later, it was found in Spain and California on grapevine, almond, pistachio, citrus and various woody hosts (Aroca *et al*., 2010; Inderbitzin *et al*., 2010). Recently, Abdollahzadeh *et al.* (2013) isolated the species for the first time from mango trees.

This study adds a new *Neofusicoccum* species associated with diseased grapevines, namely *N. algeriense.* With four isolates, it was the second most frequently isolated species on two cultivars of *Vitis vinifera* from the same locality. More sampling is necessary to understand the role of this species in grapevine diseases, its host range, distribution and variability.

A relationship between vascular lesions and the *Neofusicoccum* species isolated was highlighted in this study. Cross sections of trunks and arms of symptomatic grapevines showed seriously damaged tissues, which appeared in various shapes. *Neofusicoccum* spp. were most frequently isolated from wedge-shaped necroses and yellow soft wood rot, and at a much lower incidence from central brown necrosis and black streaking. This is consistent with the finding of previous reports (Luque *et al*., 2009; Úrbez-Torres *et al*., 2011). According to Van Niekerk *et al.* (2011), the presence of Botryosphaeriaceae spp. in the yellow soft wood rot may be a result of their saprophytic nature.

Neofusicoccum parvum and *N. algeriense* were mainly isolated from wedge-shaped necrosis. According to Úrbez-Torres *et al.* (2011) and Van Niekerk *et al.* (2011), *N. parvum* was frequently isolated from wedge-shaped necrosis and black streaking of declining grapevines in California and South Africa. Previously, Van Niekerk *et al.* (2004) reported this species from asymptomatic tissue and pruning debris. Further studies are needed to confirm the degree of involvement of Botryosphaeriaceae species in wood deterioration. Furthermore, Wunderlich *et al.* (2011) isolated Botryosphaeriaceae species from reproductive structures of grapevine over different phenological stages, confirming that such species can infect different *Vitis vinifera* tissue types throughout all stages of the growing season. They suggested considering Botryosphaeriaceae species as more than trunk disease pathogens.

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